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Neutropenia in three patients with rheumatic disorders. Suppression of granulopoiesis by control-sensitive thymus-dependent lymphocytes.

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J Clin Invest. 1979;64(1):72-82. https://doi.org/10.1172/JCI109465.

Research Article

A man with polymyalgia rheumatica (patient 1) and two patients (2 and 3) with Felty's syndrome had neutropenia at the time of diagnosis. Bone marrow samples in each patient were cellular but showed an "arrest" of granulocyte maturation at the myelocyte stage. Agar colony growth of marrow cells from each patient was subnormal but increased after removal of sheep erythrocytes rosette-forming cells (thymus-dependent [T] cells) from marrow cell suspensions before culture. Preincubation of marrow cells with cortisol also enhanced colony growth. Maximum enhancement with cortisol occurred at 1 mM (patient 1), 1 microM (patient 2), and 10 nM (patient 3). Cortisol failed to enhance colony growth after removal of T cells from marrow cell suspensions. Peripheral blood lymphocytes (PBL) and PBL-conditioned medium from all three patients inhibited colony growth of normal human marrow cells. Cortisol treatment of PBL or T depletion from PBL abrogated the inhibition in coculture and with conditioned medium. Prednisone therapy resulted in the disappearance of suppressor T-cell function concomitant with hematologic improvement in patients 2 and 3, but suppressor T cells persisted in patient 1, who did not respond to prednisone. We conclude that cortisol-sensitive T lymphocytes inhibited granulopoiesis in vitro probably by elaboration of a soluble factor or factors. Our results suggest (a) that neutropenia in these patients resulted, at least in part, from T-cell […]

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Neutropenia in Three Patients with Rheumatic Disorders

SUPPRESSION OF GRANULOPOIESIS BY CORTISOL-SENSITIVE THYMUS-DEPENDENT LYMPHOCYTES

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ABSTRACT A man with polymyalgia rheumatica (patient 1) and two patients (2 and 3) with Felty's syndrome had neutropenia at the time of diagnosis. Bone marrow samples in each patient were cellular but showed an "arrest" of granulocyte maturation at the myelocyte stage. Agar colony growth of marrow cells from each patient was subnormal but increased after removal of sheep erythrocyte rosette-forming cells (thymus-dependent [T] cells) from marrow cell suspensions before culture. Preincubation of marrow cells with cortisol also enhanced colony growth. Maximum enhancement with cortisol occurred at 1 mM (patient 1), 1 μM (patient 2), and 10 nM (patient 3). Cortisol failed to enhance colony growth after removal of T cells from marrow cell suspensions. Peripheral blood lymphocytes (PBL) and PBL-conditioned medium from all three patients inhibited colony growth of normal human marrow cells. Cortisol treatment of PBL or T depletion from PBL abrogated the inhibition seen both in coculture and with conditioned medium. Prednisone therapy resulted in the disappearance of suppressor T-cell function concomitant with hematologic improvement in patients 2 and 3, but suppressor T cells persisted in patient 1, who did not respond to prednisone. We conclude that cortisol-sensitive T lymphocytes inhibited granulopoiesis in vitro probably by elaboration of a soluble factor or factors. Our results suggest (*a*) that neutropenia in these patients resulted, at least in part, from T-cell suppression of granulopoiesis, (*b*) that the effectiveness of prednisone therapy was a result of its inhibition of suppressor T cells, and (*c*) that reponses to glucocorticoid therapy may be predicted in such patients with the agar culture technique and cortisol dose responses in vitro.

INTRODUCTION

Regulation of the human immune response is a complex process involving collaboration between thymus-dependent (T) lymphocytes (T cells), lymphocytes capable of synthesizing immunoglobulin (bone marrow-dependent [B] cells), and mononuclear phagocytes. Various subsets of mononuclear leukocytes (1–3), especially T cells (1, 2, 4–8), may serve to enhance or suppress many phases of the immune response. The enhancing ("helper") and suppressor functions of T cells can be mediated through the variety of soluble factors produced by T cells in vitro (1, 8–12). Observations recently reviewed by Waldmann et al. (1, 2) indicate that some syndromes of immunologic failure may be mediated by suppressor T cells or their soluble products. We believe that there are remarkable similarities between the suppressor/helper effects of T-cell subpopulations on the immune apparatus and the effects of T cells on hemopoiesis. For example, T cells (13–15) or their soluble products (14) may promote or enhance differentiation of murine pluripotent (13–16) and committed (17–21) stem cells. Conversely, lymphocyte suppressors of hemopoiesis have been described and may mediate various syndromes of hemopoietic failure (22–29).

We have studied the effect of lymphocytes from three neutropenic patients on granulocyte colony growth in agar. In the bone marrow and peripheral blood of these patients we have found T-cell suppressors of granulopoiesis. Furthermore, like the T-cell suppressors that

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Received for publication 12 June 1978 and in revised form 23 February 1979.

TABLE I
Clinical and Laboratory Data

Age/Sex	Patient 1 72/Male	Patient 2 54/Female	Patient 3 63/Male
Diagnoses	Polymyalgia rheumatica	Felty's syndrome Sjogren's syndrome Chronic aggressive hepatitis	Felty's syndrome Chronic hepatitis Post splenectomy (6 yr)
Peripheral blood			
Neutrophils, *mm*	527* (450‡)	495* (2,800‡)	306* (3,619‡)
Monocytes, *mm*	560* (490‡)	500* (585‡)	204* (1,000‡)
Bone marrow			
Cellularity	Normal	Normal	Normal
Erythroid:granulocyte ratio	1:6	1:5	1:6
Erythroid and megakaryocytic maturation	Normal	Normal	Normal
Granulocytic maturation	Myelocyte arrest* Myelocyte arrest‡	Myelocyte arrest* Normal‡	Myelocyte arrest* Normal‡

* Before prednisone therapy.
‡ During prednisone therapy.

may mediate certain immunologic deficiency states, the granulopoietic suppressor T cells in these three patients are cortisol sensitive, and their suppressive function is mediated, at least in part, by a soluble inhibitory activity.

METHODS

Patients. Three neutropenic patients were studied (Table I). Bone marrow morphology was similar in all patients (Table I). Only patient 3 had received blood transfusions in the past. Patient 2 had two uncomplicated pregnancies. Prednisone therapy (60 mg/d) effectively relieved muscle pain and weakness in patient 1, but doses as high as 100 mg/d effected no hematologic change (Table I). After 4 mo of prednisone therapy the patient died of bacterial septicemia. In patients 2 and 3, prednisone therapy (30 and 60 mg/d, respectively) resulted in improvement of granulopoiesis (Table I). Discontinuation of prednisone therapy in patient 3 was followed by neutropenia within 7–10 d. Resumption of prednisone therapy was followed by normalization of neutrophil counts and abnormal granulopoiesis within 7 d. All bone marrow and blood studies were performed after obtaining signed, informed consent. Bone marrow cells were obtained from the posterior iliac crests. In each patient, 4 ml was aspirated from each of three sites.

Volunteers. Peripheral blood cells were obtained from 12 normal adult volunteers (ages 22–46 yr), five nonneutropenic patients with rhematoid arthritis, and three nonneutropenic patients with systemic lupus erythematosus. All eight volunteers with rheumatic diseases were clinically stable, and none were taking glucocorticoids. Three patients with rheumatoid arthritis were taking aspirin. Bone marrow cells were obtained from the posterior iliac crests of 14 paid, normal volunteers.

Preparation of marrow and peripheral blood cells. Marrow cells were aspirated directly into heparinized (preservative-free) syringes. Single marrow cell suspensions were prepared as previously described (30). Peripheral blood mononuclear leukocytes (PBML)[1] and light-density marrow cells (LDBMC) were obtained by Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) (31) separation of heparinized whole blood or marrow (31). Complete medium for marrow cell suspensions was McCoy's 5A medium supplemented with 15% fetal calf serum, amino acids, and antibiotics (Grand Island Biological Co., Grand Island, N. Y.). Complete medium for peripheral blood cells was RPMI-1640 supplemented with amino acids antibiotics, and 20% fetal calf serum (Grand Island Biological Co.).

Experimental design. The inhibitory effects of T lymphocytes from the patients and controls were assessed as follows. First, autologous marrow cells were cultured in agar before and after T-cell depletion. Next, T-depleted and T-enriched cell suspensions were cultured for 3 d in RPMI-1640; the cells were used as "effectors" in coculture experiments, and the lymphocyte-conditioned media were assayed for soluble inhibitory activity.

Separation of adherent and phagocytic cells. Adherent cells were removed with nylon fiber columns as previously described (30). Phagocytic cells were removed from the mononuclear cell suspensions with carbonyl iron (Dow Chemical Co., Midland, Mich.) (32). 50 mg carbonyl iron was mixed with 3 ml mononuclear leukocytes (10^7 cells/ml) in a 30-ml round bottom centrifuge tube and incubated for 60 min at 37°C. Cells were gently resuspended and placed on a magnet. The resulting phagocyte-depleted PBML were poured off and placed twice more upon a magnet. The cells were then washed once in medium and resuspended.

Separation of T cells. T cells were identified and counted by E-rosette formation with washed sheep erythrocytes (SRBC) (33, 34). Cell suspensions were depleted of T cells by E-rosette formation with washed SRBC (Prepared Media Laboratories,

[1] *Abbreviations used in this paper*: ALG, equine anti-human lymphoblast globulin; CSA, colony-stimulating activity; LDBMC, light-density marrow cells; PBML, peripheral blood mononuclear leukocytes; PHA, phytohemagglutinin; PPD, purified protein derivative; SRBC, sheep erythrocytes.

Tualatin, Ore.) followed by Ficoll-Paque centrifugation of the cell mixture. The nonrosetting cells were removed from the interface, washed twice, and resuspended in a volume of complete medium equal to the original (prerosette) volume; this was done to avoid stem cell enrichment. Control cells (not T depleted) were also subjected to Ficoll-Paque centrifugation without prior SRBC mixing. The nonrosetting peripheral blood cells were washed and resuspended at $1-4 \times 10^6$/ml in complete RPMI-1640.

Cortisol treatment of bone marrow and peripheral blood cells. 10-fold concentrated stock solutions of hydrocortisone sodium succinate (Solu-Cortef, Upjohn Pharmaceuticals & Chemicals, Kalamazoo, Mich.) were made in double-strength medium. LDBMC from each patient were cultured in agar (see below) over underlayers containing colony-stimulating activity (CSA) or CSA with cortisol (35). The final concentration of cortisol in underlayers ranged from 0.1 mM to 1 nM. In two cases, short-term cortisol exposure was also used; LDBMC, T-depleted LDBMC, and equine anti-human lymphoblast globulin (ALG)-treated LDBMC from patients 1 and 2 were preincubated for 60 min at 37°C in 7.5% CO_2 in air with 1 mM–1 μM cortisol. The cells were thrice washed and resuspended in complete medium (McCoy's) before the agar culture.

Phagocyte-depleted PBML and T-depleted cells from all patients were incubated for 1 h at 37°C in complete medium (RPMI-1640) with and without cortisol (patient 1, 1 mM; patient 2, 10 μM; patient 3, 0.1 μM). The cells were washed and resuspended in complete medium for use in coculture experiments.

Lymphocyte cultures. All PBML and fractions thereof were cultured for 3 d before use in coculture. Phagocyte-depleted PBML obtained from all 3 patients, 10 normal volunteers, 5 patients with rheumatoid arthritis, and 3 patients with systemic lupus erythematosus were suspended ($2-4 \times 10^6$/ml) in complete medium (RPMI-1640). 2-ml aliquots were placed in 35-mm tissue culture plates and incubated for 60–72 h at 37°C in humidified 7.5% CO_2 in air. At the end of the culture period, cells were teased from the dish with a sterile policeman, and the suspension was poured off into a 15-ml tube and centrifuged at 400 *g* for 10 min. The fresh supernatant medium was poured off and tested (without filtration or freezing) against normal, human marrow cells in agar culture (see below). The remaining supernatant medium was stored at −20°C. The cells in the "button" were resuspended to $4-6 \times 10^6$/ml and used in agar cocultures.

"Standard" CSA. Mononuclear leukocytes from a volunteer with a strongly positive purified protein derivative (PPD) skin test were obtained by a Ficoll-Paque centrifugation, thrice washed, and then resuspended at 4×10^6/ml in complete medium which contained 25 μg/ml PPD (kindly supplied by the Central Veterinary Laboratory of the Ministry of Agriculture, Fisheries and Food, New Haw, Weybridge, Surrey, England). 2-ml aliquots of the cell suspension were placed in 35-mm culture dishes (Corning Glass Works, Corning, N. Y.) and incubated for 6 d at 37.5°C in 7.5% CO_2 in air. Medium was decanted, filtered through 0.2-micron filters (Nalgene Labware Div., Nalge Co., Div. of Sybron Corp., Rochester, N. Y.), and stored at −20°C in 3-ml polyethylene culture tubes. Because our antigen-induced CSA contains PPD, it may be of importance to note that 3 of 14 marrow volunteers and patient 3 had positive PPD skin tests.

Agar cultures (granulocyte/monocyte colony-forming units [CFU-C]) in the neutropenic patients. Double layer agar cultures were performed according to a modification of the techniques of Pike and Robinson (36). Underlayers were prepared with 0.5% agar (Bacto-agar, Difco Laboratories, Detroit, Mich.) in complete McCoy's 5A medium. Underlayer agar contained CSA (0.1 and 0.2 ml/plate) with and without cortisol (10 mM–1 pM).

The patients were studied at least twice, once before and once during prednisone therapy. On both occasions overlayer agar (0.3% in complete medium) contained 2×10^5/ml LDBMC, LDBMC depleted of T cells by the E-rosette method described above, and LDBMC preincubated for 60 min with cortisol.

Cocultures. Coculture experiments with peripheral blood lymphocytes (effectors) from the patients and controls (10 normal volunteers and 8 nonneutropenic patients with rheumatic disorders) and nonadherent LDBMC (targets) from normal volunteers were carried out at lymphocyte:marrow ratios of 1:2. Heat-inactivated fetal calf serum was used in all coculture experiments. Cocultures were carried out in 8–10 replicate plates upon underlayers, one-half of which contained CSA. CSA was not added to the other half; this was done to detect CSA production by interacting cells in coculture.

The overlayer agar was allowed to gel and was then incubated for 8 d in 7.5% CO_2 in air at 37.5°C. Colonies (≥40 cells/aggregate) and clusters (>8 to <40 cells/aggregate) were enumerated with a dissecting microscope. To confirm the granulocytic nature of the colonies, random individual colonies were picked out of agar with a finely drawn Pasteur pipet which contained a small amount of distilled water (37). The colonies were placed on glass slides which were immediately air dried by spinning at 2,000 rpm in a cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, Pa.). Slides were stained with Wright's and alpha-naphthyl acetate esterase stains.

Soluble inhibitor assay. Nonadherent nonphagocytic LDBMC from normal volunteers were prepared as described above and plated over agar underlayers containing either 0 or 0.1 ml standard CSA. The agar underlayers also contained either 0.1 or 0.2 ml medium conditioned by unstimulated lymphocytes from each patient.

Serum inhibitor assay. 0.1 ml fresh serum from each patient was placed in underlayer agar with and without standard CSA. 2×10^5 autologous LDBMC and 2×10^5 LDBMC from normal volunteers ($n = 5$ for serum from patient 1, $n = 2$ from patients 2 and 3) served as target cells in each plate.

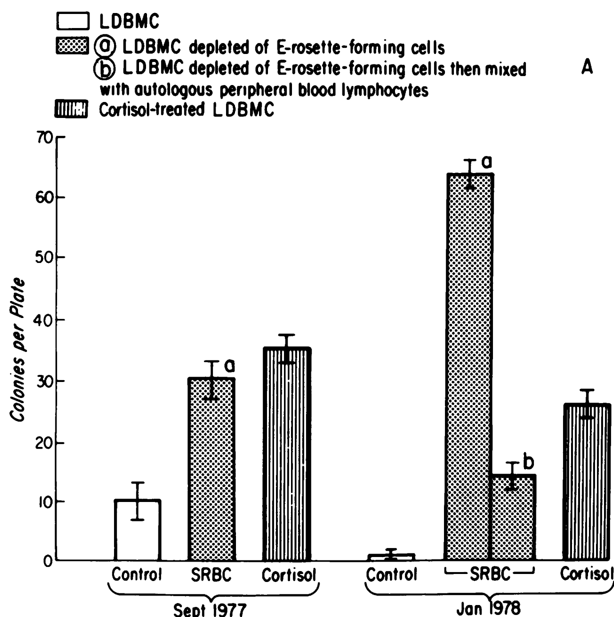
Additional studies. To determine the capacity of T-enriched cells to elaborate CSA, PBML from patient 2 were cultured in RPMI-1640 with 20 μl/ml phytohemagglutinin (PHA) (Difco Laboratories). After 72 h of culture, the PHA-treated cells were washed thrice and used in coculture (arm B) experiments. The PHA-treated lymphocyte-conditioned medium was tested against normal phagocyte-depleted LDBMC. Colony growth was compared with that observed using conditioned medium prepared with unstimulated phagocyte-depleted PBML from the same patient. All control cultures in this experiment contained 1 μl PHA/ml.

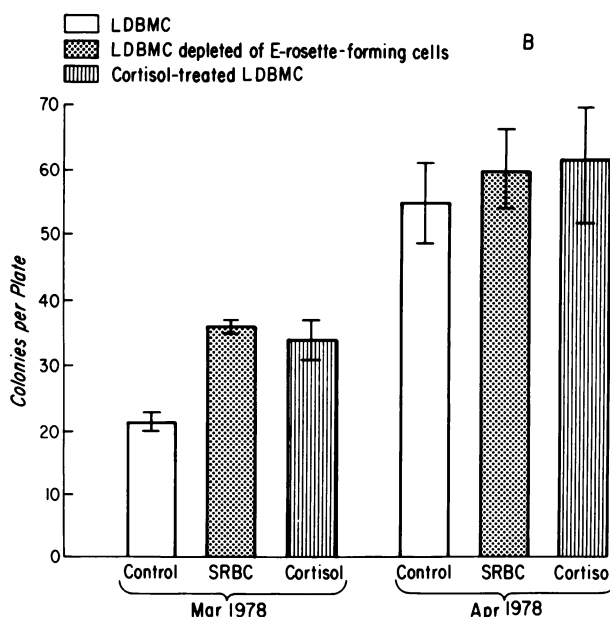
To determine the toxicity of lymphocyte-conditioned medium, a "washout" experiment was done. 0.2 ml conditioned medium from phagocyte-depleted PBML of patient 2 was added to a 1-ml suspension of normal LDBMC (10×10^6/ml) in complete medium. The suspension was incubated at 37°C for 2 h. The cells were washed and plated in agar. Observed colony growth was compared with colony growth of cells incubated in medium alone.

RESULTS

Autologous agar colony growth. Agar colony growth of normal LDBMC in our laboratory ranges from 48 to 320 colonies/2×10^5 cells. Colony growth was decreased in all patients at the time of diagnosis (Fig. 1).

In patient 1, pretreatment of LDBMC with ALG and cortisol resulted in more than threefold enhancement





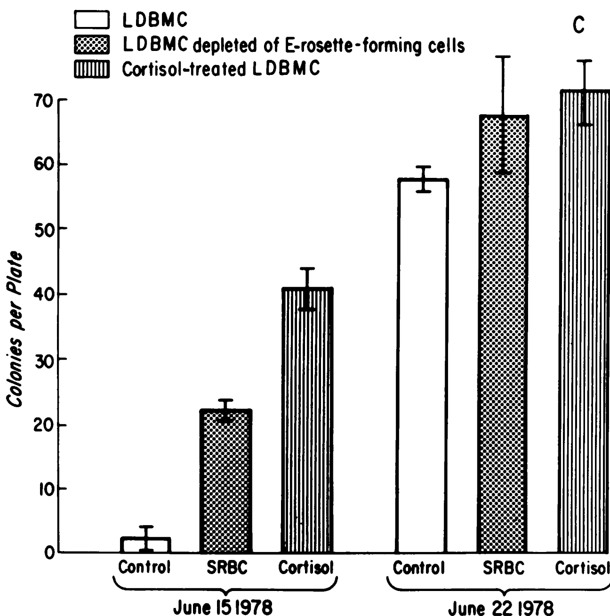


FIGURE 1 (A) The effects of T-cell depletion and short-term (1 h) cortisol exposure on agar colony growth in patient 1. Bars and vertical lines represent mean±SD in five replicate plates. The first study (September 1977) was done before prednisone therapy, the second study (January 1978) was done during therapy (60 mg prednisone daily). Colony growth is expressed as colonies per plate. Control and cortisol-treated cells were plated at cell concentrations of 2×10^5/ml. T-depleted (SRBC) plated contained 1.29×10^5 cells (September 1977) and 1.35×10^5 cells (January 1978). Base-line (control) colony growth was not enhanced during prednisone therapy in vivo. Cortisol treatment of LDBMC and removal of E-rosette-forming cells from the marrow cells (*a*) enhanced colony growth ($P < 0.001$). The source and quantity of CSA was the same in all experiments on both dates. Autologous peripheral blood lymphocytes which were added to T-cell-depleted marrow (*b*) inhibited colony growth ($P < 0.001$). (B) The effects of T-cell depletion and short-term (1 h) cortisol exposure on colony growth in agar in patient 2. The first study (March 1978) was done before prednisone therapy, the second study was done during therapy (60 mg prednisone daily). Control and cortisol-treated cells were plated at cell concentrations of 2×10^5/ml. T-depleted plates contained 1×10^5 cells (April 1978). Base-line colony growth was significantly enhanced by prednisone therapy in vivo ($P < 0.001$). In March 1978 T-cell depletion and cortisol treatment enhanced colony growth. This effect was not seen during prednisone therapy. (C) The effects of T depletion and cortisol exposure on agar colony growth of cells from patient 3. Results are similar to those of patient 2. The first study was done before prednisone therapy, and the second was performed during therapy (60 mg/d). Base-line colony growth was significantly enhanced by prednisone therapy in vivo. Although T depletion and cortisol treatment enhanced colony growth before prednisone therapy, this effect was not seen during prednisone therapy.

of colony growth (Fig. 1A). Depletion of sheep rosette-forming cells (T cells) decreased the plated cell number from 2×10^5 to 1.3×10^5 (September 1977) and to 1.5×10^5 (January 1978). As shown in Fig. 1A, colony growth was enhanced by removal of T cells in patient 1. Similar studies with LDBMC from five normal volunteers have shown neither enhancement nor inhibition of colony growth as a result of T depletion. The studies in patient 1 were repeated in January 1978 during prednisone therapy. At that time, base-line colony growth had not increased, and colony growth was again enhanced by T depletion, ALG pretreatment, and cortisol pretreatment (1 mM) (Fig. 1).

Before prednisone therapy, colony growth in patients 2 and 3 (Fig. 1B and C) was also abnormally low. In patient 2 as in patient 1, cortisol (1 mM) pretreatment

TABLE II
*Dose-Response Effects of Peripheral Blood Lymphocytes from Patient 3 on Colony Growth by T-Depleted Autologous B Cells**

Ratio of lymphocytes to marrow cells‡	Before prednisone therapy	Ratio of lymphocytes to marrow cells§	During prednisone therapy
Control (no added lymphocytes)	50±8		148±6
3:1	13±2	4:1	202±8
2:1	8±2	2:1	192±8
1:1	6±2	1:1	178±12

* Mean±SD colonies/2 × 10^5 LDBMC.
‡ 70% T lymphocytes.
§ 40% T lymphocytes.

and T depletion resulted in enhancement of colony growth. 2 wk (patient 2) and 7 d (patient 3) after the initiation of prednisone therapy the neutrophil counts were normal (Table I) and base-line colony growth had more than doubled (Fig. 1B and C). At that time neither T depletion nor cortisol pretreatment enhanced colony growth. In addition, adding back nonadherent PBML to autologous marrow failed to decrease colony growth in patient 3 (Table II).

The dose-response curves for cortisol on agar colony growth are shown in Fig. 2. Unfractionated and T-depleted cells were plated over agar underlayers containing 0.1 mM–1 nM cortisol. As shown in Fig. 2D, 0.1 μM cortisol (or greater) inhibited agar colony growth when tested against normal marrow cells both before and after T-cell depletion. In all three patients, cortisol enhanced colony growth. This enhancement was seen even when the volume of CSA was doubled, at which point colony growth had reached a plateau. In patient 1, growth enhancement was noted at 0.1–10 mM (Fig. 2A). These levels are not achievable in vivo with standard (60–120 mg prednisone daily) glucocorticoid therapy. Maximum enhancement occurred in patient 2 at 1 μM and in patient 3 at 10 nM. These levels are achievable

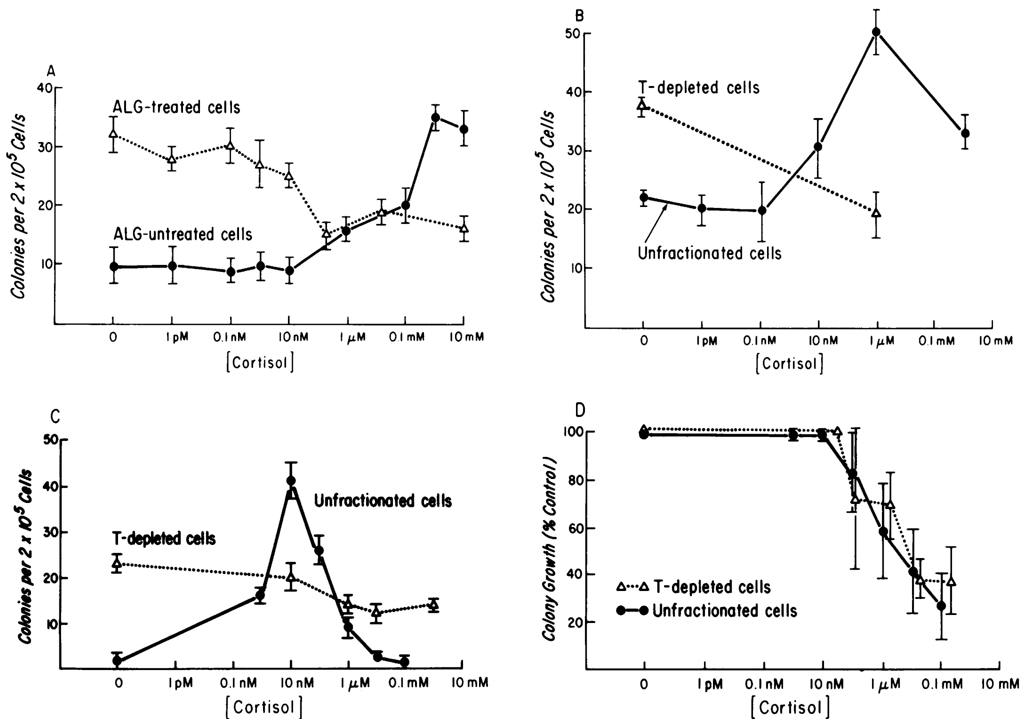


FIGURE 2 Dose-related colony growth enhancement by cortisol in patients 1 (A), 2 (B), and 3 (C), as well as in four normal volunteers (D). Points and vertical lines indicate mean±SD in three to five replicate plates except in D where they indicate mean percentage of control±SD. Maximum colony enhancement in patient 1 (A) was noted at 1 and 10 mM. These levels are not achievable with standard doses of oral glucocorticoids (e.g., 60–100 mg/d, prednisone). This patient did not respond to glucocorticoid therapy. In patients 2 (B) and 3 (C) maximum growth enhancement was seen at 1 μM and 10 nM, respectively. In these patients, base-line colony growth and neutrophil counts increased during oral glucocorticoid therapy. Cortisol inhibited colony growth after T cells were removed by treatment of marrow cells with ALG (0.6 mg/ml for 60 min) (A) or removal of E-rosette-forming cells (B and C). The effect of cortisol on normal colony growth is one of inhibition (D).

in vivo with standard glucocorticoid therapy. Cortisol inhibited colony growth of cells from all three patients after T depletion. Cortisol normally inhibits colony growth in agar (Fig. 2D) (35). That suppressor lymphocytes were present in the peripheral blood of these patients is documented by our observation that addition of peripheral blood lymphocytes to T-depleted marrow cells in patients 1 (Fig. 1A) and 3 (Table II) abrogated the granulopoietic enhancement effected by T depletion from the marrow cell suspension.

Coculture experiments. PBML and nonadherent, phagocyte-depleted PBML from patient 1 inhibited colony growth in all five normal volunteers (Fig. 3). T-cell depletion and cortisol pretreatment of the PBML abrogated the inhibitory response in all cases. Target colony growth increased to 100% of control in four of five volunteers after cortisol pretreatment of effector cells. Peripheral blood lymphocytes (nonadherent) from three noneutropenic patients with systemic lupus erythematosus and from five nonneutropenic patients with rheumatoid arthritis did not inhibit normal colony growth. Normal human peripheral blood lymphocytes in coculture did not inhibit colony growth in standard CSA-stimulated cultures. Cortisol pretreatment of normal human peripheral blood lymphocytes did not enhance colony growth of normal marrow cells in coculture

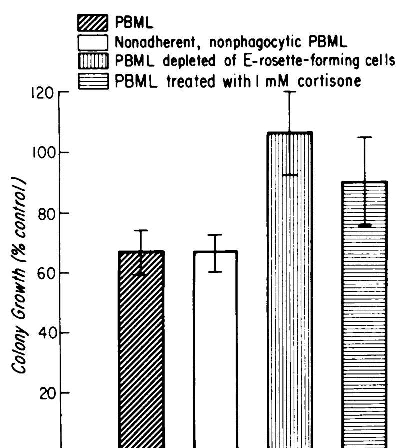


FIGURE 3 Colony growth of normal human marrow cells cocultured with mononuclear leukocytes obtained from the peripheral blood of patient 1. Marrow cells from five normal volunteers were obtained on the same day and were cultured for 7 d. Because colony growth ranged from 36 to 81 colonies per 2×10^5 cells, group colony growth is expressed as the percentage of control. Bars and vertical lines represent group mean ±SD. Both unfractionated and monocyte-depleted peripheral blood cells inhibited colony growth ($P < 0.001$), but when T cells were removed or when cells were treated with cortisol, no inhibitory effect was noted. Cells of each of five volunteers were also mixed with peripheral blood leukocytes of a normal volunteer. No inhibition of colony growth was seen. Indeed, at lower CSA levels, mononuclear leukocytes stimulated colony growth.

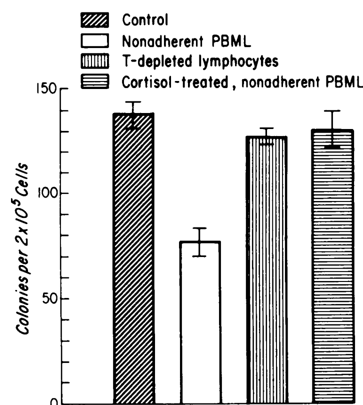


FIGURE 4 Colony growth of normal human marrow cells cocultured with nonadherent mononuclear leukocytes or T-depleted cells obtained from the peripheral blood of patient 2. The effector cells were cultured for 3 d in RPMI-1640. The targets were nonadherent LDBMC obtained from a normal volunteer. Bars and vertical lines represent mean±SD. The T-enriched fraction inhibited colony growth by 45% ($P < 0.001$), but this inhibition was abrogated by cortisol. No inhibition was seen with T-depleted cells. Results with target cells obtained from a second volunteer were similar.

(0 of 5 targets). 88% of the nonadherent, nonphagocytic PBML from patient 2 rosetted with SRBC. The T-enriched lymphocytes from patients 2 and 3 were plated in coculture with normal marrow cell targets from two volunteers. T-enriched lymphocytes inhibited colony growth of cells from both volunteers in both cases (Fig. 4, Table III). T-depleted lymphocytes and cortisol-pretreated T-enriched lymphocytes failed to inhibit colony growth. However, when T-enriched lymphocytes were stimulated with PHA, enhancement of colony growth occurred. This enhancement was mediated by CSA (Table III).

Monocyte-macrophage-depleted target cells were used in this assay to avoid interactions between the test substance (lymphocytes or conditioned medium) and endogenous CSA-producing cells normally present in marrow cell suspensions (38). In patient 3, T-depleted LDBMC were cultured with autologous PBML, T-depleted PBML, and cortisol (0.1 μM) -treated PBML. As in patients 1 and 2, T depletion and cortisol treatment of PBML abrogated inhibition of colony growth in coculture (Fig. 5).

Assay for soluble inhibitors. Serum from all three patients stimulated colony growth of normal phagocyte-depleted LDBMC. Phagocyte-depleted PBML-conditioned medium from patient 1 inhibited agar colony growth of marrow cells from each of five volunteers (Fig. 6). Preincubation of PBML with cortisol (10 mM) for 60 min before culture prevented the production (or release) of the inhibitory activity (Fig. 6). T-depleted lymphocyte-conditioned medium was not inhibitory. Similar results were found in experiments with periph-

TABLE III

*Effect of T-Enriched Lymphocytes and Lymphocyte-Conditioned Medium on Agar Colony Growth**

		Lymphocytes		Lymphocyte-conditioned medium‡	
Donor	Effector cells	No exogenous CSA	Exogenous CSA	No exogenous CSA	Exogenous CSA
Normal volunteer	Unstimulated	22±3	140±6 ($P < 0.005$ vs. below)	3±1	59±11 ($P < 0.005$ vs. below)
Patient 2	Unstimulated	0	77±6	1±1	20±6
Patient 2	PHA-stimulated	125±8	138±6	37±8	55±12

* Mean (±SD) colonies per 2 × 10^5 phagocyte-depleted, nonadherent LDBMC obtained from two normal volunteers.
‡ 0.1 ml in underlayer agar. Because the lymphocyte-conditioned medium in the PHA-stimulated sample contained both PHA and CSA, all control underlayers contained PHA (1 μl/ml). Target marrow cells in coculture were obtained from a different volunteer than were target cells for conditioned medium studies.

eral blood lymphocyte-conditioned medium from patients 2 and 3 and marrow cells from normal volunteers (Fig. 7). However, brief (2-h) incubation of normal marrow cells with unstimulated lymphocyte-conditioned medium from patient 2 did not inhibit colony growth (control, 302±8 colonies/plate; lymphocyte-conditioned medium, 289±22 colonies/plate). Unstimulated lymphocyte-conditioned medium from normal volunteers and nonneutropenic patients with rheumatic diseases did not suppress agar colony growth.

DISCUSSION

With the combined techniques described herein we have documented that T cells in the marrow and peripheral blood of three severely neutropenic patients suppressed clonal granulopoiesis of autologous (Fig. 1) and allogeneic (Figs. 3, 4, and 5) B cells in agar. Furthermore, granulopoietic inhibition in vitro was mediated, at least in part, by a soluble activity found in T-enriched lymphocyte-conditioned medium (Figs. 6 and 7). The inhibition in coculture, and the production (or release) of soluble inhibitory activities was independent of the patients' sera, mononuclear phagocytes, or complement. Serum alone did not inhibit autologous or heterologous colony growth. Indeed, serum from all patients enhanced colony growth when tested against normal marrow cells. These observations support previously reported findings of granulopoietic failure in some patients with Felty's syndrome (28, 39, 40) and support the observations of Abdou et al. (28) which suggest that the granulopoietic failure in such patients may be immunologically mediated.

Although it has been known since 1967 that the immune apparatus was capable of suppressing erythro-

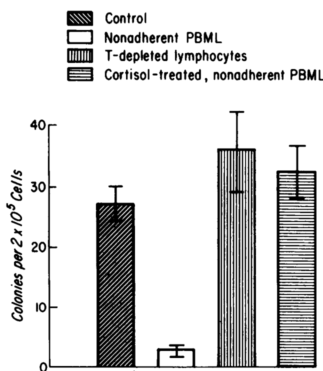


FIGURE 5 Colony growth of autologous marrow cells cocultured with nonadherent mononuclear leukocytes or T-depleted peripheral blood cells of patient 3. The target cells were nonadherent LDBMC. The effector cells in this experiment were not cultured in RPMI before the agar coculture. Bars and vertical lines represent mean±SD. The T-enriched fraction inhibited colony growth, but this inhibition was abrogated by cortisol. No inhibition was seen with T-depleted cells.

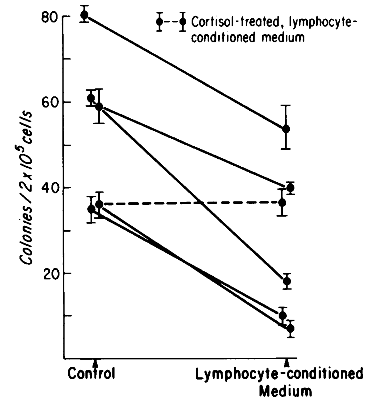


FIGURE 6 Inhibition of colony growth by 0.1 ml conditioned medium prepared by 3-d culture of lymphocytes from the peripheral blood of patient 1. Target cells were nonadherent LDBMC from five normal volunteers. Points and vertical bars represent mean±SD in each of five normal marrow cell cultures. There was inhibition of colony growth in each case. Depletion of SRBC rosette-forming cells from the cultured lymphocytes abrogated the inhibitory activity in lymphocyte conditioned medium. When lymphocytes were preincubated with 1 mM cortisol for 60 min (interrupted line) no inhibitory activity was noted in conditioned medium.

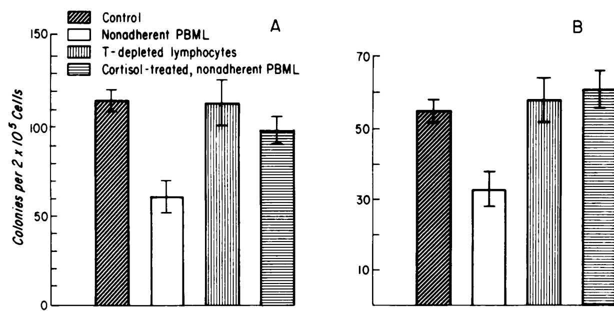


FIGURE 7 Inhibition of normal colony growth by T-enriched lymphocyte-conditioned medium (patients 2 and 3). 0.1-ml aliquots of conditioned medium were added directly to agar cultures of normal nonadherent LDBMC. Bars and vertical lines represent mean±SD. Lymphocyte-conditioned medium inhibited colony growth ($P < 0.001$) in both patient 2 (A) and patient 3 (B). T-cell depletion before lymphocyte culture and cortisol treatment of lymphocytes before culture abrogated the inhibitory activity in conditioned media.

poiesis in some patients with the syndrome of pure erythrocyte aplasia (41), only recently has it been recognized that there exist other immunologically mediated syndromes of hemopoietic failure (28, 42). For example, observations in patients with aplastic anemia of autologous hemopoietic recovery after unsuccessful marrow transplantation (43–47), immunosuppressive therapy (48–50), or plasmapheresis (51) and in vitro observations of lymphocyte-mediated (22, 23) or immunoglobulin-mediated (51, 52) suppression of hemopoiesis have supported this suggestion. However, there are both technical and theoretical concerns that must be acknowledged before in vitro abnormalities may be presumed to reflect in vivo mechanisms of pathophysiologic relevance.

The technical concerns relate to the popular technique of mixing either serum (or immunoglobulin fractions) (51, 52) or lymphocytes (22–24, 26, 27, 42, 53) from patients with marrow cells from normal volunteers in clonal assays. Whereas hemopoietic suppression in this setting has been presumed to reflect in vivo immune suppression, more recent observations in animals (54) and in patients with aplastic anemia (27) indicate that transfusion-related alloimmunization plays a major role in the development of hemopoietic suppressor lymphocytes as detected in coculture experiments. Alloimmunization may also account for stem cell antibodies in some patients with aplastic anemia (51). Thus, to implicate any immune mechanism in the pathogenesis of hemopoietic failure syndromes, studies must be performed with autologous marrow cells. In this regard it is not surprising that recently reported studies with autologous marrow cells from patients with Diamond-Blackfan anemia (26, 53) have largely refuted earlier suggestions based solely on coculture data (24) that anemia in this disorder has an immunologic basis. The three patients reported in this study exhibited T-lymphocyte-mediated suppression of granulopoiesis in both autologous and allogeneic cultures.

The theoretical concern regarding the interpretation of in vitro studies of hemopoietic failure syndromes relates to the difficulty in proving in vivo relevance, even using autologous studies. In our study we have not investigated the role of anti-neutrophil antibodies nor were granulocyte kinetic studies performed. Therefore, we can neither state that granulopoietic failure alone accounted for their neutropenia nor that inhibition of suppressor T lymphocytes was the sole mechanism by which prednisone induced remission. However, there exists evidence which suggests that these inhibitory cells were involved in the pathogenesis of neutropenia in these patients. First, there was no inhibition in cocultures of normal marrow cells with peripheral blood lymphocytes from any control patient including 10 normal volunteers and 8 nonneutropenic volunteers with collagen vascular diseases. The second, more direct line of evidence that hemopoietic suppressor T cells played a role in the pathophysiology of granulopoietic failure in these patients is found in the clinical (Table I) and cell culture changes (Fig. 1) which occurred during glucocorticoid therapy in patients 2 and 3. Before therapy, when agar colony growth was abnormally low, cortisol enhanced colony growth of unfractionated marrow cells but did not enhance colony growth after T depletion (Fig. 2). Indeed, cortisol suppressed colony growth after the T cells were removed from the suspension. Furthermore, cortisol-treated lymphocytes in vitro (Fig. 7) failed to produce or release the soluble inhibitor of granulopoiesis. Finally, during glucocorticoid therapy in patients 2 and 3, colony growth increased to a normal range, marrow T-cell numbers decreased, and no inhibitory T cells were detectable in autologous cultures (Table II). On the other hand, suppressor T cells persisted during therapy in the patient who failed to respond to prednisone. Thus, although we recognize the inherent limitations of in vitro/in vivo associations, our data suggest that the inhibitory T cells seen in vitro were responsible for granulopoietic failure in these patients and that the clinical response to glucocorticoid therapy was a result of the inhibition by prednisone of the suppressor T-cell population.

The clinical observations during therapy in patients 2 and 3 stand in contrast to those in patient 1 who did not respond to glucocorticoid therapy. Although further in vitro studies are necessary using dose-response curves in a larger number of neutropenic patients, it is reasonable to suggest that the differences in responses to therapy may relate to the doses required for an effect in vitro. For example, the cortisol concentrations (1–10 mM) effecting maximum inhibition of T suppression in patient 1 (Fig. 2A) are not achievable with high-dose,

oral prednisone therapy. On the other hand, in patients 2 and 3 (Figs. 2B and C) the observed enhancement of colony growth by cortisol occurred at therapeutically achievable levels. These data support our previously reported suggestion that glucocorticoid-responsive disorders of hemopoiesis may be identified with in vitro techniques (35).

The mechanism of granulopoietic suppression by T cells is as yet unknown. Certain technical factors must be taken into account when interpreting our observations. For example, because the T-enriched lymphocytes contained from 1 to 2% B lymphocytes, and from 15 to 30% null cells, we cannot rule out the possibility that immunocyte cooperation, especially T cell–T cell interactions (53, 55), was required for an inhibitory effect. That bone marrow samples from these patients were normally cellular with a "myelocyte arrest," that autologous colony growth increased to within our normal range after in vitro T depletion, and that short-term incubation of marrow cells with soluble inhibitor followed by "washout" did not inhibit colony growth, all suggest that T cells were not cytotoxic to granulocyte/monocyte colony-forming units (CFU-C) in vitro or in vivo. It is possible that T cells effectively inhibit granulopoiesis at the level of the myelocyte. In vivo studies in the experimental animal will be required to document such an effect.

Interestingly, although T-enriched suspensions in patient 2 produced an inhibitor, the cells were also fully capable of producing CSA when stimulated with PHA. Whether the CSA-producing and inhibitor-producing cells are the same is presently unknown, but the observation documents functional heterogeneity in our T-enriched cell populations.

Recent observations have shown that a number of cytotoxic (56) and suppressor (57–59) functions of T cells are inhibited by glucocorticoids. In fact, our observations in the setting of granulopoietic failure are congruent with observations on the role and function of cortisol-sensitive suppressor T cells in mediating some syndromes of immunologic failure (1, 2). Nevertheless, until detailed cell-fractionation studies are performed, or until the soluble T-cell inhibitors are isolated, these associations cannot be taken as evidence that T-cell suppressors of immunity are the same as T suppressors of granulopoiesis.

ACKNOWLEDGMENTS

We wish to thank Doctors John Fitchen and Scott Goodnight for helpful advice, Anne August and Jill Suttles for invaluable technical assistance, and Mark Bellis and Ann Irwin for preparation of the manuscript.

This study was supported by the Medical Research Service of the Veterans Administration (MRIS 0754) and the Leukemia Association of Oregon, Inc., Portland, Ore.

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