Dietary Dehydroepiandrosterone Inhibits Bone Marrow and Leukemia Cell Transplants: Role of Food Restriction

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Dietary dehydroepiandrosterone (DHEA) inhibits the proliferation of syngeneic bone marrow cells (BMC) infused into lethally irradiated mice. Potential mechanisms for suppression of hematopoiesis were evaluated and the findings were as follows: (i) depletion of NK, T, B or macrophage cells failed to reverse suppression by DHEA; (ii) stem cell stimulation by erythropoietin, growth hormone, interleukin-2, Friend leukemia virus, or cyclophosphamide failed to reverse suppression; (iii) supplementation of fatty acids, mevalonate, or deoxyribonucleotides, which are dependent upon glucose-6-phosphate dehydrogenase function, did not enhance BMC growth in mice fed DHEA; (iv) DHEA downstream metabolites 4-androstenedione and 17β-estradiol, as well as the synthetic steroid, 16α-chloroepiandrosterone (but not testosterone or 5-androstene-3β,17β-diol), also inhibited BMC growth. Tamoxifen antagonized the effects of 17β-estradiol but not DHEA; (v) dietary DHEA causes hypothermia, but housing of DHEA-fed mice at 34°C to maintain normal body temperature did not reverse suppression; (vi) DHEA leads to a decrease in food intake in rodents. Pair-feeding control diet to mice fed DHEA mimicked the effects of dietary DHEA; (vii) adrenalectomy and orchiectomy decrease the levels of stress and sex hormones, respectively. Neither procedure affected the ability of food restriction or DHEA feeding to inhibit hematopoiesis; (viii) growth of GR-3 NM pre-B leukemia cells in unirradiated mice was also suppressed by DHEA or food restriction. We conclude that DHEA, by reducing food intake in mice, inhibits bone marrow and leukemia cell growth. The precise mechanism(s) by which reduced food intake *per se* inhibits hematopoiesis is not known, but may involve an increased rate of cellular apoptosis. Exp Biol Med 228:1303–1320, 2003

Key words: DHEA; steroids; food restriction; hematopoiesis; leukemia; marrow grafts

Dehydroepiandrosterone (DHEA) supplementation in food is an effective preventive or therapeutic agent in several pathologic states of rodents, including those that are immune-mediated or have an immune component (1). However, little is known about its mechanism of action. Several hypotheses have been proposed to explain the beneficial effects of DHEA including (i) inhibition of glucose-6-phosphate dehydrogenase (G6PD), (ii) increase in sex hormone levels, (iii) induction of food restriction, and (iv) induction of hypothermia. Previous studies of the effect of DHEA on immune function led to various observations. Dietary DHEA causes decreased cellularity of the spleen, thymus, and lymph nodes with little or no effect on T, B, or NK cell function in unirradiated mice (2). However, DHEA inhibits the recovery of lymphocytes after sublethal irradiation (500 cGy), which results in suppression of T, B, and NK cell functions (3). These latter changes are associated with increased apoptosis of lymphocytes (4).

Bone marrow transplantation (BMT) is used for management of many human diseases, including acute and chronic leukemia, lymphoma, anemia, and certain metabolic disorders. During and after BMT, immunosuppressive agents are administered to prevent both the rejection of the transplanted cells and the "rejection of the host" by the transplanted cells, a process termed graft-versus-host disease (GVHD). GVHD can give rise to considerable morbidity and mortality (5, 6). Therapeutic agents that prevent both graft rejection and GVHD would be very beneficial in transplantation procedures. Because DHEA inhibits recovery of both NK- and T-cell function after sublethal irradiation, the initial aims of the present studies were to establish whether DHEA could be used to (i) promote bone marrow cell (BMC) engraftment by inhibiting the production or function of *host* NK cells that are largely responsible for acute rejection of donor allogeneic marrow grafts and (ii) weaken GVHD by retarding the production of donor T lymphocytes and donor and host antigen-presenting cells (APC) that largely mediate this disease. The amount of DHEA given would have to be regulated with time to allow the production of donor-type lymphocytes and APC. The num-

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bers of host-type APC (dendritic cells, macrophages, and B cells) should decrease rapidly with time after irradiation, which should also inhibit GVHD. These aims were upset by the strong inhibitory effects on hematopoiesis by donor-derived cells in mice fed DHEA. The approach may work in hosts exposed to non-myeloablative doses of irradiation and/or cytotoxic drugs, a condition more likely to result in marrow allograft rejection.

In an attempt to elucidate the mechanism by which DHEA inhibits hematopoiesis, we have analyzed two different models of proliferation: transplants of syngeneic BMC in irradiated mice and transplants of GR-3 NM pre-B leukemia cells in unirradiated mice. Dietary DHEA inhibited the proliferation of BMC as well as leukemia cells. The findings presented in this study support the idea that food restriction is the major mechanism of suppression of hematopoiesis by dietary DHEA.

Materials and Methods

Animals. All mice were obtained from a colony at the University of Texas Southwestern Medical Center at Dallas. C.B-17 SCID mice were bred and housed in a barrier facility. The other mice were housed at 22 ± 2°C in a conventionally maintained facility on a 12:12-hour light:dark cycle with lights out at 1900 hr. All mice were between the ages of 10 and 16 weeks, unless otherwise indicated. The other strains included C57BL/6 (B6), (B6 × DBA/2)F1 (B6D2F1), (CBA × B6)F1 (CBB6F1), BAB.14, and BALB/c. No differences were observed in the response to DHEA between male and female mice; however the sex of the animals is denoted in each experiment in the figures themselves.

Diets. Mice were placed on the AIN-76A diet, as pellets (Dyets Inc., Bethlehem, PA) containing either no additive, 0.25% DHEA, or 0.45% DHEA (w/w) 2 weeks or 2 to 6 days before BMC transplantation. Also where indicated, the AIN-76A diet was administered in powdered form containing additives at the percentages (w/w) shown. Mice receiving GR-3 NM leukemia cells were placed on diets at the time of cell transfer, unless otherwise indicated. All animals except pair-fed mice were given food and water *ad libitum*. The amount of food administered to pair-fed mice was determined by the weight of food consumed by DHEA-fed mice the previous day on a daily basis. Mice were fed lab chow until the time of the studies and then placed on the AIN-76A diet containing either no additive or additives as indicated (w/w).

Steroids. The steroids 5-androstene-3β,17β-diol (A-diol), 4-androstene-3,17-dione (A-dione) were purchased from SIGMA (St. Louis, MO), while 17β-estradiol (estradiol) and testosterone were purchased from Steraloids (Wilton, NH). 16α-Chloroepiandrosterone (Cl-epi) was synthesized as described (7). They were mixed with powdered AIN-76A diet at the indicated percentages (w/w) and given to mice in glass bowls.

Bone Marrow Cell Transplantation. Bone marrow cells were obtained from femurs and tibias of donor mice. Cells were filtered through nylon mesh and counted using an electronic particle counter (Coulter Electronics Inc., Hialeah, FL). Cells were washed and suspended in RPMI medium at 1×10^7 cells/ml. Recipient mice were exposed to lethal irradiation (800 cGy) in a GammaCell 40 small animal irradiator containing two ^{137}Cs sources (Atomic Energy Ltd., Ottawa, Ontario, Canada) at a rate of 67 cGy/min. This dose is lethal for mice in this conventional colony, but the lethal dose in the specific-pathogen-free facility is 900 cGy. One to 3 hrs later, mice received 5×10^6 BMC in 0.5 ml medium in a lateral tail vein (iv). Five days after BMT, engraftment was determined by measuring spleen cell proliferation, as judged by DNA synthesis. Endogenous thymidine synthesis was inhibited by intraperitoneal (ip) injection of 50 μg 5-fluoro-2′-deoxyuridine (FdU; SIGMA). Thirty minutes later mice were injected ip with 0.5 μCi of the thymidine analog [^{125}I]-5-iodo-2′-deoxyuridine ([^{125}I]dU) (Amersham, Arlington Heights, IL). Four hours after injection of [^{125}I]dU mice were sacrificed by CO_2 inhalation, and spleens were removed and soaked overnight in 70% ethanol to remove non-DNA associated radioactivity. The radioactivity of spleens was measured in a 1290 Gamma-Trac Counter (TM Analytic, Elk Grove Village, IL). Results are expressed as the geometric mean (95% confidence limits, CL) percentage of injected [^{125}I]dU uptake in groups of 4 to 7 mice (8).

Histology. Freshly excised spleens were fixed in 10% formaldehyde solution (Fisher Scientific, Fair Lawn, NJ) and imbedded in methacrylate for sectioning. Sections were stained with hematoxylin and eosin (H & E).

Lung Clearance of Radiolabeled YAC-1 Tumor Cells. YAC-1 tumor cells (5×10^7) were incubated in RPMI containing 2.5 μg/ml FdU for 20 minutes; 30 μCi of [^{125}I]dU was added, and the cells were incubated for 90 more minutes at 37°C, 5% CO_2. The cells were then washed three times in RPMI medium and adjusted to 2×10^6 cells/ml. Cell viability was determined to be greater than 95% by trypan blue exclusion; 1×10^6 cells (0.5 ml) were injected into mice (groups of 4–5) iv. Four hours after injection the mice were killed by CO_2 narcosis, the lungs were removed, and the ^{125}I radioactivity in lungs was determined. The results are expressed as the geometric mean (95% CL) % recovery of the total injected radioactivity. Administration of pan-NK antibodies (mouse PK136 anti-NK1.1 mAbs or anti-asialo GM1 serum) prevents lung clearance of YAC-1 tumor cells (9).

Bone Marrow Cell Retransplantation (Stem Cell Rescue). Mice received BMT as described above. Two days later, spleens of transplant recipients were removed, and spleen cells obtained from each mouse were filtered through nylon mesh, washed, and suspended in 1 ml medium. Secondary syngeneic recipient mice were lethally irradiated (800 cGy), and each received one-half of a spleen equivalent cells in 0.5 ml volume iv. Four or 7 days after

retransplantation, splenic proliferation was determined by [^{125}I]dU incorporation as described previously (8).

Monoclonal Antibody-Mediated Cell Depletion. Mice received antibodies injected ip as follows: PK136 anti-NK1.1 mAb (American Type Culture Collection, ATCC, Rockville, MD) and rabbit anti-asialo GM1 serum (Wako Pure Chemical Industries, Osaka, Japan) were injected ip 48 hr before BMT. YTS 168.4 anti-CD8 mAb (ATCC) was administered 4 days, and Lyt 2.2 anti-CD8 mAb (ATCC) was given 6 and 2 days before BMT. Antibody effectiveness was determined by flow cytometry of spleen cells of separate mice, staining with alternate antibodies to the same cell type. Flow cytometry of spleen cells with (i) anti-Lyt2.2 mAb to test for depletion of $CD8^+$ T cells by YTS 168.4 mAb (and *vice versa*) indicated that the $CD8^+$ T cells were depleted, (ii) staining for NK1.1 in mice treated with anti-asialo GM1 serum and for anti-asialo GM1 in mice treated with anti-NK1.1 mAb documented depletion of NK cells, and (iii) staining for F4/80 on peritoneal cells of mice treated with silica particles (see below) established the depletion of macrophages (data not shown).

Silica-Mediated Macrophage Depletion. Silica (SIGMA) was suspended in phosphate-buffered saline (PBS) and sonicated before injection. Mice received silica ip (200 mg/kg body weight) 21, 18, 15, and 12 days before BMT. This silica regimen decreases macrophage functions (10). Day 0 was the day of BMT.

Cyclophosphamide Treatment. Cyclophosphamide (Cytoxan®; SIGMA) was dissolved in PBS and administered ip (300 mg/kg) 9 days before BMT. This particular regimen stimulates stem cell function by transferred syngeneic BMC (11).

Erythropoiesis Stimulation Experiments. Erythropoietin (EPO) 5U/100g body weight was administered ip on the day of BMT, and on each of the two following days. Friend erythro-leukemia virus (FLV), Mirand strain, was injected ip into susceptible *Fv2^{ss}* BALB/c mice [500 focus-forming units (FFU)/mouse] on the day of BMT. FLV induces massive splenic erythropoiesis in these mice (12).

Growth Hormone Administration. Recombinant human growth hormone (rhGH), a generous gift of Dr. William Murphy (NCI, Frederick, MD) was administered ip at a dose of 20 μg per mouse on the day of transplant, and 2 and 4 days later.

Serum Transfer. Serum donors were lethally irradiated and received 5×10^6 syngeneic BMC. On the day of each serum transfer three donors were sacrificed by rapid decapitation, the blood collected into Eppendorf tubes, allowed to clot, and centrifuged at 1500 rpm (Eppendorf centrifuge 5415, Brinkman Inst., Westbury, NY). Supernatants (sera) were collected, measured, and brought to 0.5 ml/recipient in RPMI media and injected iv into recipients.

Interleukin-2 Experiments. Mice received daily ip injections of IL-2 (generous gift of Cetus Corp., Emeryville, CA) in RPMI media containing 5% fetal calf serum. Mice received 100 units per day from the day of the transplant until the day of assay.

Intralipid® Administration. Intralipid® 20% Fat Emulsion (Kabi Pharmacia Inc., Clayton, NC) was injected ip (0.5 ml/mouse) daily from 5 days before BMT until the time of assay.

Mevalonate Administration. Mevalonic acid lactone (SIGMA) was administered in the drinking water at 0.75 mg/ml from 3 days before transplant until the time of assay.

Deoxyribonucleoside (DRN) Administration. 2′-deoxyadenosine, 2′-deoxycytidine hydrochloride, 2′-deoxyguanosine, thymidine and 2′-deoxyuridine (U.S. Biochemicals, Cleveland, OH) were administered each at 4.2 μmol/ml in the drinking water as described (13), with fresh drinking water provided every 2 days. DRN supplement was provided from 1 day before transplant until the time of isotope assay.

GR-3 NM Cell-Induced Leukemia. GR-3 NM cells were obtained by *in vivo* serial passage of bone marrow from CBB6F1 mice containing the human p190 *bcr/abl* transgene (14). These cells produce a pre-B cell lymphoma/leukemia-like disease in recipient mice. Cells were cultured in RPMI medium containing 10% fetal bovine serum, 1 mM non-essential amino acids (GIBCO BRL, Grand Island, NY), 10 mM sodium pyruvate (GIBCO BRL), and 5×10^{-5} M 2-mercaptoethanol (SIGMA) and were maintained in culture no longer than six passages before *in vivo* transfer. Proliferation/progression of leukemia cells was determined by the induction of splenomegaly or by survival times. Mice (groups of 4–5) were sacrificed by CO_2 narcosis 10 days after transfer of 1×10^5 GR-3 NM cells iv. Spleens were removed and weighed. Other mice received 2×10^4 cells iv (groups of 5 mice). They were examined daily and allowed to die of the disease process unless they became moribund or showed lower body paralysis, in which case they were sacrificed. Mice were autopsied, and tissues were examined histologically to confirm the diagnosis of leukemia.

Tamoxifen Treatment. Tamoxifen (SIGMA) was dissolved in safflower seed oil at 100 μg/ml. Mice were injected subcutaneously (sc) into the intra-scapular fold with 0.1 ml (10 μg) of tamoxifen or vehicle alone on days 5, 4, 3, and 2 days before, and on the day of cell transfer. On day 0 mice received transfers of BMC or GR-3 NM cells. Tamoxifen (10 μg) or vehicle alone was administered every other day after day 0 for the duration of the study.

Measurement of Internal Body Temperature. Body temperature was determined by inserting a Telethermometer probe (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) 25 mm into the recta of mice that were not anesthetized (15). The temperatures were registered 60 sec after probe insertion. Mice were examined daily between 9 and 10 AM, beginning 3 days before irradiation and BMC transfer and until the day of isotope assay.

Animal Surgery. Fifteen minutes before surgery, mice were anesthetized using Ketaset® (ketamine hydro-

chloride, 100 mg/kg, Aveco Co. Inc., Fort Dodge, IA) and Xylazine (5 mg/kg, Rugby Laboratories Inc., Rockville Center, NY) injected ip. To remove the adrenal glands, a ventral incision in the abdominal wall was made to expose the adrenal glands. Due to their high vascularity, the connecting vessels were cauterized before excision of the adrenal glands. After removal, the peritoneal wall was sutured using 0.6 mm silk sutures and the skin was closed using surgical staples. In sham-operated mice the adrenal glands were exposed but not excised. Adrenalectomized mice were placed on drinking water containing 0.9% NaCl. Castration was performed by making a ventral incision in the scrotal sac to expose the testes. The testicular vessels were ligated, and the testes were excised. In sham-operated mice the testicles were exposed but not excised. In both castrated and sham-operated mice, the scrotal sac was closed using surgical staples. All animals (adrenalectomized, castrated, and sham operated) were placed on drinking water containing 0.4% neomycin sulfate (Biosol®; Upjohn, Kalamazoo, MI) as prophylaxis against infection.

Statistics. Statistical analysis across several groups was performed using the Newman-Keuls multiple group comparison procedure. When comparing between two groups, the Student's *t* Test was performed. Statistical significance was based on a P value < 0.05. Survival curves of groups of mice were analyzed by the generalized Wilcoxon comparison.

RESULTS

Can Dehydroepiandrosterone Abrogate 'Hybrid Resistance' to Parental Strain Bone Marrow Cell Grafts? The observation that recovery of natural killer (NK) cell activity after receiving a bone marrow transplant was inhibited in mice placed on a 0.45% DHEA diet (3, 4) raised the possibility that DHEA could be used to modulate the immune response to an allogeneic bone marrow graft. Sublethal irradiation (about 500 cGy for mice) results in loss of most mature lymphocytes (16), including NK cells (17). Two weeks after receiving 500 cGy, B6 male mice on the control diet had recovered their NK function, as demonstrated by the ability to clear radiolabeled YAC-1 cells from their lungs in a 4-hr assay (9). However, mice on the 0.45% DHEA diet had not recovered NK function, as indicated by high counts in the lung (Fig. 1A). These findings agree with the previous observations regarding DHEA and NK function, and suggest that mice treated in this manner would be less able to reject an allogeneic bone marrow graft. B6 BMC proliferate in syngeneic hosts as indicated by splenic [^{125}I]dU uptake 5 days after transplant. These cells are rejected by B6D2F1 hosts because of 'hybrid resistance' (8, 18). Male B6D2F1 hosts fed the control diet that received 500 cGy 2 weeks before transplant also rejected B6 cells, although not as well as normal mice, because their NK function had not fully recovered from the sublethal irradiation (Fig. 1B). We would predict from Figure 1A that

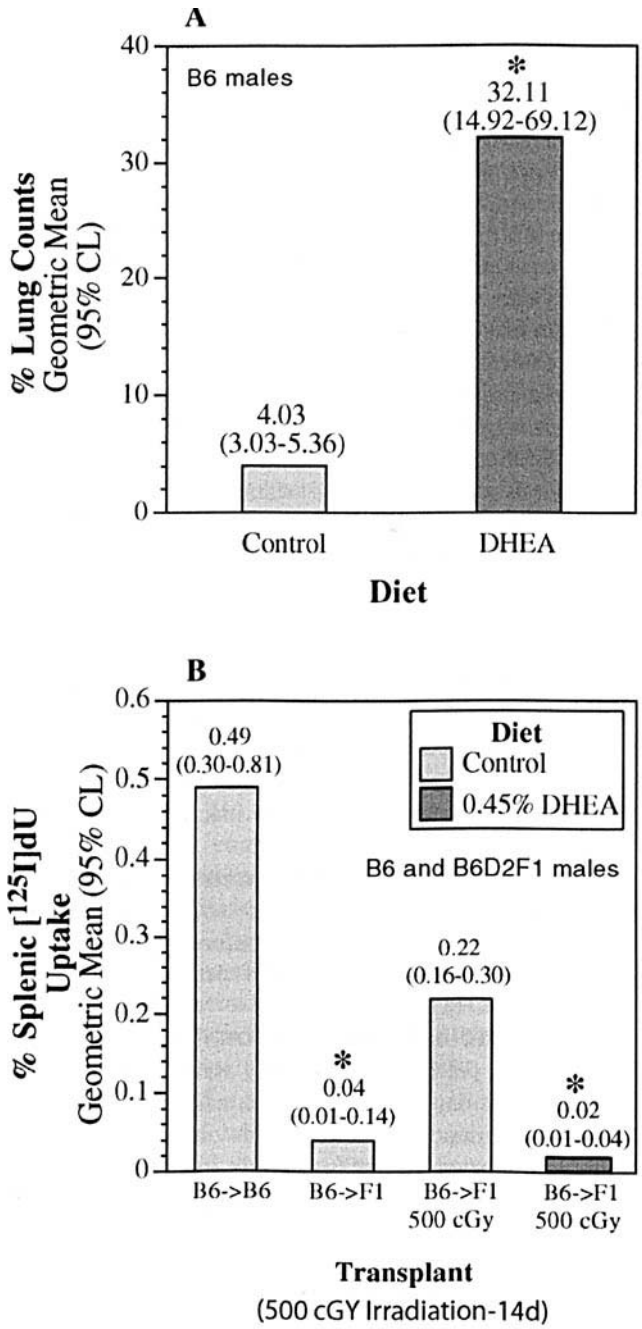

Figure 1. Suppression of NK cell function, but apparently not of 'hybrid resistance' to parental strain BMC grafts. (A) DHEA suppression of NK cell-mediated lung clearance of YAC-1 tumor cells. B6 male mice (groups of 6) were fed control AIN-76A or DHEA-containing AIN-76A diet (0.45% w/w) for 2 weeks before exposure to 500 cGy total body irradiation. The mice were infused with 1×10^6 [^{125}I]dU-labeled YAC-1 cells. Lungs were removed 4 hrs later and ^{125}I radioactivity was determined. Values represent the geometric mean (95% confidence limits) % retention of ^{125}I counts infused. DHEA greatly decreased NK cell function (i.e., clearance of infused YAC-1 cells). (B) Failure of parental B6 BMC to grow in B6D2F1 mice treated with DHEA. Male mice (groups of 6) were exposed to 0 or 500 cGy 14 days before exposure to 800 cGy and infusion of 5×10^6 B6 BMC. Splenic [^{125}I]dU uptake (%) was measured 5 days later. B6D2F1 mice on the control diet exposed to 0 or 500 cGy did not support the growth of B6 BMC, a surprising finding because NK cell function was inhibited (A). * Significantly different from controls ($P < 0.05$) by the Neuman-Keuls multiple group comparison procedure.

DHEA-treated, sublethally irradiated mice would not be able to reject histoincompatible cells. However, those mice *appeared* to do just that (Fig. 1B), as the spleens of male B6D2F1 DHEA-treated graft recipients showed virtually no proliferation.

Dehydroepiandrosterone Inhibits Growth of Syngeneic Bone Marrow Cells. The failure of B6 BMC to grow in DHEA-fed B6D2F1 mice, despite the fact that the host mice had received sublethal irradiation, was quite unexpected. This observation suggested that either the NK cell subset whose function was determined by the YAC-1 clearance assay was different from the NK cell subset responsible for stem cell rejection, or that the DHEA-fed hosts failed to provide a suitable environment for the growth of any transplanted BMC. To determine whether DHEA-fed hosts would allow growth of fully histocompatible BMC, we performed syngeneic BMC transplants into DHEA-fed hosts. Syngeneic BMC failed to proliferate well in DHEA-treated B6 male hosts, even when the graft size was increased from 5 to 20 $\times$ 10^6 cells, while transfer of 5 $\times$ 10^6 cells resulted in robust splenic proliferation by 5 days in mice receiving the control diet (Fig. 2A). The failure of syngeneic BMC to grow in DHEA-fed recipients is not likely to be a direct effect on the stem cells, because BMC from B6 male donor mice fed DHEA for a prolonged period proliferated well in control hosts not receiving DHEA (Fig. 2B).

Histologically, the spleens of B6 male BMC recipients on the control diet showed extensive areas of erythroid cells, myeloid cells, and megakaryocytes in the red pulp 5 days after receiving syngeneic BMC (data not shown). The splenic red and white pulp of mice on the 0.45% DHEA diet appeared empty (data not shown). The degree of inhibition of stem cell growth in DHEA-fed B6 male mice was directly related to the concentration of DHEA in the diet (Fig. 2C). In this experiment, mice received DHEA in the powdered diet placed in glass bowls inside the cages. Since it is difficult to administer the diet in this manner, we repeated the experiment with diet containing pellets at 0.25% or 0.45% DHEA (Fig. 2D). Both methods demonstrated that DHEA exerts its effect when fed to mice at a concentration as low as 0.25%.

Kinetics of Suppression. In all previous experiments DHEA had been administered at least 14 days before BMT, but even when diet was started 24 hrs *after* transplantation the effect of DHEA was the same as that occurring after longer feeding schedules in B6 female mice (Fig. 3A). When diet was started 48 hrs after BMT, DHEA had an intermediate effect. When the DHEA-containing diet was removed at 0, 24, 48, or 72 hrs after transplantation, the splenic proliferation was significantly different ($P < 0.05$) from both control-fed B6 female mice and mice kept on the 0.45% DHEA diet throughout (Fig. 3B). The fact that DHEA-induced suppression of hematopoiesis occurred when the diet was started after the transplant, as well as the loss of its effect when the diet was removed, suggests a rapid-acting and short-term mechanism.

We next examined whether the transplanted stem cells were dying/being sacrificed or were merely not proliferating by performing "stem cell rescue" experiments (Fig. 3C). In these experiments female B6 BMC primary recipients on control or 0.45% DHEA diets were sacrificed 2 days after BMT, and their spleens cells (one-half spleen equivalent) were retransplanted into lethally irradiated secondary hosts on the control diet. The splenic [^{125}I]dU uptake in the secondary hosts was measured 4 or 7 days after retransplantation. As expected, there was very little proliferation 4 days after transplant, due to the fact that the number of stem cells in the primary spleens was much lower than that in the original bone marrow transfer. However, at 7 days there was significantly more proliferation ($P < 0.05$) in the spleens of secondary hosts that received splenocytes from control-fed primary hosts versus splenocytes from DHEA-treated primary hosts (Fig. 3D). These findings indicate that the transplanted cells were dying/being killed within the first 2 days after the initial transplant. This observation, together with the fact that the stem cells from DHEA-treated donors are fully functional (Fig. 2B), suggests that the transplanted cells are being killed, perhaps by some effector-mediated mechanism or by apoptosis caused by a lack of stem cell stimulation. Thus, the 'environment' for the originally infused stem cells was detrimental to the survival/growth of stem cells. Because DHEA rapidly induced suppression of hematopoiesis (Figs. 2 and 3), it became unnecessary to use the 'split-dose' protocol of 500cGy followed 14 days later with 800 cGy irradiation of host mice.

Role of Effector Cells. The cells primarily responsible for the acute rejection of allogeneic BMC by nonimmunized mice are NK cells (8). However, the role of NK cells is not limited solely to rejection of incompatible donor stem cells. Under certain circumstances, activated NK cells can enhance engraftment (19). On the other hand, activated NK cells also have the ability to suppress hematopoiesis in the absence of a major histocompatibility complex (H2) mismatch (20). PK136 is a monoclonal antibody (mAb) against NK1.1, a pan-NK cell marker, which can deplete NK cells *in vivo* to inhibit the rejection of allogeneic bone marrow cells (8, 18). Rabbit anti-asialo GM1 serum is even more effective at depleting NK cells (21), but it is less specific because a small percentage of T cells and macrophages also express this antigen. Neither antibody had any effect on the inhibition of engraftment by dietary DHEA administration in B6 female mice (Fig. 4A and 4B; $P < 0.05$). In these and later experiments, DHEA feeding was started 1 to 6 days before the BMC transplant.

Cytotoxic CD8$^+$ T lymphocytes can mediate the rejection of allogeneic bone marrow cells in immunized mice and allogeneic lymphocytes in nonimmunized mice (22, 23). To examine the possible role of CD8$^+$ T cells in DHEA-induced resistance to a syngeneic BMC transplant,

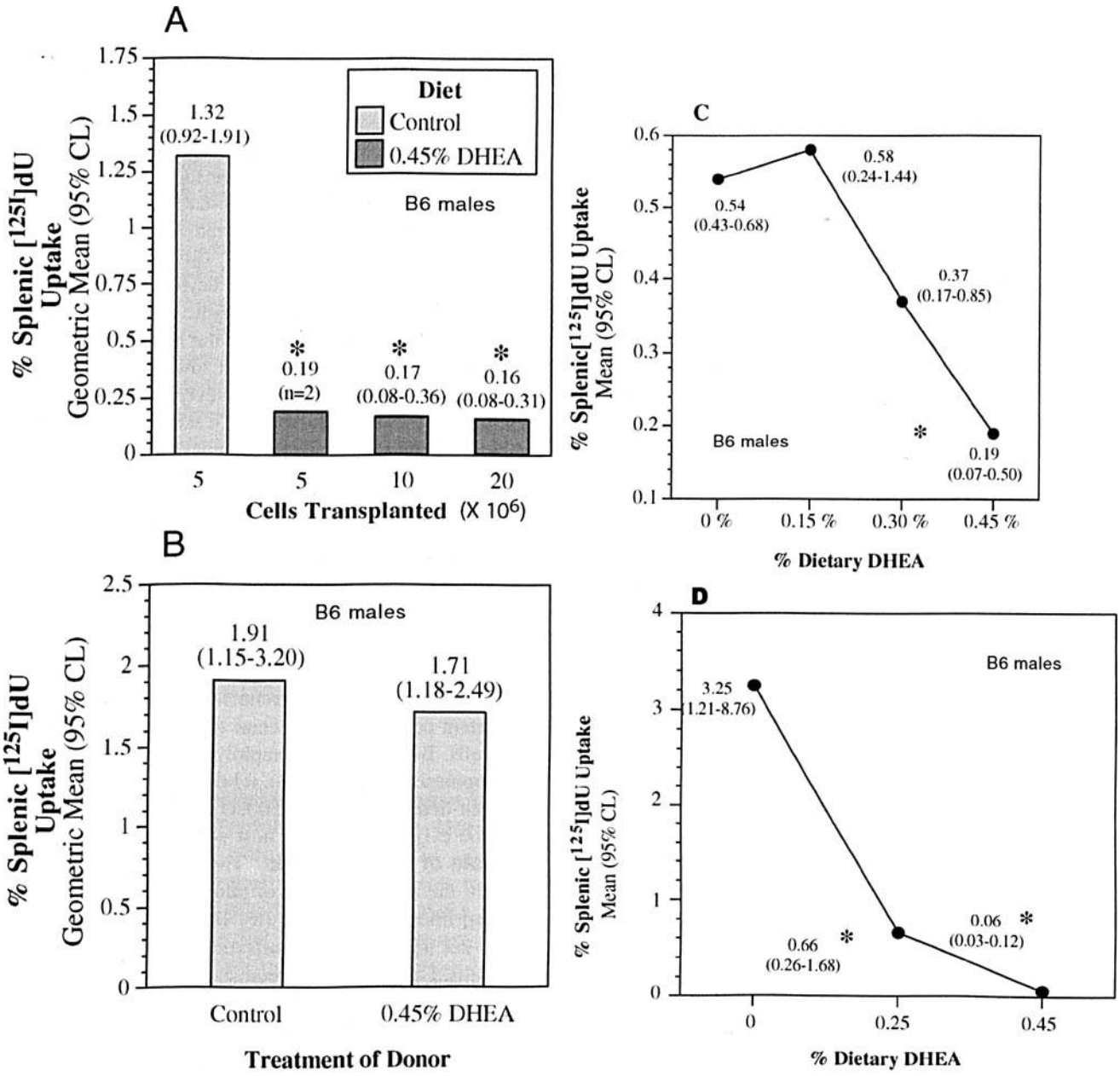

Figure 2. Differential effects of DHEA on hosts and donors of marrow transplants. (A) DHEA suppression of hematopoiesis by inocula of 5, 10, or 20 x 10^6 BMC. B6 male mice (groups of 6) exposed to 0 (control) or 500 cGy total body irradiation 14 days before exposure to 800 cGy and infusion of 5, 10, or 20 x 10^6 B6 BMC. Splenic [^{125}I]dU uptake (%) was measured 5 days later. Mice treated with DHEA did not support growth of syngeneic BMC at any inoculum size. (B) DHEA does not suppress hematopoiesis by donor stem/progenitor cells. B6 male mice were fed control or DHEA-containing diets for 4 months. Their BMC were infused into irradiated (800 cGy) B6 mice (groups of 5), and the isotope assay was performed 5 days later. There was no significant difference in growth of treated or control BMC. (C and D) Effect of DHEA dose in the diet of mice on hematopoiesis. B6 male mice were fed different amounts of either powdered (C) or pelleted (D) AIN-76A diet containing 0, 0.25, or 0.45% DHEA before irradiation (500 cGy). Fourteen days later, the mice were irradiated (800 cGy) and infused with 5 x 10^6 B6 BMC. The isotope assay was performed 5 days later. The suppression was related to the dose of DHEA. *See Figure 1.

we depleted $CD8^+$ cells using two separate antibodies (Lyt2.2 and YTS 168.4). Neither mAb reversed the ability of DHEA to suppress hematopoiesis in B6 or B10.BR male mice (Fig. 4C and 4D; $P < 0.05$).

We next examined the role of macrophages in the effects of DHEA by treating BAB.14 female mice with multiple ip injections of silica. Silica is taken up by many resident macrophages, induces the formation of oxygen radicals, and lowers intracellular pH, resulting in the death of macrophages. To test whether silica depletes macrophages in the spleen (the site of interest in our proliferation assays), the peritoneal cells from some of the treated mice were analyzed by flow cytometry. Silica treatment led to a 90% decrease in the percentage of Mac-1^+ cells in the spleen

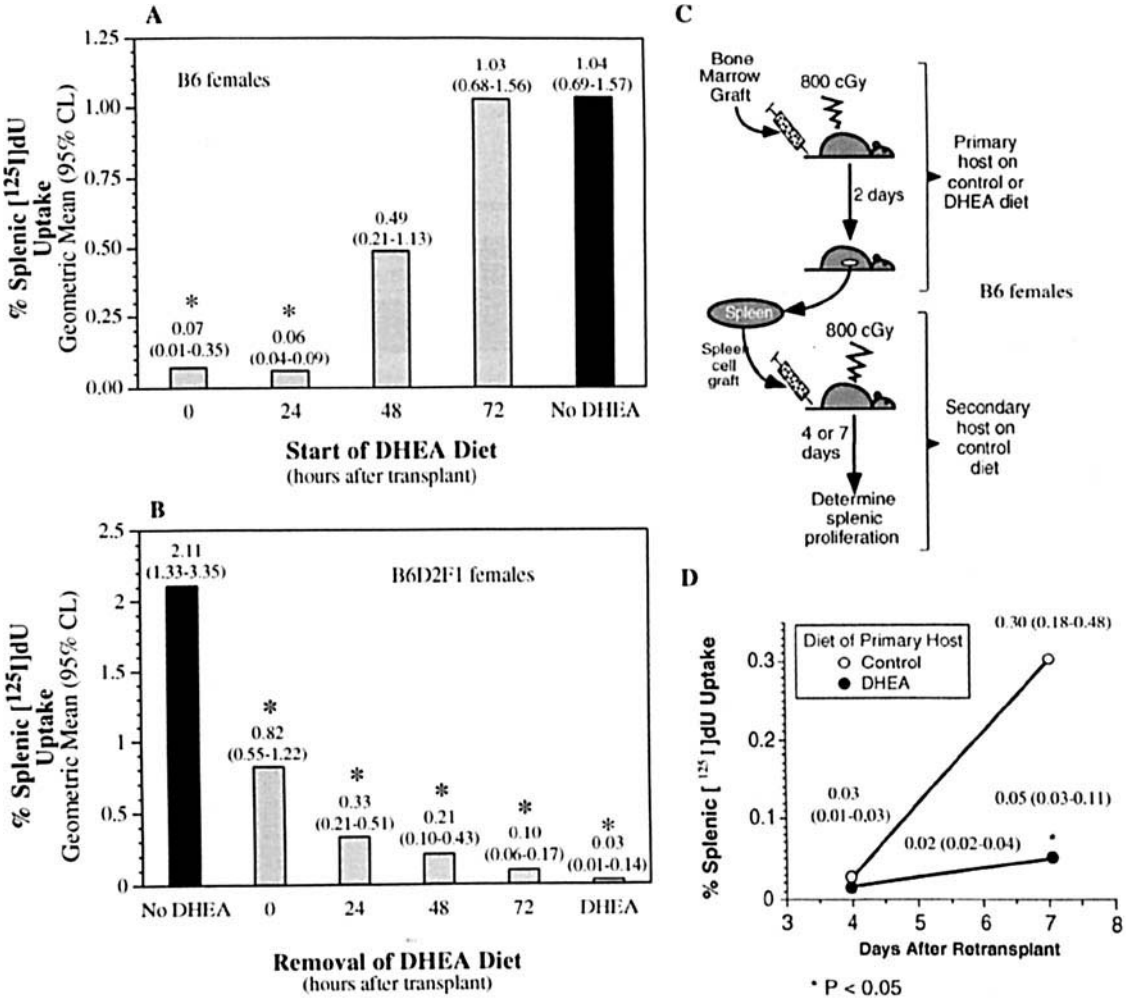

Figure 3. Rapidity of dietary DHEA effects. (A and B) Kinetics of initiation or removal of dietary DHEA to hosts (groups of 5) on growth of syngeneic B6 BMC. (A) Time of initiation. B6 female mice (groups of 5) were exposed to 800 cGy and infused with 5×10^6 B6 BMC. DHEA diet started at 0, 24, or 48 hrs after cell transfer suppressed hematopoiesis strongly, but at 72 hrs did not suppress growth of BMC. (B) Time of removal. B6D2F1 female mice (groups of 5) on the control or the DHEA diet for 14 days were irradiated (800 cGy) and infused with B6D2F1 BMC. The DHEA diet was removed at 0, 24, 48, or 72 hrs. There was significantly less growth of BMC in mice given DHEA at all times tested, but there was an inverse relationship between the time of removal of DHEA and amount of isotope uptake in the spleen. (C and D) "Stem cell rescue" experiment. (C) Protocol: B6 female mice (groups of 5) on control or the DHEA diet for 14 days were irradiated (800 cGy) and infused with 5×10^6 B6 BMC. Two days later, their spleens were removed and one-half spleen equivalent of cells was infused into each irradiated (800 cGy) B6 'secondary' recipient (groups of 5). The isotope assays were performed on days 4 or 7 days later. (D) The splenic uptake (%) of $[^{125}I]$dU was minimal at 4 days in all mice, but was much less in the DHEA-fed mice at 7 days. Thus early stages in stem cell differentiation are affected by dietary DHEA. *See Figure 1.

(data not shown). Silica had no effect on the proliferation of BMC in mice on control diet and did not effect inhibition seen in DHEA-treated animals (Fig. 4E; $P < 0.05$).

To examine the requirement of B and/or T cells, we fed 0.45% DHEA to $H2^d$ BALB/c and C.B.-17 severe combined immuno-deficient (SCID) female mice and transplanted BALB/c BMC. SCID mice, due to the inability to rearrange B and T cell receptor genes, lack mature B and T cells. Growth of BALB/c BMC in both BALB/c- and SCID-irradiated mice was poor if the mice were fed DHEA (Fig. 4F; $P < 0.05$). Thus the effect of DHEA on syngeneic BMT occurs independent of T and B cells. Due to a defective ability to repair DNA damage, SCID mice are very radiosensitive and need less irradiation (300 cGy) to ablate hematopoiesis.

Role of Complement. Certain acquired diseases, such as paroxysmal nocturnal hemoglobinuria (PNH), are characterized by damage to and depletion of hematopoietic cells, and this phenomenon appears to be mediated by the membrane attack complex (MAC) of complement (24). The sensitivity of hematopoietic cells to complement in PNH results from a deficiency of decay accelerating factor. This glycoprotein is present in the cell membranes of erythrocytes, platelets, granulocytes, and other hematopoietic cells (25). We have tested the role of complement in DHEA-induced suppression of hematopoiesis. DBA/2 mice lack the

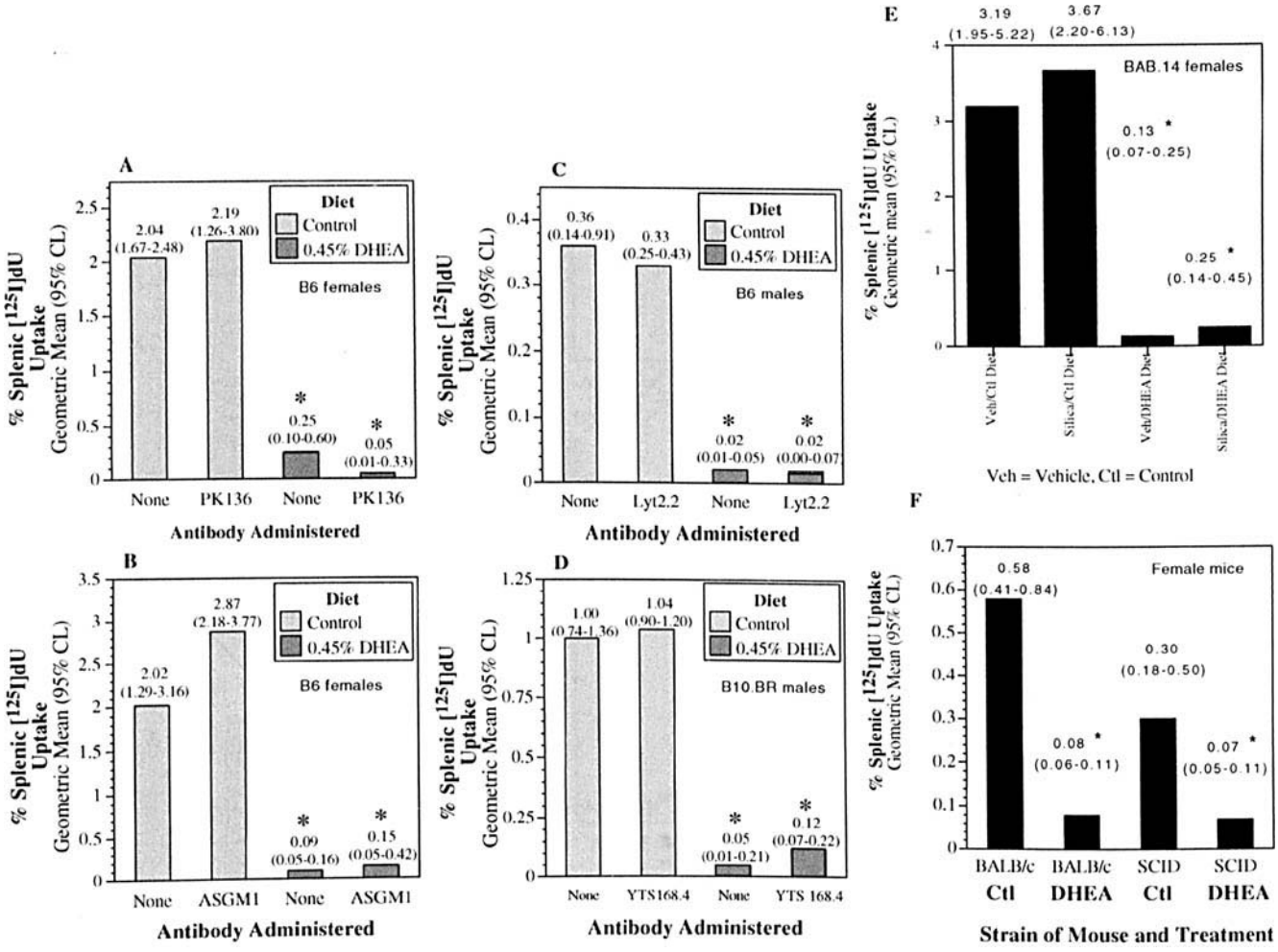

Figure 4. Requirements for effector cells on DHEA suppression of hematopoiesis. All mice were fed control or DHEA containing diets for 2 to 6 days before irradiation (800 cGy) and transfer of 5×10^6 syngeneic BMC. The isotope assays were performed 5 days later. (A and B) NK cells. B6 female mice (groups of 6) were injected ip with either 0.5 mg PK136 (anti-NK1.1) mAb (A) or 25 μl rabbit anti-asialo GM1 serum (B) 48 hrs before irradiation. (C and D) $CD8^+$ T cells. B6 male (C) or B10.BR male (D) mice (groups of 6) were injected ip with Lyt2.2 mAbs 6 and 2 days (C) or YTS168.4 mAbs 4 days (D) before irradiation. (E) Macrophages. BAB.14 female mice (groups of 5) were injected with 200 mg/kg body weight of silica particles ip 21, 18, 15, and 12 days before irradiation (800 cGy). (F) SCID mice devoid of T and B cells. Female BALB/c (groups of 5) and C.B.17 SCID (groups of 7) mice were exposed to 700 (BALB/c) or 300 (SCID) cGy irradiation. Depletion of NK cells (A and B), $CD8^+$ T cells (C and D), or macrophages (E), or the lack of T and B cells (F) did not prevent suppression of hematopoiesis by dietary DHEA. *See Figure 1.

C5 complement component due to a 2-base pair deletion in the C5 gene (26) and therefore cannot form the MAC of complement. The effect of DHEA on engraftment in DBA/2 female mice was not different from that in BALB/c male mice. The % splenic [^{125}I]dU uptake was 1.72 (1.34–2.21) in control and 0.24 (0.12–0.47) in DHEA-fed DBA/2 mice ($P < 0.05$), and was 1.87 (0.41–2.61) in control and 0.18 (0.08–0.42) in DHEA-fed BALB/c mice ($P < 0.05$). Thus, this function of complement does not play a role in DHEA-induced suppression of hematopoiesis.

Role of Stem Cell Stimulation. In other efforts to determine the mechanism of DHEA-mediated hematopoietic suppression, we treated mice with agents that stimulate stem cell function. Mice were treated with cyclophosphamide (Cytoxan®), a powerful immunosuppresive and cancer chemotherapeutic agent, 9 days before challenge with BMC. Cytoxan® inhibits the rejection of solid tissue and bone marrow transplants (27), but this particular regimen of cytoxan® *stimulates* stem cell function in irradiated rats infused with syngeneic BMC (9). Cytoxan® did not reverse the DHEA effect (Fig. 5A; $P < 0.05$) in B6D2F1 male mice.

Most of the proliferation in the mouse spleen 5 days after a BMC transplant is due to erythropoiesis (8). An important growth factor after a BMC transplant is erythropoietin (EPO) (28), and it is conceivable that DHEA administration results in decreased levels of EPO. We administered EPO (5U/100 g body weight per day) to B6 female mice and found no effect on DHEA-induced suppression of BMC growth (Fig. 5B; $P < 0.05$). We also tested whether DHEA exerts its effect *via* decreased levels of erythropoi-

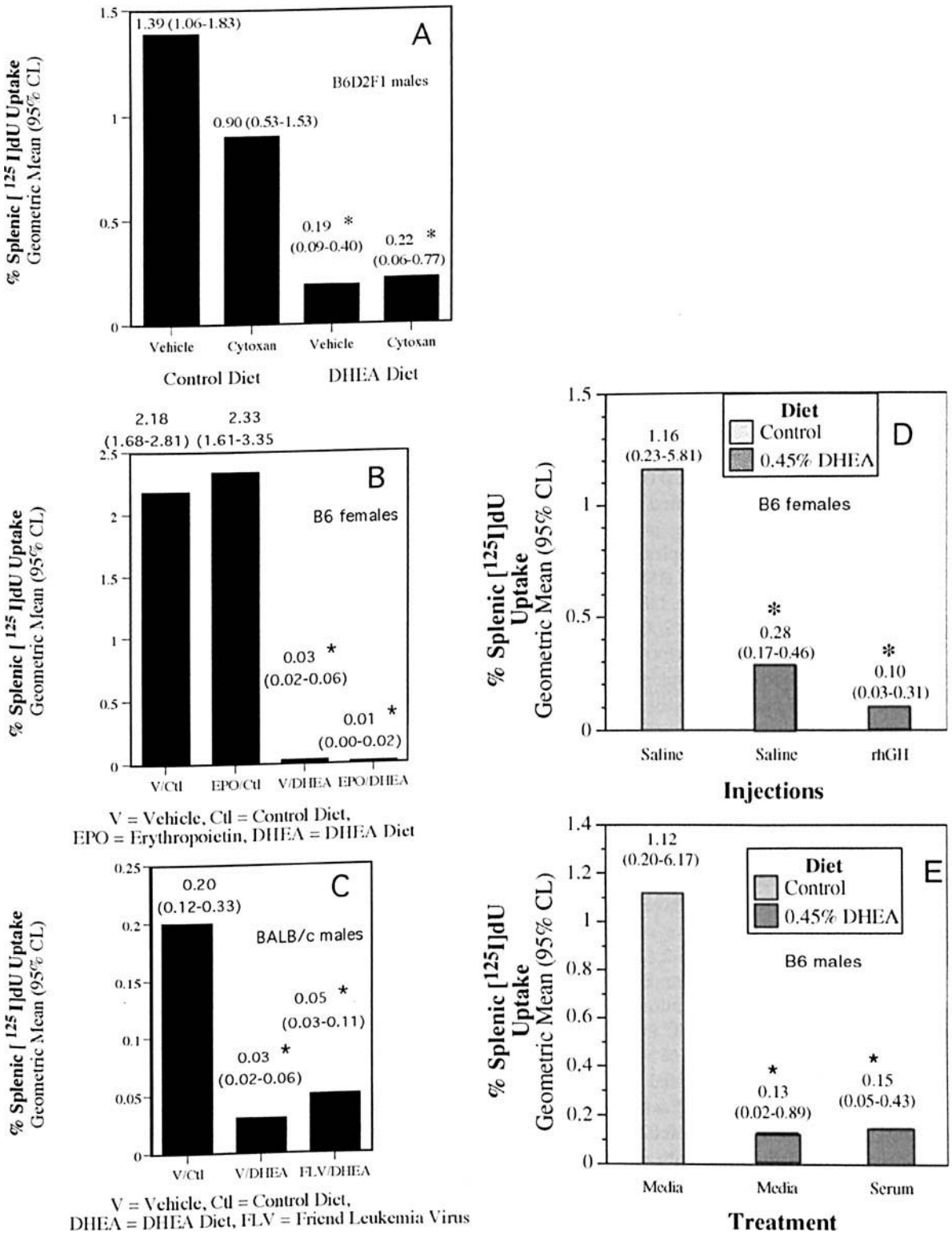

Figure 5. Stimulation of stem cell functions. All mice were fed control or DHEA-containing diets for 5 to 7 days before irradiation (800 cGy) and transfer of 5×10^6 syngeneic BMC. The isotope assays were performed 5 days later. (A) Cyclophosphamide (Cytoxan ®). B6D2F1 male mice (groups of 7) were injected with Cytoxan® (300 mg/kg body weight) ip 9 days before irradiation. (B) Erythropoietin (EPO). B6 female mice (groups of 5) were injected ip with 5 U/100 g body weight on days 0, 1, and 2 after irradiation. (C) Friend leukemia virus (FLV). BALB/c male mice (groups of 6) were infused with 500 FFU FLV along with BMC on the day of irradiation (700 cGy). (D) Growth hormone (GH). B6 female mice (groups of 5) were injected ip with 20 µg rhGH on days 0, 2, and 4 after irradiation. (E) Serum of mice undergoing bone marrow transplantation. B6 male mice (groups of 5) were injected ip with 100 to 200 µl serum from 'parallel' B6 mice (on control diet) irradiated and infused with 5×10^6 B6 BMC on days 0, 1, and 2 after irradiation. Treatments with cytoxan, EPO, FLV, rhGH, or serum from mice undergoing a BMT failed to affect the suppression of hematopoiesis by DHEA. *See Figure 1.

etin by inducing erythropoietin-independent erythropoiesis. When genetically susceptible $Fv2^{ss}$ mice are infected with Friend leukemia virus, the massive proliferation of erythropoietic cells becomes erythropoietin-independent (29). However, Friend leukemia virus had no effect on DHEA-induced suppression of hematopoiesis in $Fv2^{ss}$ BALB/c male mice (Fig. 5C; $P < 0.05$). These data suggest that the effect of DHEA is not mediated through decreased levels of erythropoietin or progenitor cell responses to erythropoietin.

Growth hormone (GH) levels are decreased after whole body irradiation in humans, and this decrease may result in the decreased likelihood of bone marrow engraftment (30). To examine whether DHEA treatment resulted in decreased hematopoietic proliferation through inhibition of GH production we administered recombinant human GH (rhGH) ip to B6 female mice. As indicated in Figure 5D, rhGH did not affect DHEA-induced suppression ($P < 0.05$).

It was still possible that an unidentified growth factor is present in control diet-fed, but lacking in DHEA-treated animals. To test this notion, serum samples from mice that received 800 cGy radiation and 5×10^6 BMC were used to test for the presence of a growth factor. DHEA-treated B6 male mice received injections of 100 to 200 μl of serum iv on days 0, 1, and 2 after BMT was performed. These animals exhibited the same decrease in splenic proliferation observed in DHEA-treated mice that received injections of vehicle, suggesting that there is no graft-enhancing factor in the serum of control graft recipients that can correct the defect caused by DHEA (Fig. 5E). However, it is possible that the timing, the amount of serum transfers, and the handling of serum transfers may be critical.

Another factor that plays an important role in bone marrow engraftment is interleukin-2 (IL-2). Bone-marrow-recipient–mice that also received IL-2 have a greater number of stem and hematopoietic precursor cells when compared with graft recipients not given IL-2 (31). We administered rhIL-2 ip (100 U/mouse/day) to B6 female mice (groups of 5) on days 0 to 5 after irradiation. IL-2 failed to affect the suppression of growth of BMC in DHEA-treated mice. The geometric mean (95% CL) % splenic [^{125}I]dU uptake values in control mice, DHEA-fed mice given no IL-2, and DHEA-fed mice given IL-2 were 0.84 (0.43–1.66), 0.02 (0.01–0.06), and 0.02 (0.01–0.07), respectively. The two groups of DHEA-fed mice with or without IL-2 were similar to each other ($P > 0.8$) and less than the group fed the control diet ($P < 0.05$).

Role of Glucose 6-Phosphate Dehydrogenase (G6PD). DHEA can inhibit the enzyme G6PD *in vitro* (32), but it is unclear whether this occurs to a great extent *in vivo* (13, 33, 34). Inhibition of G6PD would impair the synthesis of several key metabolic factors, including fatty acids, cholesterol and nucleosides. We therefore tested whether supplementation of fatty acids, in the form of Intralipid® injections ip, into B6D2F1 female mice could reverse the effect of DHEA (Fig. 6A). Inhibition of G6PD would also inhibit cholesterol synthesis by inhibiting mevalonate synthesis. Thus, supplementation of drinking water with mevalonate in B6D2F1 male mice may overcome this deficiency (Fig. 6B). Lastly, inhibition of G6PD would result in decreased cellular pools of nucleosides required for cell division. Hence we tested whether supplementation of drinking water with the deoxyribonucleosides 2′-deoxyadenosine, 2′-deoxycytidine hydrochloride, 2′-deoxyguanosine, thymidine and 2′-deoxyuridine at 4.2 μmol/ml could overcome this deficiency in B6 female mice (Fig. 6C). None of these procedures–supplementation of fatty acids, mevalonate or deoxyribonucleosides–had any effect on DHEA-induced suppression of BMC growth ($P < 0.05$). It is possible that all three supplements need to be given simultaneously. However, when this was attempted the results were inconclusive as the supplemented mice consumed less of the DHEA diet (data not shown). Thus, DHEA-induced inhibition of hematopoiesis cannot be ascribed to inhibition of G6PD activity.

Effects on Leukemia Cells. The effects of dietary DHEA on proliferation of transplanted BMC and of GR-3 NM leukemia cells were also tested. B6 female host mice (groups of 5) were placed on diets containing either no DHEA or 0.25% and 0.45% DHEA 2 weeks before irradiation and BMC transfer. Proliferation of BMC was inhibited ($P < 0.05$) in a dose-dependent manner by DHEA feeding (Fig. 7A). Dietary DHEA was started on the day of leukemia cell transfer into CBB6F1 male mice. DHEA inhibited splenomegaly ($P < 0.05$) detected on day 10 (Fig. 7B). Moreover, 0.45% dietary DHEA very significantly prolonged survival of mice ($P < 0.001$) infused with leukemia cells (Fig. 7C).

Roles of Dehydroepiandrosterone Metabolites. To determine whether DHEA itself or a metabolite mediated the observed inhibition of proliferation of normal and neoplastic hematopoietic cells, we supplemented AIN-76A diet with DHEA (0.45%), A-dione (0.45%), estradiol (0.1%), A-diol (0.45%), or testosterone (0.2%) (all w/w) and examined their effects on cell proliferation. DHEA, A-dione, and estradiol inhibited ($P < 0.05$) both normal B6D2F1 male BMC (Fig. 8A) and leukemia cell growth in male CBB6F1 hosts (Fig. 8B) whereas A-diol and testosterone did not significantly affect proliferation of normal BMC (Fig. 8A). A-diol had no effect on proliferation of leukemia cells, while 0.2% testosterone slightly inhibited leukemia cell growth (Fig. 8B). These findings suggest that DHEA could act by increasing the levels of A-dione and estradiol. However, the effect of estradiol, but not DHEA, on BMC and GR-3 NM cell proliferation was reversed ($P < 0.05$) by the estrogen receptor antagonist tamoxifen (Fig. 8C and 8D). In this experiment, the diets were started 2 days before cell transfer, while tamoxifen was injected from 5 days before until the day of cell transfer (day 0). The drug was then given every 2 days until the end of the experiment. Moreover, Cl-epi, which cannot be metabolized to sex ste-

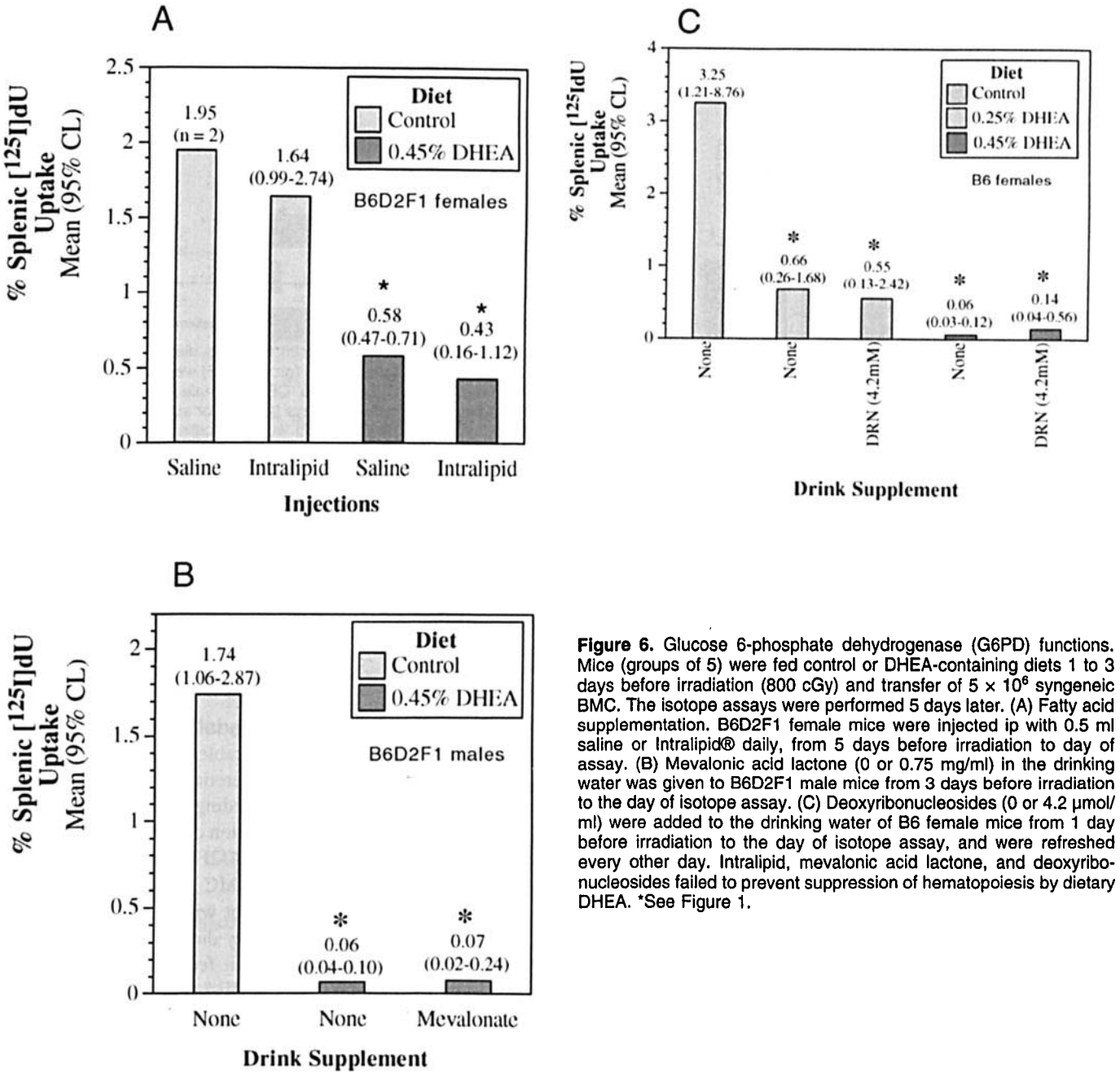

Figure 6. Glucose 6-phosphate dehydrogenase (G6PD) functions. Mice (groups of 5) were fed control or DHEA-containing diets 1 to 3 days before irradiation (800 cGy) and transfer of 5×10^6 syngeneic BMC. The isotope assays were performed 5 days later. (A) Fatty acid supplementation. B6D2F1 female mice were injected ip with 0.5 ml saline or Intralipid® daily, from 5 days before irradiation to day of assay. (B) Mevalonic acid lactone (0 or 0.75 mg/ml) in the drinking water was given to B6D2F1 male mice from 3 days before irradiation to the day of isotope assay. (C) Deoxyribonucleosides (0 or 4.2 μmol/ml) were added to the drinking water of B6 female mice from 1 day before irradiation to the day of isotope assay, and were refreshed every other day. Intralipid, mevalonic acid lactone, and deoxyribonucleosides failed to prevent suppression of hematopoiesis by dietary DHEA. *See Figure 1.

roids (35), also inhibited ($P < 0.05$) both normal BMC (Fig. 8E) and leukemia cell proliferation (Fig. 8F).

Role of Hypothermia. We recently reported that either dietary or injected DHEA induces hypothermia in mice (15). A drop in core body temperature lowers membrane fluidity among other things, and decreases in membrane fluidity can lower the rate of proliferation of normal and neoplastic cells (36). We raised the body temperature of DHEA-fed B6D2F1 male mice by housing them as well as mice on the control diet at a higher room temperature, 34°C, compared with normal housing temperature at 22°C. This housing temperature was empirically determined to bring body temperature back into the normal range of 37°C. The data in Figure 9A demonstrate the effect of the ambient temperature on body temperature. Mice housed at 22°C fed DHEA had a decrease in body temperature to 33.5°C within 3 days and less than 30°C by 8 days. DHEA-fed mice housed at 34°C maintained body temperatures indistinguishable from control mice housed at 22°C or 34°C. However, the increased body temperature of DHEA-fed mice housed at 34°C did not prevent the inhibition of BMC proliferation in spleens (Fig. 9B). This result suggests that hypothermia *per se* is not the mechanism of suppression of hematopoiesis by DHEA.

Role of Food Restriction. As described previously, food restriction plays a role in some of the actions of DHEA (4). To examine the role of food restriction in the antiproliferative action of DHEA, we first examined the effect of

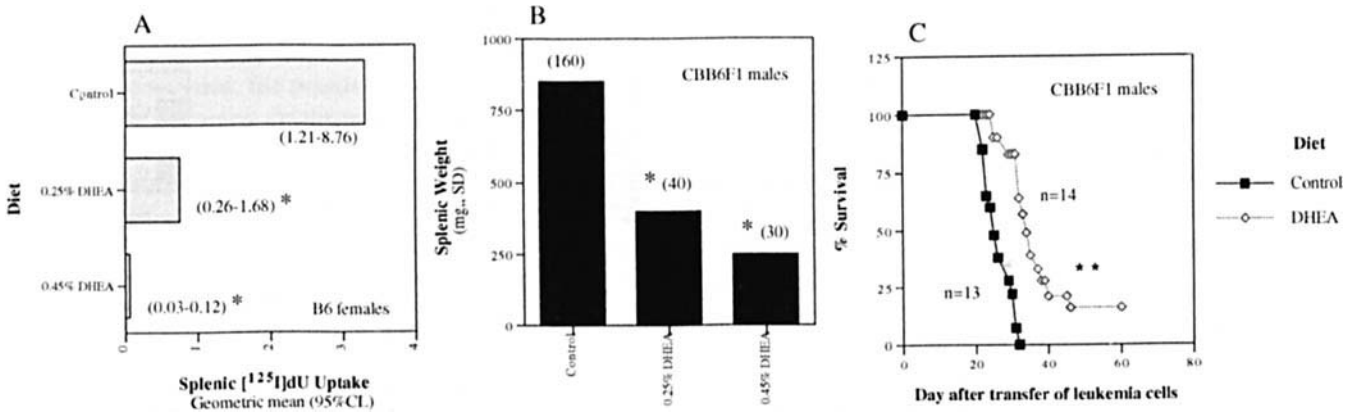

Figure 7. Inhibition of both normal and neoplastic hematopoiesis by dietary DHEA. Mice were fed 0, 0.25, or 0.45% dietary DHEA for 2 weeks before transfer of BMC or on the day of transfer of GR-3 NM leukemia cells. (A) BMT. B6 female mice (groups of 5) were irradiated (800 cGy) and infused with 5×10^6 B6 BMC. The isotope assay was performed 5 days later (B and C) Leukemia. CBB6F1 male mice were infused with 1×10^5 (B) or 2×10^4 (C) GR-3 NM cells. Spleen weights (groups of 4 mice) were determined 10 days later (B) or survival times (groups of 5 mice) were determined (C). Doses of 0.25% and 0.45% DHEA suppressed growth of BMC (A) and leukemia cells (B), and 0.45% DHEA prolonged survival of mice infused with leukemia cells (C). *See Figure 1. ** Significantly different from control groups, $P < 0.001$, by a generalized Wilcoxon comparison.

DHEA administration on food consumption. Over 8 weeks of treatment, DHEA-treated B6 mice consumed an average of 30% less food than control mice ($P < 0.05$). CBB6F1 and BALB/c mice were similarly affected (data not shown), while B6D2F1 mice were less affected (37). Even when intake was calculated per gram of body weight, DHEA-treated B6 mice consumed significantly less food than control mice. BALB/c and CBB6F1 mice also consumed less food, but B6D2F1 actually consumed more food per gram body weight than control mice (data not shown). However, B6D2F1 male mice used in the experiment presented in Figure 1B were susceptible to food restriction by DHEA after exposure to irradiation. The mean body weights of mice on the control diet were 23.4, 25.4, and 22.8 g at 0, 14, and 19 days after exposure to irradiation (includes 800 cGy at time day 14), while mean body weights of B6D2F1 mice fed DHEA were 24.3, 17.4, and 15.7 g at the same time points (5.8% vs 32.3% weight loss overall). This result suggests that irradiation abrogates the resistance of B6D2F1 mice to the food restriction induced by DHEA.

Due to the strain variability observed in the effect of DHEA on food intake, we examined the effect of DHEA on bone marrow proliferation in different strains of mice. When proliferation in B6, BALB/c, and CBB6F1 mice on control diet was standardized to 1.00 to compare across strains, DHEA inhibited significantly ($P < 0.05$) splenic proliferation after BMT in all three strains to 0.15–0.25 (data not shown). To study the role of decreased food intake in DHEA- induced inhibition of hematopoietic cell proliferation, we conducted pair-feeding experiments, where mice that were fed the control diet received the same amount of food per day as that consumed by mice on the 0.45% DHEA diet. Both 0.45% DHEA-treated and pair-fed CBB6F1 female mice exhibited significantly decreased splenomegaly 10 days after transfer of 1×10^5 leukemia cells ($P < 0.05$; Fig. 10A). Pair-feeding also prolonged survival of mice after transfer of 2×10^4 leukemia cells ($P < 0.001$; Fig. 10B). The prolongation of survival of pair-fed and DHEA-fed mice with leukemia was similar. CBB6F1 male mice pair-fed to DHEA-treated mice for 2 weeks before BMT showed the same inhibition of BMC proliferation ($P < 0.05$) as that observed in DHEA-treated mice (Fig. 10C).

Role of Adrenal or Gonadal Steroids. Limiting access to food results in considerable stress such that pair-fed mice may have increased secretion of adrenal corticosteroids. To test whether pair-feeding mimicked the effect of DHEA by increasing the secretion of adrenal steroids, we performed adrenalectomy of B6D2F1 female mice. Four days later (3 days before the BMC transplant), the mice received control AIN-76A with or without DHEA or were pair-fed to DHEA. Adrenalectomy did not reverse the inhibition of proliferation seen in pair-fed mice. Indeed, adrenalectomy of mice on 0.45% DHEA diet had significantly greater inhibition of splenic proliferation following BMT than sham-operated mice on the DHEA diet ($P < 0.05$; Fig. 10D). DHEA is produced mainly in the gonads of mice, so we also examined the effect of castration on pair-feeding induced inhibition of proliferation. Castrated B6D2F1 male mice on the DHEA diet (0.45%, w/w) and pair-fed mice demonstrated the same inhibition of proliferation of a syngeneic BMC transplant as that seen in sham-operated, DHEA-treated, or pair-fed mice ($P < 0.05$; Fig. 10E). These findings suggest that pair-feeding does not mimic the effect of DHEA by an increased secretion of adrenal or gonadal steroid hormones.

DISCUSSION

The observation that DHEA inhibits the splenomegaly associated with leukemia and that it prolongs the survival of leukemic mice (Figs. 7, 8, 10) is consistent with the anti-

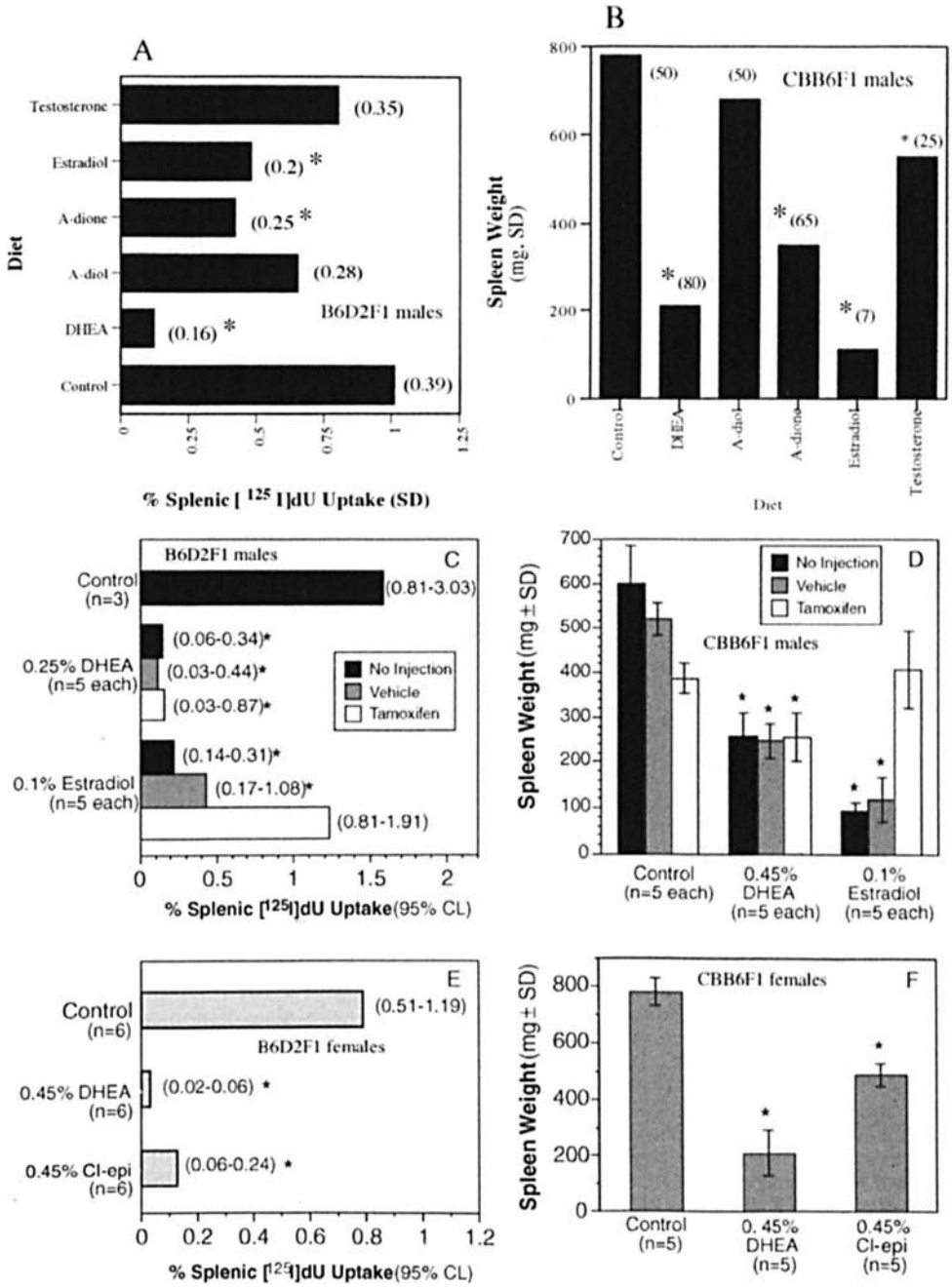

Figure 8. Effects of various steroids and tamoxifen on normal and leukemia cell proliferation. Diets containing steroids were given to B6D2F1 mice 2 days before BMC transplantation or to CBB6F1 mice on the day of GR-3 NM leukemia cell transfer. The isotope assays were performed 5 days after BMC transfer, and spleen weights (leukemia) were determined 10 days after GR-3 NM cell transfer. (A and B) DHEA downstream metabolites. AIN-76A diets containing no additive or 0.45% DHEA, A-diol, or A-dione, 0.1% estradiol, or 0.2% testosterone were fed to male B6D2F1 or female CBB6F1 female mice. DHEA, A-dione, and estradiol (but not A-diol) significantly suppressed both normal and leukemia cell proliferation. (C and D) Estradiol and tamoxifen. Mice were injected with 0 or 10 μg tamoxifen in safflower oil sc from 5 days before to the day of BMC or GR-3 NM cell transfer, and every 2 days afterward. Both DHEA and estradiol suppressed BMC growth in B6D2F1 male mice and leukemia cell growth in male CBB6F1 mice. Tamoxifen prevented suppression induced by estradiol but not DHEA. Thus, DHEA does not mediate hematopoiesis suppression by its conversion to estradiol. (E and F) 16α-Chloroepiandrosterone (Cl-epi). Diets containing 0 or 0.45% DHEA or Cl-epi were fed to mice. Both DHEA and Cl-epi suppressed BMC growth in B6D2F1 female mice and leukemia in CBB6F1 female mice. Cl-epi cannot be metabolized to sex steroids. *See Figure 1.

neoplastic role for DHEA suggested by other investigators (13, 33, 34, 38, 39). Certain DHEA metabolites can mimic this antiproliferative effect of DHEA, suggesting the possibility that DHEA may act through one of its downstream metabolites (e.g., estradiol) (Fig. 8A and 8B). However, the activity of DHEA, contrary to estradiol, is not affected by the estrogen receptor antagonist tamoxifen (Fig. 8C and 8D). Moreover, Cl-epi inhibits hematopoiesis (Fig. 8E and 8F). These findings indicate that the action of DHEA is independent of its conversion to sex steroids. The fact that

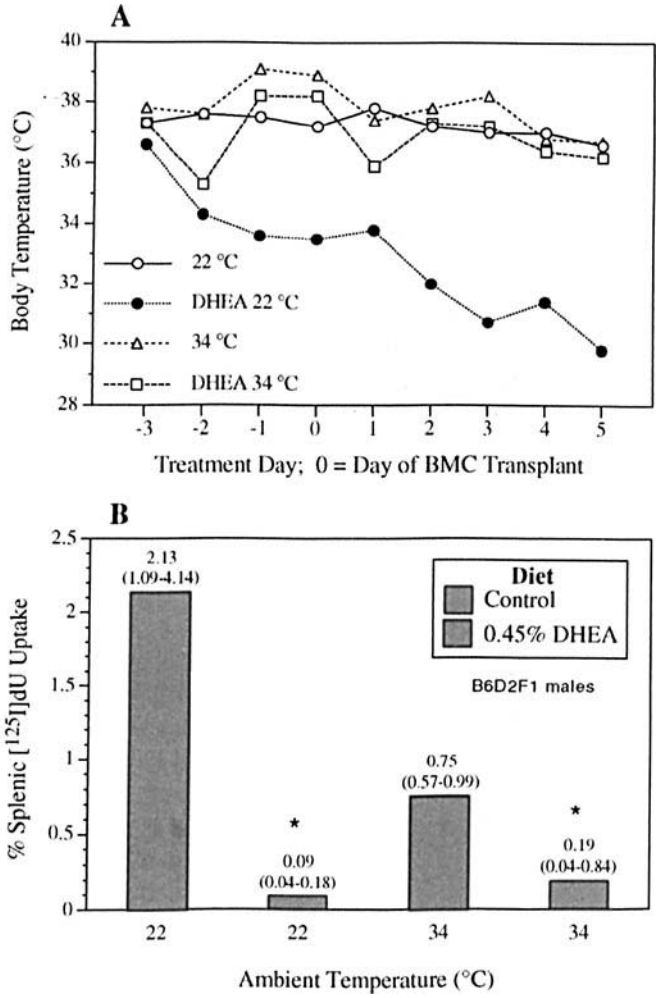

Figure 9. Hypothermia. B6D2F1 male mice (groups of 6) were placed on control or 0.45% DHEA-containing diets and housed at either 22°C or 34°C. Three days later (day 0) mice were exposed to 800 cGy and infused with 5×10^6 B6D2F1 BMC. Core body temperature was measured each day between 9 and 10 AM. The isotope assay was performed 5 days after BMC transfer. (A) Body temperature. DHEA rapidly caused hypothermia in mice housed at 22°C, but not at 34°C. (B) Hematopoiesis. In mice fed the control diet, proliferation of BMC decreased significantly at 34°C from 2.13 to 0.75 geometric mean % splenic uptake of [^{125}I]dU ($P < 0.05$). In mice fed DHEA, growth was inhibited at both 22°C and 34°C ($P < 0.05$), and was not significantly different from each other ($P > 0.05$). Thus, hematopoiesis was suppressed by DHEA even when core body temperature was maintained at or near 37°C. *See Figure 1.

estradiol has an antiproliferative effect in our models is not surprising, because estrogen inhibits hematopoiesis (40, 41). The potential mechanisms of estradiol inhibition of hematopoiesis include inhibition of IL-6 functions (42–44), inhibition of transcription factor GATA-2 function *via* the estradiol receptor (45), and induction of terminal differentiation of myelopoietic cells, which may involve vitamin D3, 17β-hydroxysteroid dehydrogenase, and/or the leukotrieine D3 receptor (46–48). Estradiol also inhibits B-cell lymphopoiesis (49–52) similar to DHEA (3, 4). The finding that mice treated with estradiol had deficiencies in lymphoid but not myeloid very early progenitor cells (53) suggests that estradiol acts directly on progenitor cells. DHEA also appears to act early in differentiation, but apparently on the 'hematopoietic microenvironment' of transferred early hematopoietic progenitor cells (Fig. 3C and 3D), but not on progenitors *per se* (Fig. 2B). To pursue these findings, it would be interesting to perform the long-term cultures of bone marrow in which one can manipulate the stroma (environment) and the marrow cells (stem cells) independently (54). Studies of estradiol benefit from the presence of known estradiol receptors. No steroid receptors are known to bind DHEA, although there are reports of intracellular DHEA "receptors" (55–57). Recent studies also suggest that DHEA binds to cell surface molecules of human T cells, human vascular smooth muscle cells, and bovine endothelial cells with high affinity (58–60), although the high-affinity binding molecules have not been characterized.

Whereas DHEA inhibits hematopoiesis and restoration of immune function after irradiation, its metabolites A-diol and especially androstene-3β,7β,17β-triol (A-triol) protect against these effects of radiation (61, 62) when injected sc. Macrophages convert DHEA into A-diol and A-triol efficiently, but convert DHEA into A-dione, testosterone, and estrogen less effectively (63). Presumably the site of metabolism of DHEA may determine some of its biological effects. Although DHEA inhibited secretion of proinflammatory cytokines (TNFα, IL-1) of stimulated murine macrophages and mitogenic responses of lymphocytes, A-triol augmented macrophage and lymphocyte responses (64, 65). A-triol also has strong anti-glucocorticoid effects (66). Therefore, it should be of interest to compare the effects of DHEA given orally versus subcutaneously on hematopoiesis.

We have also demonstrated that the antiproliferative effect of DHEA may be mediated through its effect on food intake (Fig. 10, Refs. 4, 37). Mice on a restricted diet are under considerable stress, and this may result in increased secretion of adrenal steroids such as corticosterone. If adrenal steroid levels are in fact increased, this could in turn result in inhibition of hematopoiesis. In fact, food restriction inhibits skin tumor promotion in the mouse, and this effect can be reversed by adrenalectomy (67). However, we found that adrenalectomy did not reverse the inhibition of hematopoiesis seen in either DHEA- or pair-fed animals (Fig. 10D), indicating that this effect is independent of adrenal function. In rodents, ACTH-stimulated adrenal gland cells do not appear to produce DHEA (68), although rat adrenal glands have the capacity to produce DHEA from precursor pregnenolone (69, 70). The primary sources of DHEA in rodents are the ovaries and testes. Surgical removal of the testes had no effect on the inhibition of hematopoiesis in pair-fed mice (Fig. 10E), indicating that food restriction does not exert its effect through increasing sex steroid secretion. Nevertheless, stress may still be involved, because many nonadrenal factors generated by stress can affect the hematopoietic and immune systems (71).

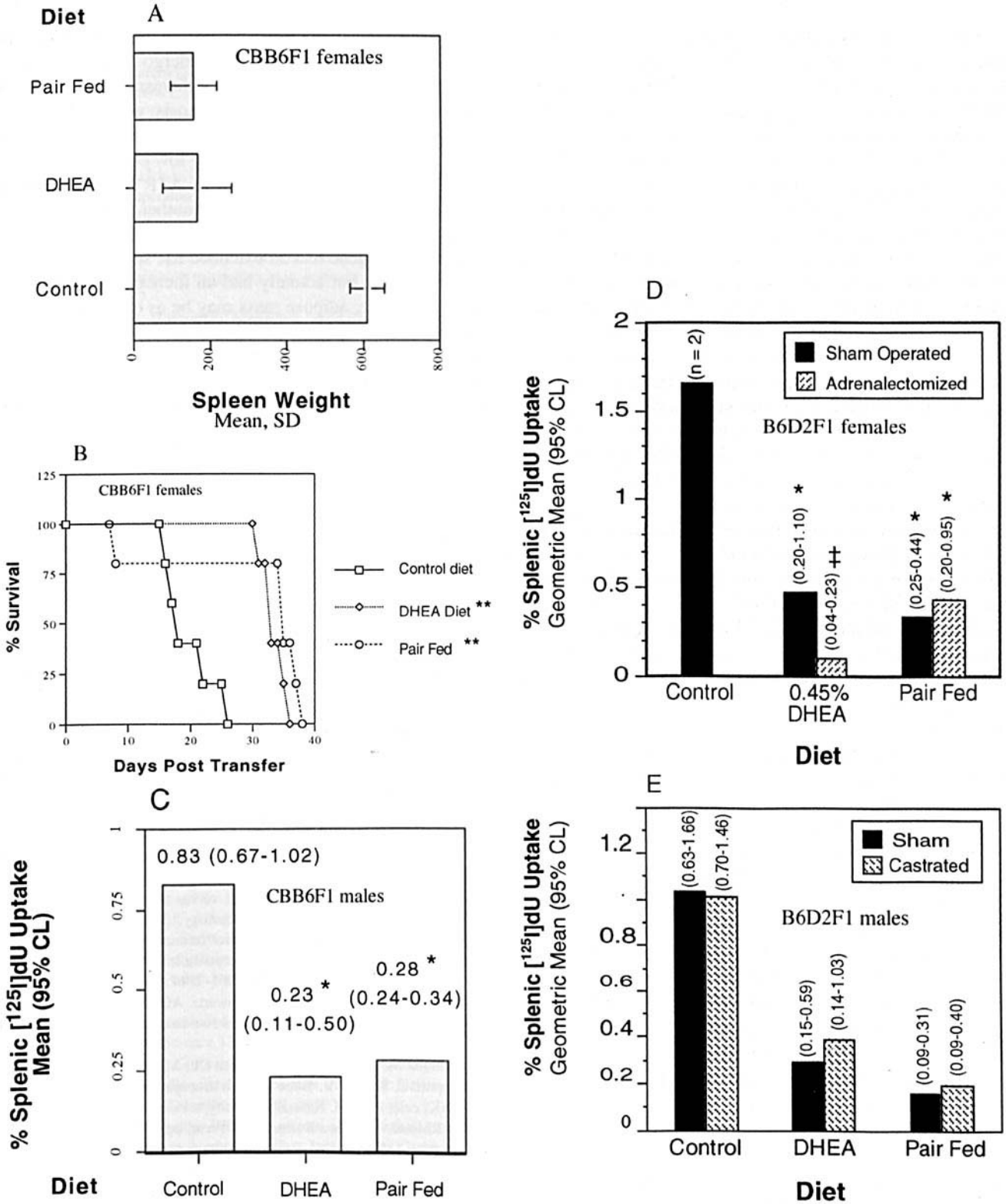

Figure 10. DHEA and pair feeding suppression of normal and neoplastic hematopoiesis: effects of adrenalectomy and orchiectomy. CBB6F1 male and B6D2F1 female or male mice were fed either the control AIN-76A diet or the 0.45% DHEA-containing diet *ad libitum* or were pair-fed the control diet based on food intake by mice fed the DHEA-containing diet on the previous day, beginning 3 days before BMC transplantation or on the first day of infusion of GR-3 NM cells. (A and B) Leukemia: DHEA and pair feeding were begun on the first day of infusion of 1×10^5 (A) or 2×10^4 (B) GR-3 NM leukemia cells in CBB6F1 mice. Spleen weights were determined 10 days later (A) or survival times were measured (B). The proliferation of GR-3 NM leukemia cells was decreased ($P < 0.05$) (A) and survival times were increased ($P < 0.001$) (B) in both DHEA and pair-fed mice. (C, D, and E) BMC proliferation: Growth of 5×10^6 CBB6F1 (C), or B6D2F1 (D, E) BMC in syngeneic mice exposed to 800 cGy. Isotope assays were performed 5 days later. Both DHEA and pair-fed mice demonstrated decreased BMC growth in spleens ($P < 0.05$) (C, D, E). Neither adrenalectomy (D) in females nor orchiectomy in males (E) prevented the effects of DHEA or pair feeding on hematopoiesis. Adrenalectomy significantly decreased growth in paired DHEA-fed mice. ‡$P < 0.05$. *See Figure 1. **See Figure 7

Our data (4, 37) and those of others (72, 73), demonstrate that dietary DHEA can inhibit food intake in mice. The ability of pair-feeding to mimic the effects of dietary DHEA on hematopoietic proliferation (Fig. 10) indicates that the observed action of DHEA can be accounted for by its effect on food intake. Food restriction has long been known to inhibit cell proliferation. The feeding state of animals affects proliferation of normal liver and hepatoma cells (74). It was proposed that food restriction inhibits cellular proliferation through suppression of DNA synthesis (74–76). Subsequent studies indicated that food restriction not only inhibits proliferation, but also eliminates preneoplastic cells through the induction of apoptosis (77). Others also have shown that the anticancer effect of food restriction may be due to modulation of the rates of apoptosis and proliferation (78). Because food restriction can induce apoptotic death of pre-neoplastic cells as well as developing hematopoietic cells, it is reasonable to propose that the hematopoiesis inhibition by DHEA is mediated, at least in part, through apoptosis (4).

There are several possible mechanisms through which DHEA might cause decreased food intake. Diet containing DHEA may be less palatable, and thus less is eaten. However, we have determined that equivalent amounts of DHEA given intraperitoneally inhibits 24-hr food intake in mice in a dose-dependent manner (15). Thus the decreased food intake with DHEA administration is independent of food palatability. Because feeding behavior is regulated in large part by neurons in the arcuate nucleus region of the hypothalamus near the third ventricle of the brain, it is possible that dietary DHEA affects food intake through alterations of hypothalamic neurotransmitter levels. In support of this hypothesis, DHEA treatment of obese Zucker rats results in both decreased food intake and increased concentrations of serotonin in the hypothalamus (79). Moreover, dietary DHEA alters neurotransmitter levels in the mouse brain (37). The differential effects of tamoxifen on DHEA- and estradiol-induced inhibition of hematopoiesis suggest that the two exert their effects through different mechanisms (Fig. 8). Administration of estrogen inhibits food intake, suggesting that both DHEA and estradiol may inhibit hematopoiesis by inducing food restriction. However, estradiol acts through the estrogen receptor, while DHEA does not. The effect of estradiol on food intake has been linked to regulation of hypothalamic neuropeptide-Y (80). The effects of DHEA on neuropeptide-Y levels as well as other appetite regulators (81) remain to be determined.

In conclusion, administration of dietary DHEA to mice greatly inhibits the proliferation of normal and neoplastic hematopoietic cells. DHEA induces mice to consume less food, resulting in caloric restriction, and this inhibition of food intake appears to mediate the observed antiproliferative effect. We suggest that administration of DHEA causes decreased food intake perhaps by affecting hypothalamic regulation of appetite (37, 79) and that food restriction resulting from reduced food intake inhibits BMC proliferation. Both food restriction and DHEA inhibit lymphocyte proliferation and induce cells to undergo apoptosis (4).

Potential insights into the mechanisms of DHEA and food-restriction induction of apoptosis were provided by observations that DHEA induced apoptosis of a cell line *in vitro*. The effects were enhanced by low concentrations of a nutrient, glucose, and depletion of ATP seemed to be involved in this process (82, 83). Another potential mechanism was reported, in that mice lacking the fat-specific insulin receptor gene had an extended life span and decreased adipose tissue, but actually had an increased caloric intake (84). Therefore, adipose mass may be as or more important than decreased caloric intake. The data presented here reinforce the potential value of food restriction in conditions such as cancer where antiproliferative effects are desirable. DHEA administration presents a potential method for mimicking the beneficial effects of food restriction, which may otherwise be difficult to accomplish in the human species accustomed to *ad libitum* food consumption.

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