MOL
PHARM

Hematotoxicity of the Chinese Herbal Medicine *Tripterygium wilfordii* Hook f in CD34-Positive Human Bone Marrow Cells

DAVID W. PYATT, YANZHU YANG, BRENDA MEHOS, ANH LE, WAYNE STILLMAN, and RICHARD D. IRONS

Molecular Toxicology and Environmental Health Sciences (D.W.P., Y.Y., A.L., W.S., R.D.I.), School of Pharmacy (B.M.); Department of Pathology, School of Medicine (R.D.I.); and Cancer Center (R.D.I.), University of Colorado Health Sciences Center, Denver, Colorado

This paper is available online at http://www.molpharm.org

MOLECULAR PHARMACOLOGY

ABSTRACT

T2, a chloroform/methanol extract of the herb *Tripterygium wilfordii* Hook f, has been used in China for the treatment of autoimmune and inflammatory diseases for many years. Recent experimental evidence has confirmed that T2 has potent anti-inflammatory and immunosuppressive activity, and a United States Food and Drug Administration-approved clinical trial is currently exploring the efficacy of T2 in the treatment of rheumatoid arthritis. Despite the potential therapeutic benefits of T2, there is ample documentation that T2 is toxic, targeting, among other things, the hematopoietic system, and its use has resulted in cases of leukopenia, thrombocytopenia, and aplastic anemia. This investigation was undertaken to characterize the in vitro effects of T2 on primary human CD34-positive (CD34+) bone marrow cells. Our results demonstrate that T2 has a potent inhibitory effect on the clonogenic response of human bone marrow cells to exogenously added hematopoietic growth factors. The inhibition of colony formation by T2 is not the result of direct cytotoxicity or increased apoptosis and indicates a functional suppression of hematopoiesis. Additional experiments demonstrate that T2 also alters transcriptional regulation in bone marrow cells by inhibiting nuclear factor-κB. This transcription factor is found in CD34+ bone marrow cells and has been recently shown to be a requirement for colony formation. These results demonstrate that therapeutic concentrations of T2 exert a significant hematotoxic effect by inhibiting growth factor response in CD34+ bone marrow cells and suggest that inhibition of nuclear factor-κB may play a role in the blood dyscrasias encountered with the use of this drug.

Tripterygium wilfordii Hook f (TWH) is a vine-like plant indigenous to large regions in southern China. The Chinese have used crude preparations and extracts of TWH as medicinal herbs for more than 2000 years. Although much of the clinical experience with this herb comes from uncontrolled studies and anecdotal reports, recent clinical trials have confirmed the efficacy of TWH in the treatment of rheumatoid arthritis and other autoimmune diseases (Tao et al., 1989; Liu and Wu, 1996). These studies have sparked renewed interest in the therapeutic utility of this plant, and TWH is an increasingly popular herb-derived remedy for many inflammatory and autoimmune conditions (Asano et al., 1998). Although the actual root of the plant has been used, the administration of various extracts, including a chloroform/methanol extract known as T2, is far more common (Tao et al., 1989; Li and Weir, 1990). T2 is commercially available from Taizhou Pharmaceutical Company in China and was used throughout the studies reported herein.

Although many published reports have demonstrated that T2 (as well as other extracts) is efficacious in the treatment of rheumatoid arthritis (RA), its precise mechanism of action is unknown. It has long been appreciated that T2 has anti-inflammatory activity, and recent reports indicate that this drug inhibits the induction of cyclooxygenase 2, thereby decreasing prostaglandin production (Tao et al., 1998). T2 is also clearly immunosuppressive, both in vitro and in vivo, as evidenced by its ability to inhibit T- and B-lymphocyte mitogen responses, Ig production, bacterial phagocytosis, and the production of several cytokines important in mediating the immune response [interleukins (IL) -1, -2, -4, -6, -8, and tumor necrosis factor-α (TNF-α) (Li and Weir, 1990; Tao et al., 1996; Chang et al., 1997)]. It is likely that these activities account for some, if not all, of the effectiveness of T2 in the treatment of rheumatoid diseases and probably other autoimmune diseases as well (Lipsky and Tao, 1997).

Despite the potential usefulness of T2 in the clinic, there is little doubt that this drug is toxic; multiple adverse side effects have been reported in all patient populations taking T2. These include GI disturbances, amenorrhea, kidney dys-

ABBREVIATIONS: TWH, *Tripterygium wilfordii* Hook f; RA, rheumatoid arthritis; IL, interleukin; TNF, tumor necrosis factor; CFU, colony forming unit; GM-CSF, granulocyte-macrophage-colony stimulating factor; SCF, stem cell factor; EPO, erythropoietin; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T cells; CsA, cyclosporine A; HPC, hematopoietic progenitor cells; GEMM, granulocyte, erythroid, macrophage, megakaryocytic.

function, leukopenia, thrombocytopenia, and aplastic anemia (Tao et al., 1989; Lipsky and Tao, 1997). Often, the side effects are transient, and recovery is usually complete upon removal of the drug. However, the potential seriousness of these conditions has prompted Chinese physicians to routinely monitor hematotoxicity in patients taking T2 for any length of time. The purpose of this study was to determine whether the hematotoxicity associated with T2 administration is more likely to be idiosyncratic or if a predictable, dose-related consequence of treatment with the drug. Our results demonstrate that treatment of CD34+ bone marrow cells with extremely low concentrations of T2 (nanograms per milliliter) results in a significant suppression of colony formation and that bone marrow suppression is the most likely dose-limiting consequence of T2 administration. Because this drug can be self-prescribed as an herbal remedy (T2 is available directly via the Internet), it is likely to be used in the absence of appropriate medical supervision. Based on the results presented herein, we believe that the potential for serious hematotoxicity is a significant health risk to anyone taking this drug.

Materials and Methods

Participants. Human bone marrow and peripheral blood was obtained from healthy human volunteers by aspiration from the posterior iliac crest and venipuncture, respectively. All protocols were approved by the University of Colorado Health Sciences Center Internal Review Board and samples were taken with informed consent.

Cell Purification. Mononuclear cells were isolated from blood and bone marrow using Histopaque-1077 (Sigma, St. Louis) and purification of individual cell subpopulations was achieved using a high magnetic gradient MiniMACS purification system (Miltenyi Biotec, Auburn, CA). CD34+ bone marrow cells devoid of B cells (CD34+CD19−) were obtained using the pan B cell antigen CD19. CD19+ cells were removed and detected by using a fluorescein isothiocyanate conjugated anti-CD19 monoclonal antibody followed by antifluorescein isothiocyanate microbeads. CD34+ CD19- cells were then obtained by using the CD34 isolation kit following manufactures instructions (Miltenyi Biotec). The purity of CD34+ bone marrow cells (>95%) was determined by flow cytometric analysis (Epics 752; Coulter Electronics, Miami Lake, FL) using anti-CD34 monoclonal antibody (HPCA-2; PharMingen, San Diego, CA) specific for a CD34 epitope distinct from that used in the purification process (QBEND-10, Miltenyi Biotec).

Treatment Solutions. T2, the chloroform/methanol extract of TWH, was obtained from Taizhou Pharmaceutical Company (Jiangsu, China). A 2 mg/ml stock solution was prepared by dissolving the tablets in 70% ethanol and filtering. Final concentrations used in these experiments were obtained by diluting the stock solution in RPMI 1640 medium. Cyclosporine (Sigma, St. Louis, MO) was prepared as a stock solution (5 mg/ml) in PBS and diluted in RPMI medium as appropriate.

Colony Forming Assays. Colony-forming unit (CFU) assays were performed as described previously (Irons et al., 1995) Briefly, CD34+ bone marrow cells were plated in 35-mm culture dishes at a concentration of 1 to 5×10^3 cells/ml in 1 ml of modified Iscove's medium containing 20% fetal bovine serum, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1.0% (w/v) methyl cellulose, and 5 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), 50 ng/ml stem cell factor (SCF), 5 ng/ml interleukin 3 (IL-3), and 5 U/ml erythropoietin (EPO). Each cytokine was used at concentrations determined experimentally to produce maximal colony formation. Cytokines and drugs were added to the methyl cellulose media before the addition of cells,

ensuring equal exposure of drugs and cytokines between treatment groups and control groups. Higher concentrations of T2 were dissolved in ethanol; therefore, a control group using an equivalent amount of ethanol was used in addition to the normal media controls. Ethanol alone had no effect of colony formation (data not shown). All cultures were maintained at 37°C in 5% CO_2 and scored on day 14 of culture. Colony identification was based on color and morphology according to standardized histological criteria. Four plates were scored for each treatment group and results expressed as the mean ± 1 S.E.M. Where applicable, significant differences ($P \leq .05$) between treated and control groups were determined using the Student's t test (Excel 4.0; Microsoft Corp., Redmond, WA).

Electrophoretic Mobility Shift Assays (EMSA). Nuclear protein was extracted using a modified Dignam protocol (Dignam et al., 1983) from 250,000 to 1 million cells per treatment group. Protein concentrations were determined using a bicinchoninic acid protein kit (Pierce, Rockford, IL) and the nuclear extracts were frozen at −80°C until used. NF-κB and activator protein-1 (AP-1) probes were made and labeled as described previously (Pyatt et al., 1996). For supershift samples, cellular extracts were preincubated with appropriate specific antibodies for 1 h at 4°C. Antihuman p50 and p65 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear protein (2–8 μg) was incubated on ice for 10 min with 1 μg of dI-dC, 4 μl of binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 10% glycerol, 0.05 mM EDTA, 1.6 mM $MgCl_2$, 1 mM dithiothreitol, and deionized water) for a total volume of 19 μl. One microliter of ^{32}P-labeled probe (~50,000 cpm) was added and the binding reaction continued for 30 min at 22°C. After complex formation, 2 μg of loading buffer (250 mM Tris · HCl, pH 7.5, 0.2% bromphenol blue, 0.2% xylene cyanol, and 40% glycerol) was added to the DNA-protein complexes and the sample was analyzed by electrophoresis on a prerinsed 6% polyacrylamide gel. The gels were dried and exposed at −80°C to Fuji X-ray film (Fuji, Tokyo, Japan) for various time points indicated in figure legends.

Competition experiments were conducted to help establish the specificity of NF-κB binding. These experiments were carried out by adding competing (NF-κB) or noncompeting [AP-1, nuclear factor of activated T cells (NFAT), mutated NF-κB] cold probes in approximately 100-fold excess, to nuclear extracts from human CD34+ cells in the EMSA.

Results

Colony-Forming Responses Are Inhibited by T2. The effects of T2 on clonogenic response in human CD34+ bone marrow cells is shown in Fig. 1. Treatment with T2 over a concentration range of 1 to 10,000 ng/ml resulted in a concentration-dependent inhibition of colony formation, with total inhibition (100%) occurring at concentrations of 500 ng/ml and higher. Suppression occurred in the presence of hematopoietic growth factors (GM-CSF, IL-3, SCF, and EPO) and was observed in myeloid, erythroid, and mixed-lineage colonies with equal potency. The concentration of T2 resulting in complete abrogation of CFU response (500 ng/ml) is approximately one-half (or lower) of that previously demonstrated to be effective in suppressing lymphocyte functions in vitro (Tao et al., 1996, 1998). Remarkably, concentrations of T2 that completely suppressed CFU activity did not result in an observable loss in cell viability. Because T2 has immunosuppressive activity, we compared the effects of T2 with cyclosporine A (CsA), a widely used clinical immunosuppressant. In contrast to T2, treatment with concentrations of CsA demonstrated previously to totally inhibit T lymphocyte activation produced no suppression of clonogenic response in CD34+ bone marrow cells (Fig. 2) (Halloran and Madrenas, 1991; Suthanthiran et al., 1996).

T2 Does Not Induce Apoptosis in CD34+ Bone Marrow Cells. Further experiments were conducted to determine whether T2 exposure increased apoptotic cell death in CD34+ bone marrow cells. Cells were treated with titered concentrations of T2, and apoptosis was measured at 24 and 48 h after treatment by the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling assay (i.e., TUNEL) As shown in Fig. 3, there is no evidence of increased apoptosis at either time point, even at T2 concentrations as high as 1000 ng/ml. This concentration of T2 completely inhibits colony formation, and the lack of apoptosis suggests that colony suppression by T2 is not simply the result of increased cell death (at least within the first 48 h). Figure 3 also illustrates the level of T2 resulting in frank cytotoxicity, as 10,000 ng/ml of T2 induced as much apoptotic cell death as the positive control, etoposide.

Pretreatment with T2 for 24 h Does Not Inhibit CFU. Experiments were performed to determine the kinetics involved in the inhibition of colony formation by T2. Cells were treated with T2 for 24 h, washed free of the drug, and resuspended in fresh media and plated in CFU culture media. These cells were virtually 100% viable (even at the highest concentration of T2 used) before plating and established normal clonogenic response to growth factors (Fig. 4). In contrast, cells from the same bone marrow, added directly to

CFU media already containing equivalent concentrations of T2, were suppressed to the same degree previously shown (Fig. 1). These results indicate that short exposure to T2 is not sufficient to suppress the clonogenic response but the drug must be present at a later time point to exert this effect.

Cytokine Induced NF-κB Nuclear Localization Is Inhibited by T2. Previous studies in this laboratory have reported that NF-κB is required for colony formation in CD34 bone marrow cells (Pyatt et al., 1999). Studies were therefore conducted to investigate the possibility that T2 may be inhibiting CD34+ cells at a transcriptional level. It has been consistently demonstrated that NF-κB is a heterodimer composed of two members of the Rel family of mammalian transcription factors, p50 and p65 (for reviews, see Baeuerle and Baltimore, 1996; Baeuerle and Henkel, 1994). The first step was to establish the presence of NF-κB in cytokine-treated human CD34+ cells. As can be seen in Fig. 5A, competition experiments confirmed specificity for NF-κB binding to its consensus sequence. Nonradiolabeled NF-κB probe, but not nonradiolabeled probe for AP-1 or NFAT, was able to totally compete off NF-κB from the radio labeled probe. Additional experiments using an NF-κB probe with a single point mutation was partially inhibitory. The fact that protein binding was slightly decreased with the addition of a mutated NF-κB
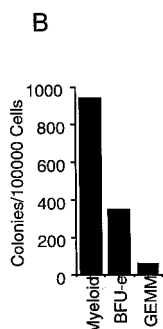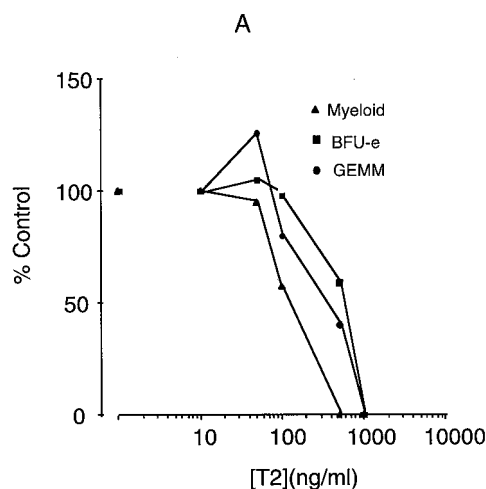
Fig. 1. T2 inhibits colony formation in treated CD34+ bone marrow cells. Human CD34+ bone marrow were purified (>96% pure) and cultured in methyl cellulose medium containing various cytokines (IL-3, GM-CSF, EPO, and SCF) and titered doses of T2. Myeloid, erythroid, and GEMM colonies were scored. Colonies numbers were expressed as percentage of control and one representative experiment is shown in A. The total number of colonies per 100,000 cells plated is shown in B.
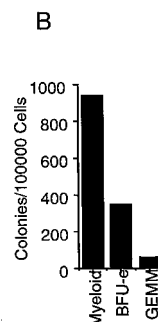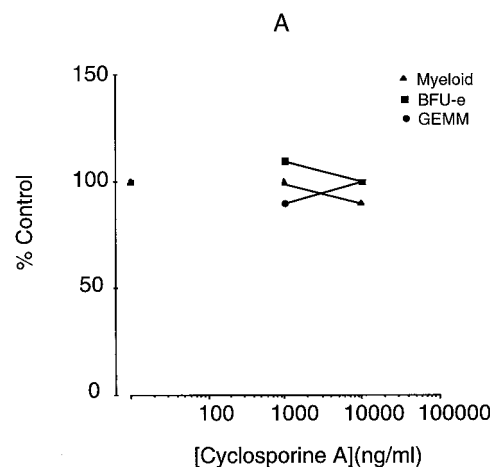

Fig. 2. CsA has no effect on colony formation in CD34+ bone marrow cells. Cells were purified and cultured in methylcellulose medium containing various cytokines (IL-3, GM-CSF, EPO, and SCF) and titered doses of CsA. Myeloid, erythroid, and GEMM colonies were scored. Colony numbers were expressed as percentage of control and one representative experiment is shown in A. The total number of colonies per 100,000 cells plated is shown in B.

probe suggests the possibility of nonspecific binding; however, the mutated probe was only one base different than the radiolabeled probe, which may not have been sufficient to completely prevent NF-κB from binding. Supershift EMSA using antibodies specific for the Rel family members demonstrated the presence of p50 and p65 by the appearance of shifted bands or a loss in binding (Fig. 5B). The remaining protein left bound after the addition of anti-p65 antibody is probabaly a p50:p50 homodimer, because it was shifted with p50. Taken collectively, these data demonstrate specificity for binding to the NF-κB consensus sequence and positively identify the presence of p65 and p50 in the bound protein complex.

A comparison of the effects of T2 and CsA on NF-κB in human CD34+ bone marrow cells is shown in Fig. 6A. Treatment with T2 resulted in suppression of NF-κB activation by growth factors at concentrations similar to those resulting in the inhibition of CFU response, whereas CsA failed to inhibit activation of NF-κB in these cells at concentrations known to be immunosuppressive. Surprisingly, opposite results were obtained from comparable experiments conducted in T cells. In CD4+ T cells, T2 had no effect on NF-κB activation, whereas CsA was inhibitory (data not shown). This apparent inconsistency strongly suggests that signaling pathways governing NF-κB in CD4+ lymphocytes and CD34+ bone marrow cells are different, and that susceptibility to NF-κB inhibition by T2 and/or CsA is cell-specific. These data further suggest that the differential effect of T2 and CsA on NF-κB activity may explain in part the observed differences in CFU inhibition between these two drugs (Figs. 1 and 2).

T2 Does Not Inhibit NF-κB by Directly Altering the Protein. One trivial possibility to explain the inhibition of NF-κB by T2 is that the drug directly alters the protein itself, thereby influencing its ability to bind to the DNA probe. Therefore, we conducted experiments to investigate this possibility. Nuclear extracts from growth factor (GM-CSF, IL-3, SCF, and EPO)-stimulated hematopoietic progenitor cells (HPC) were incubated for 1 h with 1 mg/ml concentrations of T2 before running the EMSA gel. As shown in Fig. 6B, direct treatment with a concentration of T2 1000 times higher that those used in earlier experiments had no effect on the ability of this protein to bind to the DNA probe. These results suggest that NF-κB inhibition by T2 is not caused by a direct alteration of the protein itself; rather, T2 seems to inhibit the translocation or activation of this transcription factor in viable CD34+ HPC.

Discussion

T2, an extract of TWH, is currently under formal evaluation in the United States for the treatment of RA; however, neither the precise mechanism of action or the full range of toxicity associated with this traditional Chinese herbal drug is known. Both experimental and clinical evidence indicate that T2 exerts potent immunosuppressive and anti-inflammatory effects that are believed to play a role in its clinical effectiveness (Asano et al., 1998). The toxicity of T2 (and other extracts) is also well documented and has been re-
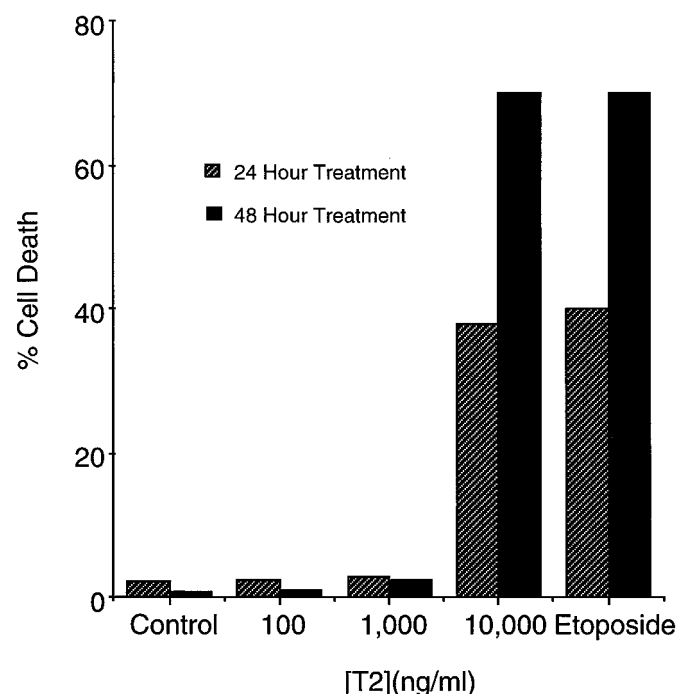

Fig. 3. Treatment with T2 does not induce apoptosis in human CD34+ human bone marrow cells. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed on human CD34+ bone marrow cells (>98% pure) after a 24- and 48-h incubation with titered doses of T2. The control cells were incubated with buffer only or 50 μM etoposide. One representative experiment is shown.
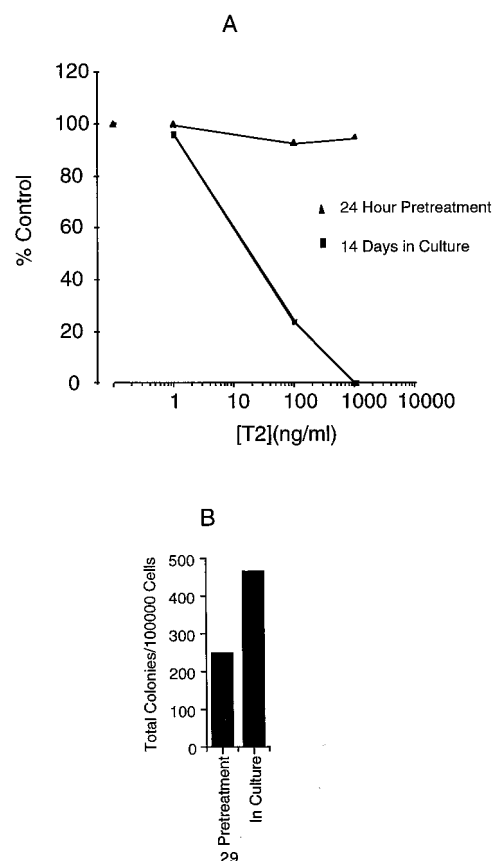

Fig. 4. Pretreatment with T2 has no effect on colony formation in treated CD34+ bone marrow cells. Human CD34+ bone marrow were purified (>96% pure) and cultured in methylcellulose medium containing various cytokines (IL-3, GM-CSF, EPO, and SCF) and treated at different time points with titered doses of T2. Total colonies were scored and expressed as percentage of control and one representative experiment is shown. The total number of colonies per 100,000 cells plated is shown in B.

ported for as long as the plant has been in use (more than 2000 years). Of particular relevance to these studies are the consistent reports of hematotoxicity after treatment with the drug. Treatment with T2 has resulted in clinically significant cases of thrombocytopenia, leukopenia, and aplastic anemia (Tany and Ersenbrand, 1992), indicating that the hematopoietic system is a sensitive target for toxicity. We undertook this investigation to characterize the effects of T2 on cytokine dependent clonogenic proliferation and transcriptional regulation of CD34+ human bone marrow cells. CD34 is an antigen found on the surface of all human HPC with colony-forming potential, and CD34+ cells are critical to normal hematopoiesis as well as long-term engraftment of the bone marrow after transplantation. The assay we employed to test the effects of T2 on CD34+ human bone marrow cells is the CFU assay. In this assay, HPC are stimulated with exogenously added growth factors that induce clonogenic proliferation in the responding cells, resulting in morphologically recognizable colonies of cells. CFU assays are routinely used to monitor the bone marrow function in patients receiving chemotherapy and are used to determine the repopulating potential of bone marrow grafts used in bone marrow transplantation.

Our results clearly demonstrate that T2 suppresses the clonogenic response of primary human HPCs at concentrations ranging from 5 to 500 ng/ml. These levels of T2 have been demonstrated previously to result in immunomodulation of both T and B lymphocytes and are likely to be achieved in patients treated with T2 (Tao et al., 1991, 1996, 1998). Dose-dependent suppression of CFU was observed in cells stimulated with GM-CSF, IL-3, SCF, and EPO. This combination of growth factors was chosen because it supports multilineage differentiation. T2 clearly inhibited myeloid, burst-forming unit-erythroid, and multilineage [i.e., granulocyte, erythroid, macrophage, megakaryocytic (GEMM)] colony formation in a relatively nonspecific manner. Most of the colonies formed in this type of assay are committed progenitor cells, although some cells (e.g., GEMM) are considered to be early progenitor cells that have multilineage potential.
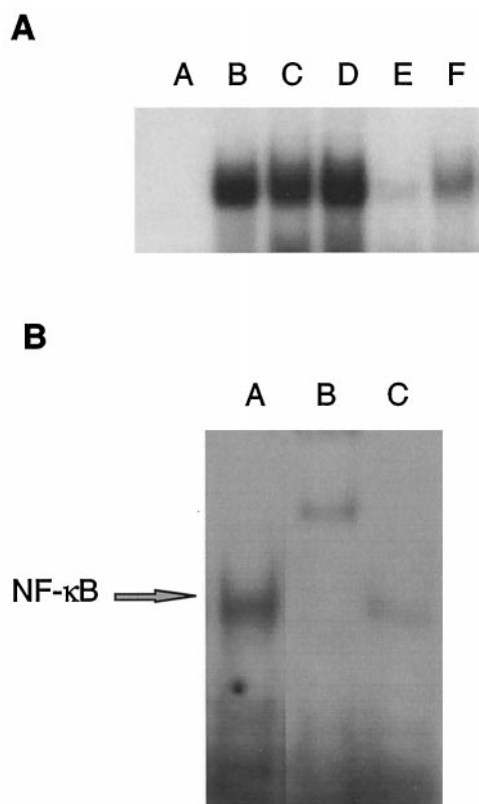

Fig. 5. A, competition experiments were conducted to confirm specificity of NF-κB binding: Lane A, negative control (probe with no nuclear extract added); lane B, nuclear extracts from cells treated with IL-3, GM-CSF, EPO, and SCF and incubated for 24 h in RPMI complete medium; lane C, nuclear extracts from group B with unlabeled AP-1 probe; lane D, nuclear extracts from group B with unlabeled NFAT probe; lane E, nuclear extracts from group B with unlabeled NF-κB probe; lane F, nuclear extracts from group B with cold mutated NF-κB probe. B, supershift EMSA experiments were conducted to identify discreet Rel family members binding to the NF-κB consensus sequence with antibodies specific for human p50 and p65. Lane A, nuclear extracts from cells treated with IL-3, GM-CSF, EPO, and SCF and incubated for 24 h in RPMI complete media; lane B, nuclear extracts from group A with anti-p50 antibody added; lane C, nuclear extracts from group A with anti-p65 antibody added. One representative experiment is shown.
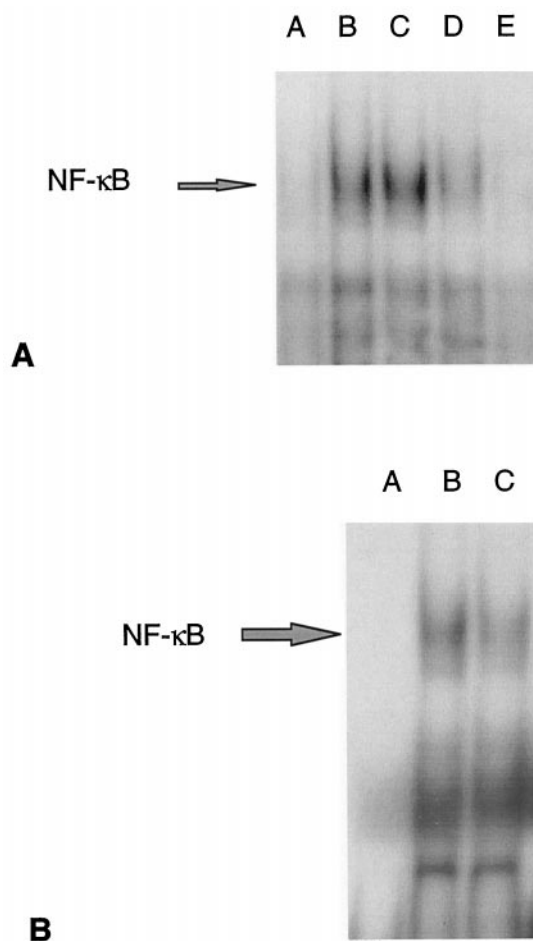

Fig. 6. A, T2 but not CsA inhibits cytokine activation of NF-κB in CD34+ human bone marrow cells. Lane A, media control; lane B, positive control; cells were treated with IL-3, GM-CSF, EPO, and SCF and incubated for 24 h in RPMI complete medium; lane C, cells treated with IL-3, GM-CSF, EPO, and SCF and 10,000 ng/ml CsA for 24 h in RPMI complete medium; lane D, cells treated with IL-3, GM-CSF, EPO, SCF, and 1000 ng/ml T2 for 24 h in RPMI complete medium; lane E, cells treated with IL-3, GM-CSF, EPO, SCF, and 10,000 ng/ml T2 for 24 h in RPMI complete medium. B, direct effect of T2 on ability of NF-κB to bind to DNA. Nuclear extracts from IL-3, GM-CSF, EPO, and SCF treated bone marrow cells were split into two groups and treated with PBS (lane B) or 10,000 ng/ml μM T2 (lane C) for 1 h before running the gel. Lane A is the probe with no protein added. One representative experiment is shown.

Surprisingly, T2 does not seem to be directly cytotoxic to bone marrow cells even at the highest dose tested, because no decrease in cell viability or evidence of increased apoptosis was observed (Fig. 3). This data demonstrates that T2 directly blocks the ability of very early multilineage as well as lineage-specific committed HPC to respond to growth factors and form colonies. This hematopoietic suppression may manifest itself in a loss of hematopoietic regenerative potential in patients receiving T2 and could result in aplastic anemia.

The mechanism underlying T2's profound inhibition of bone marrow cell function is not currently known. Recent studies from our laboratory have shown that NF-κB, a transcription factor originally isolated in T cells, is required for normal clonogenic response in CD34+ bone marrow cells (Pyatt et al., 1999). We therefore tested the hypothesis that T2 might be acting at the molecular level and blocking the activation of NF-κB in these cells. Consistent with this hypothesis, T2 inhibited NF-κB in CD34+ HPCs (Fig. 6A). The precise role of NF-κB in bone marrow cell function is not clear, although NF-κB is required for the expression of several gene products relevant to hematopoiesis, including IL-1B (Hiscott et al., 1993), TNF-α (Ziegler-Heitbrock et al., 1993), IL-6 (Muñoz et al., 1996), macrophage CSF (Peng et al., 1995), GM-CSF (Musso et al., 1996), granulocyte-CSF (Dunn et al., 1994), EPO (Lee-Huang et al., 1993), interferon-γ (Young, 1996), c-myc (Lee et al., 1995) and c-myb (Toth et al., 1995). In addition, NF-κB is activated by cytokines known to regulate hematopoiesis, such as TNF-α (Lowenthal et al., 1989), IL-1α and IL-1β (McKean et al., 1995), interferon-γ (Brown et al., 1997), macrophage-CSF (Oster et al., 1992), GM-CSF (Oster et al., 1992), transforming growth factor-β1 (Hong et al., 1997), and IL-3 (Besançon et al., 1998). The role of NF-κB as a secondary messenger for cytokine response as well as the requirement for NF-κB in colony formation suggest that it is a critical regulatory factor in human hematopoiesis. We believe the inhibition of NF-κB by T2 in CD34+ cells plays a role in the bone marrow suppression reported herein and may be involved in the blood dyscrasias seen after T2 treatment.

T2 shares many molecular characteristics with the immunosuppressant CsA. Our data demonstrates that CsA has no effect on CFU activity and furthermore does not inhibit NF-κB in HPC (Figs. 2 and 6A). This suggests that CsA does not present the risk of bone marrow toxicity observed with T2 and is consistent with published reports that CsA has no intrinsic myeloid toxicity even at the relatively high doses given after transplants (Tao et al., 1996). Additional experiments conducted in our laboratory compared the effects of T2 and CsA on NF-κB in CD4+ lymphocytes. Consistent with many published reports, CsA blocked NF-κB activation in T cells. However, T2 failed to suppress NF-κB activation in CD4+ lymphocytes even at concentrations exceeding those known to suppress lymphocyte function (data not shown). These results suggest that T2 and CsA do not exert their respective effects on T lymphocytes or bone marrow cells via the same mechanism. Moreover, they illustrate that NF-κB regulation is probably cell-type specific.

T2 is a complex mixture consisting of at least eight different biologically active glycosides, diterpenoids, ketones, and alkaloids (Tao et al., 1991). The two components believed to be responsible for T2-induced immunosuppression are triptolide and tripdiolide, which make up ~0.1% of the T2 extract together (Tao et al., 1995). At a 500 ng/ml concentration of T2 (which completely inhibits all CFU activity), these two ingredients would be present at ~0.5 ng/ml. At this point, we have no way of knowing if these components are also responsible for the bone marrow suppression reported herein. However, this concentration is consistent with other studies reporting the EC_{50} values of these two chemicals for other toxic endpoints at 0.5 to 2.0 ng/ml (Wei and Adachi, 1991; Tany and Ersenbrand, 1992; Tao et al., 1995). It would be extremely useful to know if these compounds (or others) in T2 are responsible for the bone marrow toxicity. This is especially relevant in light of the fact that different extracts have different triptolide/tripdiolide compositions and have different EC_{50} values for various immunosuppressive assays (Tao et al., 1995). If the compounds responsible for the immunosuppression could be distinguished from those that suppress the bone marrow, then perhaps different extraction procedures could be developed to generate a safer product.

It has been suggested in the literature that T2 may have some clinical benefit in the treatment of multitude of ailments ranging from RA and other autoimmune disorders to a male contraceptive agent (Zhen et al., 1995; Chang et al., 1997; Shamon et al., 1997). However, the results of both clinical trials and experimental animal studies reveal that T2 does not have a very large therapeutic index. This is evidenced by the fact that in one recent clinical trial, a patient who mistakenly took three times the recommended study dosage (180 mg/day) developed aplastic anemia and had to be removed from the study (Tao et al., 1989). In another study, twice the recommended dose reported to be effective at inhibiting tumor growth was fatal in mice (Shamon et al., 1997). We believe this low therapeutic index, in combination with poor public awareness of the hazards of this drug, represents a potentially dangerous situation. This is exacerbated by T2 availability (it can be purchased over the Internet) as well as the fact that some herbal 'remedies' contain extracts of TWH that are not even listed as ingredients, so people may not even be aware they are taking it. Based on the pattern of toxicity published previously and our current experimental results, we feel that T2 should be restricted to investigational use only and that routine blood work conducted under the supervision of a licensed physician should be a mandatory safeguard for use of this drug.

References

Asano K, Matsuishi J, Yu Y, Kasahara T and Hisamitsu T (1998) Suppression effects of Tripterygium wilfordii Hook f, a traditional Chinese medicine, on collagen arthritis in mice. *Immunopharmacology* **39:**117–126.

Baeuerle P and Baltimore D (1996) NF-κB: Ten years after. *Cell* **87:**13–20.

Baeuerle P and Henkel T (1994) Function and activation of NFκB in the immune system. *Annu Rev Immunol* **12:**141–179.

Besançon F, Atfi A, Gespach C, Cayre YE and Bourgeade MF (1998) Evidence for a role of NF-κB in the survival of hematopoietic cells mediated by interleukin 3 and the oncogenic TEL/platelet-derived growth factor receptor b fusion protein. *Proc Natl Acad Sci USA* **95:**8081–8086.

Brown MC, Tomaras GD, Vincenti MP and Taffet SM (1997) Two forms of NF-κB1 (p105/p50) in murine macrophages: Differential regulation by lipopolysaccharide, interleukin-2, and interferon-gamma. *J Interferon Cytokine Res* **17:**295–306.

Chang D-M, Chang W-Y, Kuo S-Y and Chang M-L (1997) The effects of traditional antirheumatic herbal medicines on immune response cells. *J Rheumatol* **24:**436–441.

Dignam JD, Lebovitz RM and Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:**1475–1489.

Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA and Shannon MF (1994) Requirement for nuclear factor (NF)-κB p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood* **83:**2469–2479.

Halloran P and Madrenas J (1991) The mechanism of action of cyclosporine: A perspective for the 90's. *Clin Biochem* **24:**3–7.

Hiscott J, Marois J, Garoufalis J and D'Addario M (1993) Characterization of a functional NF-κB site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol Cell Biol* **13:**6231–6240.

Hong Y-H, Peng H-B, Fata VL and Liao JK (1997) Hydrogen peroxide-mediated transcriptional induction of macrophage colony-stimulating factor by TGF-β1. *J Immunol* **159:**2418–2423.

Irons RD, Le AT, Som DB and Stillman WS (1995) 2′3′-dideoxycytidine-induced thymic lymphoma correlates with species-specific suppression of a subpopulation of primitive hematopoietic progenitor cells in mouse but not rat or human bone marrow. *J Clin Invest* **95:**2777–2782.

Lee H, Arsura M, Wu M, Duyao M, Buckler AJ and Sonenshein GE (1995) Role of rel-related factors in control of c-*myc* gene transcription in receptor-mediated apoptosis of the murine B cell WEHI 231 line. *J Exp Med* **181:**1169–1177.

Lee-Huang S, Lin J-J, Kung HF, Huang PL, Lee L and Huang PL (1993) The human erythropoietin-encoding gene contains a CAAT box, TATA boxes and other transcriptional regulatory elements in the 5 flanking region. *Gene* **128:**227–236.

Li XW and Weir MR (1990) Radix *Tripterygium wilfordii*—A Chinese herbal medicine with potent immunosuppressive properties. *Transplantation* **50:**82–86.

Lipsky PE and Tao X-L (1997) A potential new treatment for rheumatoid arthritis: thunder god vine. *Semin Arthritis Rheum* **26:**713–723.

Liu PL and Wu XC (1996) Clinical and experimental study on sustained-release tablet of *Tripterygium wilfordii* in treatment of rheumatoid arthritis. *Chung-Kuo Chung Hsi i Chieh Ho Tsa Chih* **16:**10–13.

Lowenthal JW, Ballard DW, Bohnlein E and Greene WC (1989) Tumor necrosis factor a induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. *Proc Natl Acad Sci USA* **86:**2331–2335.

McKean DJ, Bell M, Huntoon C, Rastogi S, Van Norstrand M, Podzorski R, Nilson A and Paya C (1995) IL-1 receptor and TCR signals synergize to activate NF-κB-mediated gene transcription. *Int Immunol* **7:**9–20.

Musso M, Ghiorzo P, Fiorentini P, Giuffrida R, Ciotti P, Garré C, Ravazzolo R and Bianchi-Scarrà G (1996) An upstream positive regulatory element in human GM-CSF promoter is recognized by NF-κB/Rel family members. *Biochem Biophys Res Commun* **223:**64–72.

Muñoz C, Pascual-Salcedo D, Castellanos MD, Alfranca A, Aragonés J, Vara A, Redondo JM and de Landazuri MO (1996) Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: Modulation of NF-kappaB and AP-1 transcription factors activity. *Blood* **88:**3482–3490.

Oster W, Brach MA, Gruss H-J, Mertelsmann R and Herrmann F (1992) Interleukin-1B (IL-1b) expression in human blood mononuclear phagocytes is differentially regulated by granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, and IL-3. *Blood* **79:**1260–1265.

Peng HB, Rajavashisth TB, Libby P and Liao JK (1995) Nitric oxide inhibits macrophage-colony stimulating factor gene transcription in vascular endothelial cells. *J Biol Chem* **270:**17050–17055.

Pyatt DW, Stillman WS, Yang Y and Irons RD (1999) An essential role for NF-κB in human CD34(+) bone marrow cell survival. *Blood* **93:**3302–3308.

Pyatt DW, Zheng JH, Stillman WS and Irons RD (1996) Inorganic lead activates NF-κB in primary human CD4+ T lymphocytes. *Biochem Biophys Res Commun* **227:**380–385.

Shamon L, Pezzuto J, Graves J, Mehta R, Wangcharoentrakul S, Sangsuwan R, Chaichana S, Tuchinda P, Cleason P and Reutrakul V (1997) Evaluation of the mutagenic, cytotoxic, and antitumor potential of triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii*. *Cancer Lett* **112:**113–117.

Suthanthiran M, Morris R and Strom T (1996) Immunosuppressants: Cellular and molecular mechanisms of action. *Am J Kidney Dis* **28:**159–172.

Tany W and Ersenbrand G (1992) *Tripterygium wilfordii* Hook, in *Chemistry, Pharmacology, and Use in Traditional and Modern Medicine* pp 991–995, Springer Verlag, Berlin.

Tao X, Davis LS and Lipsky PE (1991) Effect of an extract of the Chinese herbal remedy *Tripterygium wilfordii* hook F on human immune responsiveness. *Arthritis Rheum* **34:**1274–1281.

Tao X, Davis LS, Hashimoto K and Lipsky PE (1996) The Chinese herbal remedy, T2, inhibits mitogen-induced cytokine gene transcription by T cells, but not initial signal transduction. *J Pharmacol Exp Ther* **276:**316–325.

Tao X, Schulze-Koops H, Ma L, Cai J, Mao Y and Lipsky PE (1998) Effects of *Tripterygium wilfordii* Hook F extracts on induction of cyclooxygenase 2 activity and prostaglandin E$_2$ production. *Arthritis Rheum* **41:**130–138.

Tao X-L, Xiao Y-L, Zhu Q-L, Sun Y, Dong Y, Hu D-W, Shi Y-P, Dai H and Zhang N-Z (1989) A prospective, controlled, double-blind, cross-over study of *Tripterygium wilfordii* Hook F in treatment of rheumatoid arthritis. *Chin Med J* **102:**327–332.

Tao X, Cai JJ and Lipsky PE (1995) The identity of immunosuppressive components of the ethyl acetate extract and chloroform methanol extract (T2) of *Tripterygium wilfordii* hook F. *J Pharmacol Exp Ther* **272:**1305–1312.

Toth CR, Hostutler RF, Baldwin AS Jr and Bender TP (1995) Members of the nuclear factor κB family transactivate the murine c-*myb* gene. *J Biol Chem* **270:**7661–7671.

Wei Y-S and Adachi I (1991) Inhibitory effect of triptolide on colony formation of breast and stomach cancer cell lines. *Acta Pharmacologica Sinica* **12:** (5):406–410.

Young HA (1996) Regulation of interferon-γ gene expression. *J Interferon Cytokine Res* **16:**563–568.

Zhen Q, Ye X and Wei Z (1995) Recent progress in research on *Tripterygium*: A male antifertility plant. *Contraception* **51:**121–125.

Ziegler-Heitbrock HWL, Sternsdorf T, Liese J, Belohradsky B, Weber C, Wedel A, Schreck R, Bauerle P and Strobel M (1993) Pyrrolidine dithiocarbamate inhibits NF-κB mobilization and TNF production in human monocytes. *J Immunol* **151:**6986–6993.

Send reprint requests to: Richard Irons, Ph.D., D.A.B.T., University of Colorado Health Sciences Center, 4200 E. 9th Ave., Box C238, Denver, CO. E-mail: richard.irons@uchsc.edu