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### Research Article

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# Alternate-Day Prednisone Therapy and Human Lymphocyte Subpopulations

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**ABSTRACT** The mechanisms and kinetics of the immunosuppressive effects of alternate-day prednisone were investigated in a group of patients with a variety of inflammatory diseases receiving a range of alternate-day prednisone doses from 5 to 120 mg. Total circulating lymphocyte and monocyte counts, as well as proportions of lymphocyte subpopulations defined both by surface markers and by in vitro functional capacities, were studied. At 8 a.m. of the day on prednisone, just before drug administration, lymphocyte and monocyte counts, proportions of lymphocyte subpopulations, as well as in vitro lymphocyte blastogenic responses to various mitogenic and antigenic stimuli were normal. 4 h after the administration of prednisone, there was a profound lymphocytopenia and monocytopenia, with a differential depletion of thymus-derived lymphocytes as well as various functionally defined lymphocyte subpopulations. Lymphocyte kinetic studies using radioactive chromium-labeled autologous lymphocytes showed that the lymphocytopenia was due predominantly to a transient depletion of the recirculating portion of the intravascular lymphocyte pool. All these parameters returned to normal by 8 a.m. of the following day (off prednisone) and remained normal throughout the day. This very transient lymphocytopenia and monocytopenia after prednisone, with normal cell numbers, proportions, and functions throughout the remainder of the 2-day cycle, was associated with suppression of disease activity, yet did not affect cutaneous delayed hypersensitivity in these patients nor increase the likelihood of infectious complications. This drug-associated cyclic and transient monocytopenia and selective lymphocytopenia is best explained by a redistribution of recirculating lymphocytes

to other body compartments, particularly the bone marrow.

## INTRODUCTION

Alternate-day corticosteroid therapy has become a widely employed and highly effective mode of therapy in a variety of conditions, including childhood nephrotic syndrome (1), chronic asthma (2), renal transplantation (3, 4), as well as various inflammatory or autoimmune diseases (5-8). It is a particularly attractive mode of therapy since the serious cushingoid side effects associated with daily prednisone therapy have been shown to be absent or markedly diminished (9, 10). In addition, cutaneous delayed hypersensitivity remains intact (10), and the increased incidence of infectious complications frequently encountered with long-term, daily prednisone therapy is not found with alternate-day regimens (10, 11).

The diseases in question are thought to be mediated by inflammatory and/or immunological mechanisms. It is assumed but not proved that suppression of disease activity by alternate-day corticosteroid therapy results from the suppression of various measurable parameters of the immune response. If the suppression of the immune response is indeed correlated with the suppression of disease activity, it is unclear how such immunosuppression occurs, in light of intact cutaneous delayed hypersensitivity and apparent lack of increased susceptibility to infection in patients on alternate-day corticosteroid therapy. The present study is aimed at elucidating the extent and the kinetics of the measurable immunosuppression associated with alternate-day prednisone therapy to understand better this dichotomy between clinical immunosuppression and apparently intact host defenses.

Recently, we have demonstrated that a single intravenous dose of hydrocortisone in normal human beings causes a profound and transient lymphocytopenia and

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TABLE I  
Patients in Alternate-Day Prednisone Study

Patient	Age	Sex	Diagnosis	Prednisone dose	Duration of therapy
	yr			mg	mo
Low dose					
J. T.	45	M	Fever of unknown origin*	5	36
L. K.	59	F	Temporal arteritis	15	4
E. S.	59	F	Erythema nodosum	20	48
D. T.	50	F	Granulomatous hepatitis	20	13
H. Y.†	58	M	Granulomatous hepatitis	20	7
Medium dose					
N. D.	35	M	Rheumatoid arthritis	25	84
A. J.‡	52	M	Granulomatous hepatitis	30	36
P. S.	25	F	Lupus erythematosus	30	22
L. D.	43	F	Sarcoidosis	40	2
W. H.	59	M	Fever of unknown origin	40	17
E. K.	59	F	Fever of unknown origin	40	39
High dose					
T. J.	47	M	Pulmonary hemosiderosis	60	1
C. T.	17	F	Chronic idiopathic neutropenia	60	½
L. S.§	66	M	Granulomatous hepatitis	60	18
W. H.§	38	M	Granulomatous panniculitis	100	2
M. S.	43	F	Sarcoidosis	120	9
C. N.	43	M	Polyarteritis nodosa	120	60

\* Cf. Subjects in Methods section.

† These two patients had lymphocyte transformation studies done as well as <sup>51</sup>Cr-labeled lymphocyte kinetic studies. They were receiving the above doses of prednisone during the lymphocyte transformation studies. Both were receiving 60 mg on alternate days during the labeling studies.

§ Only the effect of prednisone on the kinetics of <sup>51</sup>Cr-labeled lymphocytes was studied in these two patients.

monocytopenia, maximal at 4–6 h after injection, with a return to normal by 24 h (12). In addition to the absolute lymphocytopenia, we also noted a differential depletion of lymphocyte subpopulations as measured by a proportionately greater decrease in thymus-derived (T)<sup>1</sup> lymphocytes, and a selective depletion of lymphocyte subpopulations functionally defined by in vitro responses to various mitogens and antigens. A selective depletion of circulating T lymphocytes has also been shown to result from single doses of orally administered prednisone (13).

<sup>1</sup> Abbreviations used in this paper: B, bone marrow-derived; ConA, concanavalin A; HBSS, Hanks' balanced salt solution; MEM-S, Eagle's minimum essential medium modified for suspension culture; PHA, phytohemagglutinin; PWM, pokeweed mitogen; RBC, erythrocytes; SK-SD, streptokinase-streptodornase; SRBC, sheep erythrocytes; T, thymus-derived; TCA, trichloroacetic acid; TT, tetanus toxoid; WBC, leukocytes.

To investigate the mechanisms and kinetics of the immunosuppressive effects of single-dose alternate-day corticosteroid therapy, patients on various doses of alternate-day prednisone were studied at different times during their day on and off the drug, and the following parameters were measured: absolute numbers of circulating lymphocytes and monocytes, relative proportions and absolute numbers of circulating T lymphocytes, and the function of lymphocyte subpopulations as determined by in vitro blastogenic responses to stimulation with various mitogens and antigens. In addition, the effects of alternate-day prednisone administration on the kinetics of radioactive chromium-labeled lymphocytes were also studied.

## METHODS

*Subjects.* 17 patients, 9 men, 8 women, ranging in age from 17 to 66 yr, with a variety of nonneoplastic diseases, were studied while on alternate-day prednisone therapy. The diagnoses, prednisone doses, and duration of therapy at the time of study are listed in Table I. Three patients (J. T.,

W. H., and E. K.) are given the diagnosis of fever of unknown origin. They have been closely followed for 3½–11½ yr at the National Institute of Allergy and Infectious Diseases for frequent, intermittent, febrile illnesses without a specific diagnosis. Extensive and repeated workups over the years have failed to reveal the causes of their fevers. Their fevers have been successfully suppressed with alternate-day prednisone. Attempts to taper and discontinue the drug in these patients have resulted in return of fever.

For purposes of the lymphocyte studies, the patients have been divided into three groups: those receiving a low dose (5–20 mg) of prednisone, those receiving a medium dose (25–40 mg), and those receiving a high dose (60–120 mg) on alternate days. In the subsequent figures and tables in which the data are reported for patients in these prednisone dosage groups, every lymphocyte study was done on each individual patient listed in the particular dosage groups in Table I except for patients L. S. and W. H. Only the effects of prednisone on the kinetics of radioactive chromium-labeled lymphocytes were studied in these two patients. None of the patients were taking any other medications during the study. The patients were studied on consecutive days starting with the day of prednisone administration (on day). Venous blood was drawn at 8 a.m. (0 h) and prednisone was given orally in a single dose immediately thereafter. Blood was then drawn at 12 noon and again at 8 a.m. and 12 noon of the subsequent day (off day). We chose to perform the lymphocyte studies at 0 and 4 h after prednisone since we have previously determined that maximal circulating lymphocytopenia occurs from 4 to 6 h after a single dose of prednisone. Similar studies were done for comparison on 41 normal controls of both sexes, ranging in age from 19 to 55 yr, who were not receiving any medications.

*Cutaneous delayed hypersensitivity testing.* Intradermal testing with a panel of common skin test antigens was performed to evaluate cutaneous delayed hypersensitivity. The antigens used were Dermatophytin "O", 1:100 (Hollister-Stier Laboratories, Spokane, Wash., lot H4886M), mumps skin test antigen (Eli Lilly and Company, Indianapolis, Ind. Lot 7FF43A), tuberculin PPD 5TU (Connaught Laboratories, Toronto, Canada, lot 12115.1), and streptokinase-streptodornase (SK-SD) (Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., lot 5-2201-663, diluted in phosphate-buffered saline, pH 7.4, to 40 U streptokinase and 10 U streptodornase/ml). Skin tests were performed with 0.1 ml of each solution and were read at 48 h. A finding of induration of 10 mm or greater was considered a positive reaction. Skin tests were done either on the day on or the day off prednisone with no noticeable related trend in response. Several patients had the skin tests repeated on both the day on and day off prednisone with the tests separated by a few weeks interval. There were no differences in responses regardless of what day skin tests were applied.

*Total leukocyte and differential counts.* Leukocyte counts were performed with a Coulter Counter (Model F<sub>n</sub>, Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.), and differential counts were performed on peripheral blood smears stained with Wright's stain. 200 cells/smear were counted by the same observer throughout the study.

*Preparation and culture of lymphocyte suspensions.* Mononuclear cells (lymphocytes and monocytes) were obtained by Hypaque-Ficoll density gradient centrifugation (14) of heparinized venous blood obtained at 8 a.m. and 12 noon of both the day on and the day off prednisone. The mononuclear cells were washed three times in Eagle's mini-

mum essential medium modified for suspension culture (MEM-S) (Grand Island Biological Co., Grand Island, N. Y.). For culture, MEM-S was supplemented with 0.02 M L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulfate (National Institutes of Health Media Supply Section), and 15% homologous AB serum (Antibodies Inc., Washington, D. C.). Cultures were done in microtiter plates (Cooke Laboratory Products, Cooke Engineering Co., Alexandria, Va.). Each well contained 0.2 ml of cells in a concentration of  $0.5 \times 10^6$  lymphocytes/ml. Quadruplicate cultures were incubated at 37°C in 5% CO<sub>2</sub> in air, at 100% humidity. 10 µl of mitogen or antigen at various concentrations were added to each well. The mitogens used were: phytohemagglutinin (PHA) MR 68, lot K4402 (The Wellcome Research Laboratories, Beckenham, Kent, England), optimal dose 1 µg/ml of culture; concanavalin A (ConA), lot 2145 (Nutritional Biochemicals Corporation, Cleveland, Ohio), optimal dose 10 µg/ml of culture; pokeweed mitogen (PWM), lot 180690 (Grand Island Biological Co.), optimal dose 20 µl of 1:10 dilution in distilled water/0.2 ml of culture. The antigens used were: SK-SD, optimal dose 24 U streptokinase, 6.75 U streptodornase/ml of culture; tetanus toxoid (TT), lot L 0.246 (Eli Lilly and Co.), optimal dose 1.5 flocculation U/ml of culture. Cultures containing mitogens were incubated for 3 days and those containing antigens were incubated for 5 days. In addition, in several experiments, mitogen cultures were incubated for 4 and 5 days as well, to control for any changes in the kinetics of the cultures caused by prednisone administration. 4 h before harvesting, 0.4 µCi of tritiated thymidine (6.7 Ci/mM, New England Nuclear, Boston, Mass.) was added to each well. The cells were collected from the wells onto fiberglass filters by a semiautomated micro-harvesting device (15). The filters were washed with 10% trichloroacetic acid (TCA) and 95% ethanol, and placed in 10 ml of Aquasol (New England Nuclear). The TCA-precipitable radioactivity was counted in a liquid scintillation counter (Model LS-350, Beckman Instruments, Inc., Fullerton, Calif.). The arithmetic mean of the counts per minute of quadruplicate cultures was determined and the degree of stimulation expressed as the difference in counts per minute per  $10^6$  lymphocytes between stimulated and unstimulated (control) cultures ( $\Delta$  cpm).

*Proportion of circulating T lymphocytes.* The percentage of circulating lymphocytes that formed rosettes with sheep red blood cells (SRBC) was determined (16). In this assay T lymphocytes, but not bone marrow-derived (B) lymphocytes, bind SRBC to form rosettes. To 0.25 ml of the lymphocyte suspension ( $4 \times 10^6$  cells/ml) in Hank's balanced salt solution (HBSS) (National Institutes of Health Media Supply Section) was added 0.25 ml of 0.5% SRBC, which had been neuraminidase treated (17). The mixed cell suspension was incubated at 37°C for 5 min and centrifuged at 200 g for 5 min at 4°C, and the supernatant HBSS was removed. 0.5 ml of fetal calf serum (Gray Industries, Inc., Fort Lauderdale, Fla.), absorbed twice with SRBC, was added and the pellet incubated at 4°C overnight. One-half the volume of supernate was then removed and the cells were gently resuspended by shaking. One drop of cell suspension was put on a glass slide and a cover slip applied. 200 lymphocytes were counted by the same observer throughout the study with phase contrast optics at 400× magnification on a Zeiss microscope (Carl Zeiss, Inc., New York). The observer did not know which patient was being studied nor whether it was the day on or off prednisone. All lymphocytes binding more than three SRBC were considered positive. The total number of

T lymphocytes per cubic millimeter for each individual was determined by multiplying the percent rosettes by the total lymphocyte count.

**Radioactive labeling of lymphocytes.** The effect of alternate-day prednisone therapy on the migration and distribution of radioactive chromium ( $^{51}\text{Cr}$ )-labeled autologous lymphocytes was studied in four patients (H. Y., A. J., L. S., and W. H.). Four normal volunteers receiving no prednisone were used as controls. The protocol for the study was as follows: At 8 am (the day off prednisone for the patient studies), 450 ml of citrate dextrose anticoagulated blood were collected in a sealed, sterile plastic bag to which two satellite bags were connected (Fenwal Laboratories, Inc., Morton Grove, Ill.). Using this system, separation, labeling, and washing of WBC, as well as reinfusion of labeled WBC and unlabeled erythrocytes (RBC) could be accomplished without breaking sterile conditions or exposing the blood components to the air. Separation and labeling of cells were carried out by a slight modification of a previously described method (18). 100 ml of 6% dextran in 0.9% saline (Abbott Laboratories, North Chicago, Ill.) was added to the bag of whole blood and the cells were allowed to sediment at room temperature, at 1 g, for 45 min. The leukocyte-rich supernate was expressed to a satellite bag with a plasma extractor (Fenwal Laboratories), and the platelets were removed by differential centrifugation. The WBC in 20 ml of plasma-dextran were incubated with 500  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  (sodium radiochromate, E. R. Squibb & Sons, Inc., Radiopharmaceutical Dept., Princeton, N. J.) for 30 min at 37°C. 50 mg of ascorbic acid (Eli Lilly and Co.), was then added to reduce the remaining unbound chromate, and so prevent in vivo labeling of other cells after reinfusion. The cells were washed twice with and resuspended in 50 ml of Cr-free autologous plasma-dextran and reinfused over 1-3 min. The viability of the reinfused labeled cells was greater than 95%, as determined by the trypan blue dye exclusion test. The total time from withdrawal of blood to reinfusion of labeled cells was approximately 3 h.

Total lymphocyte-associated radioactivity as well as the specific activity of lymphocytes (cpm/ $10^6$  lymphocytes) were determined from an 8-ml aliquot of labeled cells taken from the bag before reinfusion, and from 20-ml samples of blood drawn at 15 min, 1, 3, 6, 12, and 24 h after the reinfusion. In the patient studies, prednisone was administered after the 24 h blood sample, and further samples were obtained at 4, 8, 12, 24, 28, and 48 h after prednisone. Another prednisone dose was given 48 h after the previous dose and the same blood-drawing protocol as above was repeated, i.e., with samples obtained at 4, 8, 12, 24, and 28 h after drug administration. Normal control subjects were studied in the same manner except that they did not receive prednisone.

The lymphocytes were separated from each sample by the Hypaque-Ficoll method (14). The Hypaque-Ficoll-separated mononuclear cells will hereafter be referred to as "lymphocytes" with the awareness that these cells are approximately 85% lymphocytes, 15% monocytes, and less than 1% granulocytes. During the study, several Hypaque-Ficoll-separated cell samples drawn 24 h or more after the reinfusion of labeled cells were also plated on glass Petri dishes to remove monocytes by adherence, and it was found that, indeed, more than 95% of the radioactivity was associated with the lymphocytes. This was probably due to the fact that most of the reinfused labeled monocytes had left the circulation by this time. Hence, we will refer to the activity of these Hypaque-Ficoll-separated mononuclear cells as

"lymphocyte-associated radioactivity." The yield of lymphocytes in the Hypaque-Ficoll preparations was approximately 85% of the lymphocytes in the whole blood samples. This was consistent and reproducible, and there was no difference in the percentage of lymphocyte yield in Hypaque-Ficoll preparations compared to whole blood in the samples drawn either before or after prednisone administration. Cells were counted in a Coulter Counter, and radioactivity was measured in an automatic gamma counter (Series 1185, Nuclear-Chicago Corp., Des Plaines, Ill.). The total lymphocyte-associated radioactivity remaining in the circulation at each time point was calculated by converting the lymphocyte-associated radioactivity in the 20-ml sample to the amount in the total intravascular blood compartment, assuming blood volume as 7% of body weight (19).

Body surface counts were measured at each time point over the spleen, sternal bone marrow, and liver, with a Tri-Carb scintillation spectrometer with a shielded 2-in thallium-activated sodium iodide crystal (Model 3002, Packard Instrument Co., Inc., Downers Grove, Ill.) (20).

As described above, we chose to label and reinfuse dextran-sedimented WBC rather than separated lymphocytes for several reasons. First, it allows for minimal manipulation of cells before reinfusion, thus lessening the chance of damaging or altering cells. Second, Hypaque-Ficoll separation of lymphocytes before reinfusion, with subsequent labeling of just lymphocytes, would require removing cells from the sterile, sealed bags, allowing a slight but definite chance of contamination that we felt obligated to avoid in these studies. Third, removal of granulocytes and monocytes by passage of cells over nylon or glass bead columns before labeling was undesirable, since this procedure is known to remove selectively certain lymphocyte populations (21, 22).

## RESULTS

**Cutaneous delayed hypersensitivity.** Table II lists the results of cutaneous delayed hypersensitivity testing in each patient. 15 of 17 patients had positive skin tests

TABLE II  
Cutaneous Delayed Hypersensitivity in Patients on  
Alternate-Day Prednisone Therapy

Patient	Prednisone dose	Candida	Mumps	SK-SD	Tuberculin PPD
	<i>mg</i>				
J. T.	5	Neg	+*	ND†	Neg
L. K.	15	+	Neg	+	Neg
E. S.	20	Neg	Neg	+	Neg
D. T.	20	Neg	Neg	+	+
H. Y.	20	Neg	+	Neg	+
N. D.	25	ND	+	+	Neg
A. J.	30	+	Neg	+	Neg
P. S.	30	Neg	Neg	+	Neg
L. D.	40	+	Neg	+	Neg
W. H.	40	Neg	+	+	Neg
E. K.	40	+	ND	+	+
T. J.	60	+	+	+	Neg
C. T.	60	+	+	Neg	Neg
L. S.	60	Neg	Neg	+	+
W. H.	100	+	Neg	+	+
M. S.	120	Neg	Neg	Neg	Neg
C. N.	120	Neg	Neg	Neg	Neg

\* Positive test is the production of 10 mm or more of induration.

† ND, not done.

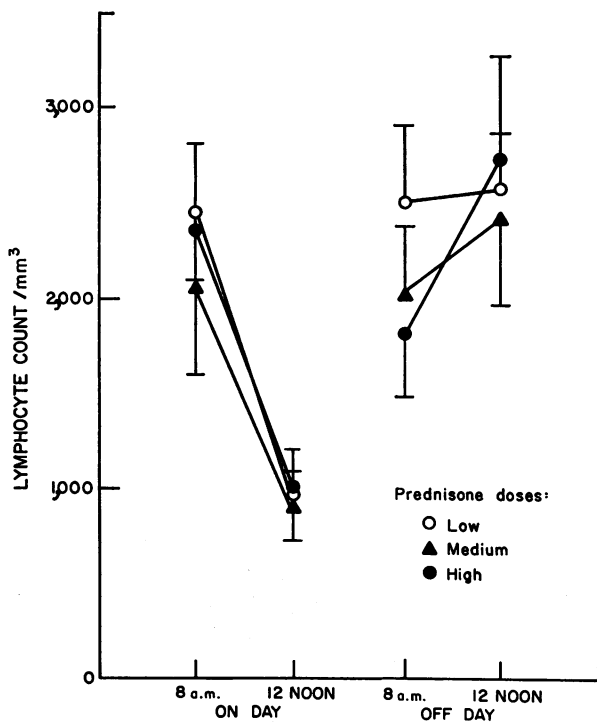


FIGURE 1 Absolute circulating lymphocyte counts on alternate-day prednisone therapy. Mean levels ( $\pm$ SEM) at various times during the day on and the day off prednisone.

to one or more of the four antigens tested. The only two anergic patients (M. S. and C. N.) were receiving 120 mg of prednisone on alternate days; M. S. has sarcoidosis and C. N. is chronically debilitated with polyarteritis nodosa.

**Circulating lymphocytes and monocytes.** The group of normal controls receiving no therapy had mean  $\pm$ SEM lymphocyte counts of  $2,393 \pm 134/\text{mm}^3$  at 8 a.m. and  $2,696 \pm 186/\text{mm}^3$  at 12 noon. The effect of alternate-day prednisone therapy on the total numbers of circulating lymphocytes is shown in Fig. 1. There was no significant difference (Student's *t* test) between the mean total lymphocyte count at 8 a.m. of normal controls and the mean total lymphocyte count at 8 a.m. of either the on day ( $P > 0.2$  for all three prednisone dose groups) or the off day ( $P > 0.2$  for the low and medium-dose groups;  $P > 0.05$  for the high-dose group) of patients receiving alternate-day prednisone. However, 4 h after prednisone there was a significant decrease in circulating lymphocyte counts of patients in all dose groups (low dose,  $P < 0.02$ , medium dose,  $P < 0.05$ , high dose,  $P < 0.05$ ). The lymphocyte counts in all three dose groups returned to normal by 8 a.m. of the off day and remained so through 8 a.m. of the following on day.

The effect of alternate-day prednisone on the total numbers of circulating monocytes is depicted in Fig. 2.

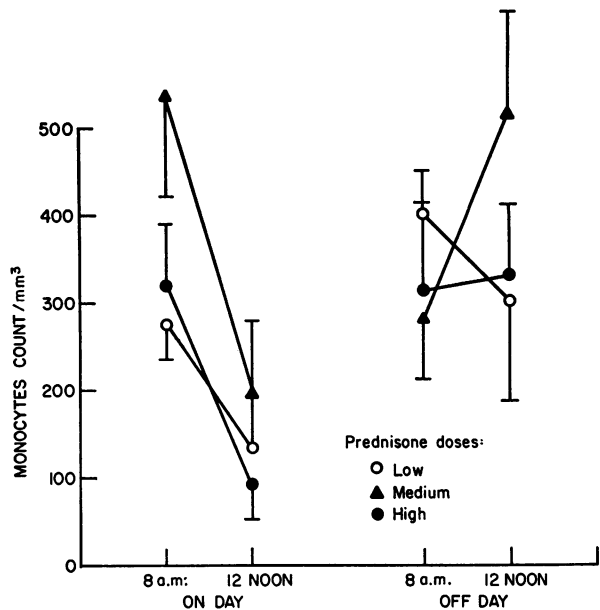


FIGURE 2 Absolute circulating monocyte counts on alternate-day prednisone therapy. Mean levels ( $\pm$ SEM) at various times during the day on and the day off prednisone.

The 10 normal controls receiving no drug had a mean  $\pm$ SEM monocyte count of  $305 \pm 37/\text{mm}^3$  at 8 a.m. and  $359 \pm 35/\text{mm}^3$  at 12 noon. There was no significant difference ( $P > 0.2$ ) between the mean total monocyte count at 8 a.m. of normal controls and the mean total monocyte count at 8 a.m. of either the on day or the off day of patients receiving alternate-day prednisone in the low and high-dosage groups. The mean monocyte count of the medium-dose group was slightly higher ( $P < 0.05$ ) than that of normal controls. 4 h after prednisone, there was a slight but not significant decrease in mean monocyte count in the low-dose prednisone group, while in the medium and high dose groups there were significant decreases in mean monocyte counts ( $P < 0.05$ ) at 12 noon, which returned to normal by 8 a.m. of the off day and remained normal through 8 a.m. of the following on day.

**Circulating T lymphocytes.** Table III shows the effect of alternate-day prednisone therapy on both the proportion and total numbers of circulating T lymphocytes. At 8 a.m. of the on day, both the proportion and total numbers of circulating T lymphocytes in all three dose groups were no different from normal controls. 4 h after prednisone there was a significant decrease in both the proportion and total numbers of circulating T lymphocytes in all three groups, indicating not only an absolute T lymphocytopenia, but also a selectively greater depletion of the T lymphocyte population as compared to the decrease in total lymphocyte count. Both the proportion and total numbers of T lymphocytes returned to nor-

TABLE III  
Effect of Alternate-Day Prednisone Therapy on Circulating T Lymphocytes

	Day	Time	T cells		Total T cells	
			%		no./mm <sup>3</sup>	
Normal controls (n = 10)*	—	8 a.m.	71.0±1.7‡		1,700±90	
		12 noon	71.0±2.2	NS‡	1,900±170	NS
Low-dose prednisone (5–20 mg) (n = 5)	On	8 a.m.	74.4±1.2		1,800±330	
		12 noon	62.4±3.6	P < 0.02	600±150	P < 0.01
	Off	8 a.m.	70.4±1.8		1,800±350	
		12 noon	71.4±1.0	NS	1,800±230	NS
Medium-dose prednisone (25–40 mg) (n = 6)	On	8 a.m.	73.7±3.4		1,600±380	
		12 noon	56.5±6.1	P < 0.05	500±140	P < 0.05
	Off	8 a.m.	72.8±2.9		1,500±310	
		12 noon	72.8±2.7	NS	1,800±400	NS
High-dose prednisone (60–120 mg) (n = 4)	On	8 a.m.	73.8±2.8		1,800±430	
		12 noon	38.0±3.1	P < 0.001	400±100	P < 0.05
	Off	8 a.m.	71.0±1.4		1,300±240	
		12 noon	72.5±1.3	NS	1,900±450	NS

\* (n) is the number of normal controls or patients studied in each group.

‡ Mean ± SEM.

§ Not significant.

|| P values are obtained from a Student's *t* test comparing mean counts at 12 noon with mean counts at 8 a.m. on a given day.

mal by 8 a.m. of the off day and remained so through 8 a.m. of the following on day.

**Lymphocyte blastogenic responses.** Table IV shows the effect of alternate-day prednisone therapy on lymphocyte blastogenic responses to various mitogens and antigens. The data given are for optimal stimulatory doses of mitogens and antigens. In several subjects in whom a range of stimulatory doses was examined, there were no essential differences in the patterns of suppression. In addition, mitogen cultures harvested at 4 and 5 days likewise showed no differences in the patterns of suppression. At 8 a.m. of the on day of prednisone, there was no significant difference between normal controls and any of the three patient groups in the lymphocyte blastogenic responses to the mitogens PHA, ConA, PWM, and the antigen SK-SD. All three patient groups had significantly lower mean lymphocyte blastogenic responses to TT than normal controls (low dose group,  $P < 0.02$ ; medium dose group,  $P < 0.05$ ; high dose group,  $P < 0.05$ ). 4 h after prednisone, at the point of maximal lymphocytopenia, there was a differential decrease in lymphocyte blastogenic responses to various mitogens and antigens of the lymphocytes that had remained in the circulation. To obtain a more reliable

determination of the actual drug-induced changes in response to the various mitogens and antigens 4 h after prednisone, the following analysis was performed: the ratio of the blastogenic responses at 8 a.m. and 12 noon of the on day was compared with the ratio of the responses at 8 a.m. and 12 noon of the off day for each individual patient. In this way, the off day data served as an internal control for normal variability in lymphocyte blastogenic responses between 8 a.m. and 12 noon for each patient. Hence, the ratio ( $r$ ) of (cpm at 12 noon of on day/cpm at 8 a.m. of on day)/(cpm at 12 noon of off day/cpm at 8 a.m. of off day) was calculated for each patient. The mean  $r \pm \text{SEM}$  was obtained for each stimulus in each dosage group. Thus, mean per cent change in stimulation =  $100\% - \text{mean } r$ . To determine the statistical significance of these changes, a Student's *t* test was performed where  $t = \text{mean } \% \text{ change} / (\pm \text{SEM})$  (23). Table V lists the mean change and the statistical significance of these changes. When the changes in blastogenic responses between 8 a.m. and 12 noon of the on and off day were compared, there was no decrease in PHA responses in any of the three prednisone dose groups during the on day. There was a significant decrease in response to ConA and SK-SD in all

TABLE IV  
Effect of Alternate-Day Prednisone Therapy on Lymphocyte Transformation

	Day	Time	Mitogens			Antigens	
			PHA	ConA	PWM	SK-SD	TT
			<i>Δcpm</i>				
Normal controls ( <i>n</i> = 41)*	—	8 a.m.	216,700‡ (±11,700)	52,700 (±4,500)	58,500 (±4,000)	75,800 (±7,200)	64,500 (±5,700)
		12 noon	208,000 (±15,100)	57,400 (±5,900)	57,800 (±4,800)	79,400 (±9,100)	63,100 (±6,900)
Low dose prednisone (5–20 mg) ( <i>n</i> = 5)	On	8 a.m.	196,400 (±28,300)	41,900 (±13,000)	64,500 (±17,200)	50,100 (±14,000)	23,900 (±5,500)
		12 noon	194,400 (±24,700)	32,300 (±15,700)	55,600 (±21,200)	21,843 (±5,500)	23,600 (±6,000)
	Off	8 a.m.	185,000 (±22,700)	37,300 (±13,100)	71,500 (±15,500)	54,000 (±18,900)	24,100 (±5,700)
		12 noon	193,700 (±18,900)	39,000 (±11,200)	66,800 (±13,600)	59,900 (±21,500)	25,800 (±3,400)
Medium dose prednisone (25–40 mg) ( <i>n</i> = 6)	On	8 a.m.	212,300 (±9,500)	37,200 (±8,400)	66,700 (±11,700)	78,100 (±15,700)	28,200 (±6,200)
		12 noon	195,500 (±12,000)	15,600 (±2,600)	38,700 (±10,300)	51,800 (±18,300)	6,300 (±1,200)
	Off	8 a.m.	184,800 (±20,300)	47,900 (±10,800)	73,000 (±9,600)	83,700 (±13,600)	22,400 (±6,300)
		12 noon	209,300 (±28,700)	54,400 (±8,600)	74,900 (±12,700)	83,100 (±13,400)	23,400 (±5,500)
High dose prednisone (60–100 mg) ( <i>n</i> = 4)	On	8 a.m.	169,000 (±35,200)	33,400 (±23,200)	45,700 (±8,700)	47,100 (±28,100)	24,800 (±16,100)
		12 noon	152,200 (±43,200)	12,800 (±9,700)	19,500 (±5,000)	13,100 (±6,400)	8,400 (±6,700)
	Off	8 a.m.	169,900 (±28,300)	37,000 (±16,600)	43,100 (±9,500)	45,100 (±33,900)	27,800 (±17,600)
		12 noon	169,900 (±24,200)	37,300 (±14,100)	45,400 (±7,300)	49,100 (±32,800)	27,900 (±18,400)

\* *n* in parenthesis is the number of normal controls or patients studied in each group.

‡ Mean (±SEM) of radioactivity in stimulated cultures minus radioactivity in unstimulated cultures. Every normal control and every patient in each dosage group was tested with each mitogen and antigen. The mean responses shown are for the number (*n*) of subjects in each group.

three groups and to PWM and TT in the medium and high-dose groups during the on day. As seen in Table IV, the blastogenic responses, when suppressed at 12 noon of the on day, returned to normal by 8 a.m. of the off day and remained so through 8 a.m. of the following on day.

**Labeled lymphocytes.** After reinfusion of labeled cells, there was a rapid equilibration of labeled lymphocytes with the intravascular volume as well as with the extravascular lymphocyte compartments as described in previous studies (18). By 1 h after infusion, the percent of total injected lymphocyte-associated radioactivity remaining in the circulation was less than 50% for patients and controls. To evaluate more appropriately the effect of prednisone on the labeled cells in a steady state after equilibrium with the extravascular lymphocyte compartments had been reached, the amount of lympho-

cyte-associated radioactivity left in the circulation at any given time, as well as the specific activity of lymphocytes, was expressed as the percent of the 1-h value (19).

Fig. 3 illustrates the effect of alternate-day prednisone on the circulating labeled lymphocytes. The upper panel shows that over the 4 days of study, the lymphocyte counts of the untreated normal controls remained relatively constant. In contrast, the patients had a sharp decrease in circulating lymphocyte counts 4 h after each prednisone dose with return to normal counts by 24 h. This is in accord with the findings in the other patients on alternate-day prednisone (Fig. 1). The lower panel of Fig. 3 illustrates that there was no difference between the patients and controls in the lymphocyte-associated radioactivity remaining in the circulation throughout the study. Hence, prednisone administration did not af-



fect the labeled lymphocytes in the circulation at the time of drug administration. The middle panel of Fig. 3 shows that the lymphocyte specific activity of the control subjects followed a linear decay curve, while that of the patients showed a marked increase precisely concomitant with the prednisone-induced lymphocytopenia, with a return of the specific activity to values similar to the decay curve of the controls as the lymphocyte counts returned to normal. This exact phenomenon was seen during the following cycle of alternate-day prednisone dosage. Hence, the lymphocytopenia caused by prednisone was of the nonlabeled lymphocytes, since the amount of lymphocyte-associated radioactivity was unchanged, while the specific activity increased during the decrease in lymphocyte counts.

Surface body counting over various organs (spleen, sternal bone marrow, and liver) indicated that after the initial equilibration of labeled lymphocytes between the intravascular and extravascular lymphocyte compartments, there was a gradual linear decrease in counts over each organ (Fig. 4), similar to that reported elsewhere (19). There was no apparent difference in the distribution of labeled lymphocytes between the organs counted in the controls and patients, and there was no appreciable change in the pattern of surface counting after administration of prednisone. Hence, unlabeled and not labeled lymphocytes were depleted by prednisone administration. Likewise, there was no redistribution of labeled lymphocytes already present in the various organs at the time of prednisone administration that could be detected by surface body counting.

## DISCUSSION

The precise relationships between alternate-day prednisone therapy, clinical immunosuppression, and the suppression of disease activity are uncertain. In the present study several points concerning the circulating lymphocytes and monocytes of patients on alternate-day prednisone therapy are clear. At 8 a.m. of the on day, immediately before administration of prednisone,

TABLE V  
Percent Decrease in Lymphocyte Transformation 4 h after Prednisone on Alternate-Day Therapy

In vitro stimulus	Prednisone dose group					
	Low (n = 5)*		Medium (n = 6)		High (n = 4)	
	Δ%	P	Δ%	P	Δ%	P
PHA	3(±9)‡	NS	19(±8)	NS	14(±19)	NS
ConA	40(±9)	<0.02	61(±7)	<0.001	65(±13)	<0.001
PWM	21(±15)	NS	43(±8)	<0.01	56(±13)	<0.05
SK-SD	50(±11)	<0.01	40(±13)	<0.05	76(±4)	<0.001
TT	14(±15)	NS	78(±3)	<0.001	73(±5)	<0.001

\* (n) is number of patients studied in each group.

‡ Mean percent change (%SEM).

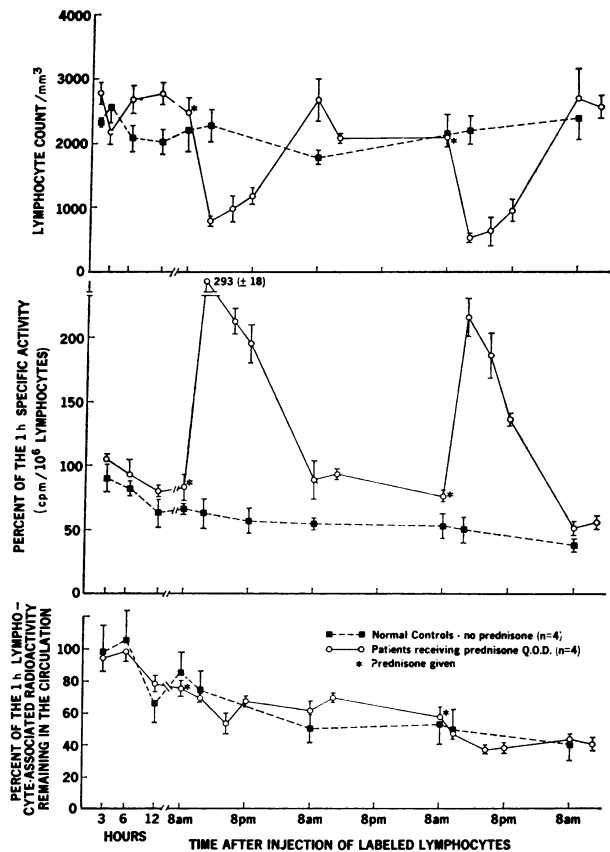


FIGURE 3 Effect of alternate-day prednisone administration on the kinetics of reinfused  $^{51}\text{Cr}$ -labeled autologous lymphocytes. Labeled cells are reinfused at time 0 on the horizontal scale. Data on the vertical scale for the lymphocyte specific activity and the lymphocyte-associated radioactivity remaining in the circulation at a given time are given as the percent of the 1-h value for each subsequent time point. The means ( $\pm$ SEM) for the four normal controls and four patients are given. Patients H. Y., A. J., and L. S. were receiving 60 mg and patient W. H. 100 mg of prednisone on alternate days.

the total circulating lymphocyte and monocyte counts of patients in all three dosage groups were normal. After prednisone administration, there was a profound but transient lymphocytopenia and monocytopenia, which was maximal at 4 h. By 8 a.m. of the off day, the counts returned to normal and remained normal throughout the off day, through 8 a.m. of the following on day when the next cycle of alternate-day dosage began. Hence, in these patients on alternate-day prednisone therapy, chronic treatment, even as long as several years did not result in a cumulative lymphocytopenia and monocytopenia. There continued to be cyclic transient decreases in circulating counts after therapy, with counts decreased for only a few hours every 2 days. This same type of transient lymphocytopenia and monocytopenia was noted after intravenous administration of a single dose of hydrocortisone to normal volunteers (12), while

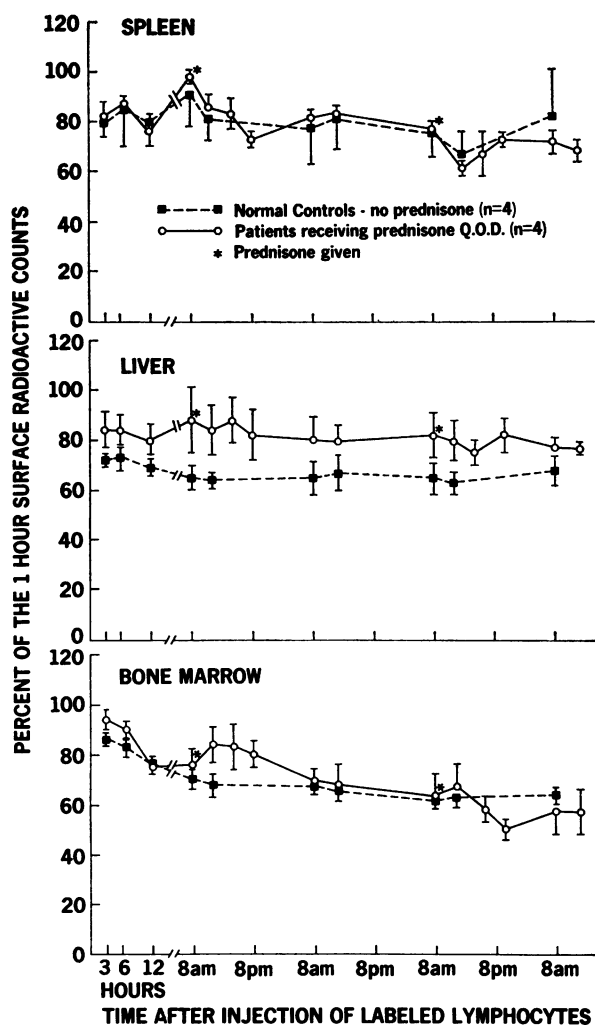


FIGURE 4 Effect of alternate-day prednisone administration on the organ distribution of reinjected  $^{51}\text{Cr}$ -labeled autologous lymphocytes detectable by surface body counting. Labeled cells are reinjected at time 0 on the horizontal scale. Data on the vertical scale for the organ activity are given as the percent of the 1-h value for each subsequent time point. The means ( $\pm$ SEM) for the four normal controls and four patients are given.

similar effects have been reported after oral prednisone in volunteers (13). A similar decrease in circulating lymphocytes has been noted 6 h after prednisone in a group of children with chronic asthma on alternate-day prednisone therapy (24).

The transient lymphocytopenia in the present study was characterized by a more selective decrease in proportion of circulating T lymphocytes, indicating a particular sensitivity of that lymphocyte population to the lymphocytopenic effects of corticosteroids. This phenomenon is similar to the selective depletion of circulating T lymphocytes noted previously with hydrocortisone (12) and prednisone (13) in normal volunteers.

In addition to surface markers, lymphocyte subpopulations can be functionally characterized by their differential response to *in vitro* stimulation by various mitogens (25, 26). In the present study, in addition to normal total lymphocyte counts and normal T cell proportions, functional subpopulations were normal as measured by *in vitro* responses to the mitogens PHA, ConA, and PWM. Coincident with the transient lymphocytopenia after prednisone, there was a differential decrease in responses to mitogens. When the lymphocytes remaining in the circulation at the point of maximal lymphocytopenia were cultured *in vitro* at the standard concentration ( $0.5 \times 10^6$  lymphocytes/ml), the response to PHA was minimally but not significantly reduced, while the responses to ConA in all three patient groups and PWM in the medium and high-dose groups were markedly diminished, indicating a depletion of functional lymphocyte subpopulations.

The mean responses of patients' lymphocytes to the antigen SK-SD were normal. The mean responses to the antigen TT were slightly diminished, possibly reflecting a greater length of time from most recent tetanus boosters among these patients compared to the normal controls. Nevertheless, the *in vitro* lymphocyte responses to SK-SD in all patient groups and to TT in the medium and high dose groups were markedly diminished 4 h after prednisone, with a return to normal in the same pattern as the mitogen responses. We have previously shown that this corticosteroid-induced depression of *in vitro* antigen response could not be totally reconstituted by adding back autologous monocytes to monocyte-deficient cultures and hence probably represents depletion of antigen-reactive cells (12).

The fate of these depleted lymphocytes during this period of corticosteroid-induced lymphocytopenia is uncertain. It is highly unlikely that these cells are destroyed, both because of the rapid return to normal of the levels of circulating cells and because it has been clearly shown that *in vitro* incubation of human lymphocytes with suprapharmacological levels of corticosteroids does not cause cell lysis or affect viability (27, 28). It has been suggested that lymphocytes are redistributed out of the circulation into other body compartments (12, 13). This theory is based on convincing evidence in mice that corticosteroid treatment results in a redistribution and accumulation of T lymphocytes in the bone marrow (29-31). However, the mouse is a "steroid-sensitive" species and it has been emphasized by Claman (32) that the mouse is particularly sensitive to the lymphopenic effects of corticosteroids, while the guinea pig more closely resembles man in its relative resistance to steroid-induced effects on lymphocytes. In this regard, it has recently been shown that in the guinea pig, corticosteroid treatment similarly results in a lymphocytopenia with a concomitant accumulation

of lymphocytes in the bone marrow (33). This evidence is suggestive that a redistribution of circulating lymphocytes into the bone marrow after prednisone may explain the current findings. Labeling studies in the rat have shown that corticosteroids cause a homing of T lymphocytes to the bone marrow (34). This was done by reinfusing  $^{51}\text{Cr}$ -labeled thoracic duct or thymic lymphocytes and examining whole organs for labeled cells during corticosteroid treatment.

The extent to which lymphocyte labeling studies in human beings can provide information about the localization of reinfused peripheral blood lymphocytes after corticosteroid administration is somewhat limited. There are several reasons for this limitation. Peripheral blood lymphocytes consist of two populations of cells. One is a short-lived nonrecirculating population of cells that do not migrate from the circulation and the other is a long-lived recirculating population of cells that freely move into and out of the circulation in equilibrium with the vastly larger extravascular portion of this recirculating pool contained in the thoracic duct lymph, spleen, lymph nodes, and bone marrow (35, 36). When lymphocytes are labeled and reinfused, the labeled recirculating cells become markedly diluted out during the rapid equilibration with the intravascular and extravascular recirculating pools. Precise quantitation by surface body counts of changes in organ distribution is difficult and at best only a gross estimation of true radioactivity in these organs (18). The numbers of lymphocytes that must be labeled as well as the intensity of labeling required to detect changes in distribution within this enormous pool of cells by this method would be unacceptable in human experimentation. However, these labeling studies have provided important information concerning the relative effects of alternate-day prednisone administration on these different lymphocyte pools within the circulation. After the reinfusion of labeled lymphocytes in the normal control subjects, there was an initial rapid equilibration of labeled lymphocytes with the intravascular and extravascular pool, followed by a gradual linear decrease in amount of labeled lymphocytes and specific activity of lymphocytes over the 4-day study period (Fig. 3). The lymphocyte counts remained constant. It is reasonable to assume that the infused labeled recirculating lymphocytes, capable of migrating into and out of the circulation via certain distinct channels (37, 38), have been diluted into the vastly larger total body recirculating pool, and so the labeled lymphocytes remaining in the circulation belong predominantly to the nonrecirculating pool of lymphocytes, which are incapable of migrating out of the circulation (35). Due to the equilibration and dilution mentioned above, the labeled recirculating lymphocytes reinfused have been largely replaced in the circulation by

unlabeled recirculating lymphocytes from the extravascular pool. This theory is strongly supported by the data in Fig. 3. After prednisone administration, despite the profound lymphocytopenia, there is no change in the amount of lymphocyte-associated radioactivity in the circulation, while the lymphocyte specific activity markedly increases. This clearly demonstrates that the prednisone has no effect on the numbers of labeled cells, presumably nonrecirculating lymphocytes, in the circulation at that time.

We can conclude from these data that these nonrecirculating labeled lymphocytes in the circulation are truly a distinct population from the unlabeled recirculating cells that have been depleted by prednisone. If this were not true, and these nonrecirculating labeled cells were depleted by prednisone, then the total lymphocyte-associated radioactivity would have decreased, which is clearly not the case (Fig. 3). The prednisone-induced lymphocytopenia appears to be entirely within the unlabeled recirculating pool. The fact that the lymphocyte specific-activity returns to the normal linear decay slope when the lymphocyte counts return to normal (Fig. 3) indicates that unlabeled recirculating lymphocytes from the extravascular pool must be replacing the unlabeled recirculating lymphocytes depleted by prednisone.

Hence, these labeling studies, though not sufficiently sensitive to indicate precisely where lymphocytes are redistributed after prednisone administration, do demonstrate that the lymphopenic effects of the drug are predominantly on the recirculating lymphocytes capable of normally migrating into and out of the circulation in equilibrium with the much larger extravascular portion of the recirculating pool. Prednisone administration does not deplete the nonrecirculating lymphocytes that are not capable of normally migrating out of the circulation.

13 of our 15 patients had intact cutaneous delayed hypersensitivity and none have had problems with infectious complications of corticosteroid therapy. Thus, these alternate-day prednisone regimens, transiently suppressing specific lymphocyte subpopulations on alternate days, are not appreciably affecting critical host defense mechanisms. However, in several patients in whom tapering of steroid dosage was attempted, flareup of disease activity occurred. Hence, the drug is clearly responsible for suppression of disease activity.

The precise relationship between the transient depletion of these lymphocyte populations and the suppression of disease activity is unclear at present. Should we begin to find direct associations between lymphocyte subpopulations and various immunologically mediated diseases, this selective suppression will assume more clinical relevance.

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