Human Lymphocyte Subpopulations

EFFECT OF CORTICOSTEROIDS

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ABSTRACT Normal subjects given 60 mg of prednisone orally at 8:00 a.m. developed a transient lymphopenia at 2:00 p.m. To define the populations of lymphocytes affected the number and type of lymphocytes in the peripheral blood were assayed. "Late" and "early" spontaneous sheep red blood cell rosettes were used as markers for thymus-derived (T) lymphocytes and one of its subpopulations, respectively. Receptors for aggregated gammaglobulin and complement identified bursal-equivalent or bone marrow-derived (B) lymphocytes and one of its subpopulations, respectively. 6 h after administration of 60 mg of prednisone, the blood samples showed a decrease in proportion of T cells from 69.2±2.1% to 55.9±2.8% (average±SE) and an increase in B-cell proportion from 21.3±2.0% to 44.8± 4.1%. The changes of "early" rosettes and complement receptor lymphocytes also paralleled these. In all cases the absolute numbers of T cells and of B cells were decreased by prednisone. The density gradient distribution of the lymphocytes did not change after prednisone. These data indicate that both T and B lymphocytes are affected by the prednisone but that the T cell lymphopenia was more pronounced. The lymphopenia might reflect either sequestration in the marrow and/or transient arrest of recirculation.

INTRODUCTION

Corticosteroids are a group of drugs commanding much attention in immunological investigations. They have profound effects on lymphocytes in steroid-sensitive species (1), including differential effects on lymphocyte subpopulations in mice and rats.

This group of drugs is also a frequently employed therapeutic agent in the treatment of many human diseases, some of which are immunologically aberrant. Normal human lymphocytes, however, are considered relatively resistant to these drugs because human lymphocytes are relatively refractory to lysis by these drugs in vitro (2).

Here, we define a transient lymphopenia in normal subjects receiving prednisone or hydrocortisone and analyze the steroid-induced changes in peripheral blood lymphocytes. Methods employed include (*a*) identification and quantitation of thymus-derived (T)[1] and bursal-equivalent or bone marrow-derived (B) lymphocytes; and (*b*) density distribution of lymphocytes on discontinuous Ficoll density gradients.

METHODS

Subjects. Subjects were fully informed, consenting volunteers, most of whom belonged to the staff of our laboratories. All received the drug orally at 8:00 a.m. Blood was collected just before ingestion, 6 h later (2:00 p.m.), and at other intervals where indicated. The drug preparation used was prednisone (Parke-Davis & Co., Detroit, Mich.; biochemical name: 17α,21-dihydroxypregna-1,4,diene-3,11,20-trione). Six normal subjects (three men and three women within the same age group as those taking prednisone) served as controls. These control subjects did not take prednisone or any other medication. Blood was also taken at 8:00 a.m. and 2:00 p.m. for similar studies.

Cell counts and lymphocyte separation. Blood samples were collected in heparinized tubes. White blood cell (WBC) counts were made on hemocytometers after dilution in white cell pipettes. Differential counts were made on blood smears with Wright's stain.

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[1] *Abbreviations used in this paper:* AggG, aggregated gammaglobulin; AggGRL, aggregated gammaglobulin receptor lymphocytes; B, bursal-equivalent or bone marrow-derived; concn., concentration; CRL, complement receptor lymphocyte; HBSS, Hank's balanced salt solution; SRBC, sheep red blood cells; T, thymus-derived; WBC, white blood cell.

Lymphocytes were separated from the blood by Ficoll-Hypaque gradient (3).

Identification of T and B cells. Human T cells were recognized by their capacity to form spontaneous sheep red blood cell (SRBC) rosettes (4). Washed SRBC (Davis Laboratories, Berkeley, Calif.) were suspended in 0.5% concentration (concn.) in Hank's balanced salt solution (HBSS) with 10% fetal bovine serum which had been adsorbed with SRBC. 0.4 ml of this was mixed with 0.4 ml of lymphocytes in concn. of 5×10^6 per ml in HBSS. These were incubated at 37°C with 2 drops of a 10-fold dilution of latex particles (0.81 μm, Difco Laboratories, Detroit, Mich.) for 45–60 min, followed by centrifugation at 200*g* for 5 min. Two types of T-cell rosettes were counted: "early rosettes" and "late rosettes." To enumerate "early rosettes" ("active rosettes"[2]), the pellets were resuspended immediately. To enumerate "late rosettes," the tubes were kept at 4°C for 24 h. For early rosettes, 200 lymphocytes were counted. For late rosettes, samples were prepared in triplicate and 100 lymphocytes from each tube were counted. Results were expressed in percent of lymphocytes forming rosettes±SE. A lymphocyte surrounded by three or more SRBC is defined as a rosette. Monocytes were recognized by their ability to ingest latex particles and were excluded from the count.

Human B cells were identified by their receptors for complement (CRL, complement receptor lymphocytes) or for aggregated human gammaglobulin (AggGRL, aggregated gammaglobulin receptor lymphocytes). The method for assaying CRL was similar to that of Ross, Rabellino, Polley, and Grey (4). SRBC were sensitized by 1:200 dilution of rabbit IgM anti-SRBC antibodies (Cordis Laboratories, Miami, Fla.) and then by 1:10 dilution of human serum (fresh frozen) which had been adsorbed with SRBC. The SRBC were then resuspended in concn. of 1×10^8 per ml in HBSS. 0.4 ml of this was mixed with 0.4 ml of lymphocytes and incubated at 37°C with 2 drops of latex particles for 15 min. They were then put on horizontal rotator at 37°C for 15–30 min. Unless specified, tests were done in triplicate.

The method for detecting lymphocytes bearing receptors for aggregated gammaglobulin (AggG) was adapted from that reported by Dickler and Kunkel (5). Lymphocytes were first incubated with aggregated human globulin fraction II and then stained with fluorescein isothiocyanate-conjugated anti-human gammaglobulin antibodies (Custom Reagent Lab, San Diego, Calif.). Cells were counted with epifluorescent and phase-contrast microscope (Zeiss Ultraphadt, Carl Zeiss, Inc., New York) under ×100 oil immersion objective. Note that this method actually identified cells that have receptors for AggG as well as cells with surface immunoglobulins. They will all be grouped as AggGRL cells for abbreviation.

Density distribution of lymphocytes. Density distribution was assessed by equilibrium centrifugation on a discontinuous Ficoll density gradient. Details of the method have been reported (6). The following concn. of Ficoll solutions were used: 5, 12, 15, 19, 22, 25, and 30%. Cells distributed at the interfaces after centrifugation were designated by the concn. of the solutions immediately above it. For example, the layer between 25 and 30% is named the 25% layer. The number of cells in each layer is calculated as the percentage of the total number of cells in the 5–25% layers. We noticed that the density distribution may vary depending on the batch of Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) used. All distributions shown in Table VI are from lot 5534.

Statistics. All values shown are means±SE. The Student *t* test was employed and differences were considered significant if $P < 0.05$.

RESULTS

Lymphocyte conc. In the initial experiments six subjects were given 30 mg and four were given 5 mg of prednisone orally at 8:00 a.m. Blood specimens were obtained at time zero, 4, 5, 6 and 24 h. All counts were done in duplicate. With the 30 mg dose, a large decrease in the absolute concn. of both lymphocytes and monocytes at the fourth hour was reversed at the 24th hour (Table I). After the 5 mg dose, a similar decrease occurred, but the changes were less marked. Subsequently, only zero and sixth hour samples were assayed unless otherwise specified.

Further investigations of eight other subjects (Table II) revealed that there was invariably a lymphopenia after 30 mg of prednisone. The lymphocyte concn. at the sixth hour was only 32±4% of the preprednisone concn. at 8:00 a.m. ($P < 0.001$). The total WBC count, on the other hand, was 6,481±655/mm³ initially and 9,100±721 at the sixth hour ($P < 0.02$), as expected from the well-known capacity of prednisone to cause granulocytosis (7).

13 subjects each received a 60-mg dose of prednisone at 8:00 a.m. instead of a 30-mg dose. In nine of them the total WBC concn. was increased at the sixth hour. All 13 developed lymphopenia (32±5% of the initial count). The extent of leucocytosis and lymphopenia at 6 h was similar to that produced by the 30-mg dose (Table II). Neutrophils made up for most of the cells other than lymphocytes in all instances. The sex and age of the subjects are listed in Table III. None of the subjects were taking any medication (including oral contraceptives) other than prednisone on the day of the study.

Lymphocyte characteristics were studied in the subjects receiving 60 mg of prednisone. This was given at 8:00 a.m. Early SRBC rosettes assayed in five subjects showed no significant change in the proportions at 8:00 a.m. and at 2::00 p.m. (28.1±2.6% and 23.2±2.9%, respectively, Table III). Late SRBC rosettes were assayed in 12 subjects. All except one (S.T.) developed a lower proportion of these rosettes at 2:00 p.m. (69.2±2.1% to 55.9±2.8%, $P < 0.001$). In these 12 subjects, all had an increase in the proportion of fluorescent cells in the AggG test system (21.3±2.0% to 44.8±4.1%, $P < 0.001$). By contrast the CRL assay in eight subjects

[2] Here, the term "early rosette" is used to substitute for "active rosette" used by Wybran and Fudenberg (9). The former term contrasts these with late rosettes and bears no other connotations.

TABLE I

Effect of Prednisone on Concentrations of Total WBC Count and Mononuclear Cell Count in Peripheral Blood of Healthy Volunteers

Hours afrer administration of:	(Cell counts/mm³) ±SE		
	WBC	Lymphocytes	Monocytes
30 mg of prednisone*			
0	8,990±623	2,248±286	551±116
4	9,798±544	1,079±247	61±24
5	10,788±467	970±152	71±33
6	9,562±418	819±138	103±30
24	9,560±1,306	2,773±390	465±140
5 mg of prednisone‡			
0	9,057±1,000	2,808±321	404±64
4	10,845±1,391	1,900±109	139±6
5	9,874±828	1,982±117	270±24
6	10,635±728	2,463±229	402±61
24	9,110±754	2,590±282	513±36

Values are means±SE.
* Six subjects.
‡ Four subjects.

showed a similar trend but no significant differences in the proportion at 8:00 a.m. (10.1±1.1%) and 2:00 p.m. (13.7±1.8%, $P > 0.05$). These averages and the individual results are listed in Table III.

In three additional subjects (listed in Table IV) WBC and lymphocyte concn. were assessed 24 h after ingestion of 60 mg of prednisone; these counts returned to 123±14% and 113±30% of their respective presteroid levels (results not shown). In these patients the proportions of late rosettes and AggGRL, which were abnormal at 6 h, had returned to normal after 24 h (Table IV).

Changes in the absolute concn. of early and late SRBC rosettes, AggGRL and CRL were calculated (Table V). All four types of cells were markedly decreased poststeroid. However they were affected to different extents. Only 24±4.9% of the late rosettes remained at 2:00 p.m., whereas 52.2±9.4% of the AggGRL were still present at that time ($P < 0.02$). The decreases of

TABLE II

Effect of Prednisone on WBC and Lymphocyte Counts in Peripheral Blood

Dose of prednisone (mg)	Presteroid	Poststeroid	$\frac{\text{Poststeroid}}{\text{Presteroid}} \times 100$
	WBC count/mm³		
30*	6,481±655	9,100±721	144±8
60‡	8,200±1,251	10,723±1,168	142±13
Control§	6,433±809	6,783±616	111±13.9
	Lymphocyte count/mm³		
30*	2,197±318	677±79	32±4
60‡	3,045±385	897±123	32±5
Control§	2,866±466	2,562±463	95.1±21.3

* Eight subjects.
‡ Thirteen subjects
§ Six subjects.
At 8:00 a.m. (presteroid values) eight subjects received 30 mg of prednisone and 13 received 60 mg. All poststeroid samples were taken 6 h later (2:00 p.m.).
Values are means±SE.

TABLE III

Effect of Prednisone on Proportions of Early and Late SRBC Rosettes, AggG Receptor Lymphocytes, and CRL in Peripheral Blood

Age, Sex	Subject	Prednisone Dose	Presteroid				Poststeroid			
			Late T rosettes	Early T rosettes	AggGRL	CRL	Late T rosettes	Early T rosettes	AggGRL	CRL
40 F	B. E.	60 mg	71±0.6	—	16.5	7	63.3±0.9	—	41	8
30 F	L. R.	"	62±1.5	—	17	8	50.3±1.3	—	28	13
49 M	R. M.	"	60±1.5	—	36	12.3±0.7	36±0.6	—	72	13.7±0.9
33 F	K. S.	"	78±0.6	—	27.5	10±0.6	68±1.0	—	37	13±1.0
39 M	E. S.	"	66.7±1.5	—	10.3	9.7±0.7	47.3±0.9	—	40	10±0
31 F	F. B.	"	81.3±0.9	27	27	7±0.6	65.5	20.5	33	—
30 M	D. C.	"	79±1.2	21.5	25	10	67±0.9	21.0	58	—
27 M	S. D.	"	68±1.6	37.5	23	10±0	52.7±0.7	15.0	56	15±0.6
22 F	S. T.	"	61.3±0.9	28.5	19	6.3±0.3	69.7±2.8	31.0	41.5	—
50 M	J. D.	"	65±1.2	26.0	18.5	12.3±0.3	53±1.5	28.5	33	25.3±2
25 M	S. G.	"	65.3±1.3	—	16.0	20±0.6	50.7±0.7	—	65	—
31 F	C. C.	"	73.3±0.9	—	20.0	8.0±1.0	58±1.0	—	34	11.7±0.3
	Aver. (12)	"	69.2±2.1	28.1±2.6 (5)	21.3±2.0	10.1±1.1	55.9±2.8	23.2±2.9 (5)	44.8±4.1	13.7±1.8
	Aver. (6)	None	70.4±2.4	17.3±2.7	21.3±2.7	10.4±1.9	68.8±1.4	16.4±1.4	21.8±2.4	10.1±1.6

For each individual the values are percent of total lymphocytes±SE for three determinations. Averages and SE of entire group are given on the last line with the number of subjects shown in parentheses.

the early SRBC rosettes and CRL paralleled these changes (Table V).

The normal variation was studied in six subjects who did not receive the prednisone. Blood was taken at 8:00 a.m. and 2:00 p.m. There was minimal variation of WBC and lymphocyte concn. and T- and B-cell ratio in peripheral blood compared to those who were given prednisone (Tables II and III).

Density distribution of lymphocytes 6 h after administration of 60 mg of prednisone was assayed in five subjects. Lymphocytes remained predominantly at layers 19–25% (Table VI).

Hydrocortisone and plasma cortisol levels. One subject received 240 mg hydrocortisone orally at 8:00 a.m. Blood was collected just before drug administration and at 1, 2, 3, 4 and 6 h thereafter. Plasma cortisol levels were assayed in the UCLA Clinical Laboratories (normal levels 5–25 μg/100 ml). Serial WBC and lymphocyte concn. were also assayed. The plasma cortisol level was 23 μg/100 ml at 8:00 a.m. and reached a peak of 220 μg/100 ml at the second hour. In contrast, the absolute lymphocyte concn. declined slowly from 1,640 to 1,100/mm³ during the first 3 h, reaching a low of 266/mm³ at the fourth hour (Fig. 1). The total WBC count actually rose from 4,700/mm³ to 13,300/mm³ during the first 4 h, and the proportion of lymphocytes fell from 35 to 2%. These results were very similar to those reported elsewhere (8).

DISCUSSION

That transient lymphopenia generally follows corticosteroid administration to children was recently documented (9). In our group of adult subjects, 30-mg or 60-mg doses of prednisone depressed the lymphocyte concn. in peripheral blood (Table II). The cause of this lymphopenia in man is obscure. Here we probed the question by analyzing the changes in lymphocyte characteristics after corticosteroid challenge. Preliminary experiments in a small number of subjects after 30 mg of prednisone were equivocal (results not shown), but statistically significant differences were observed after 60 mg (Table III).

Membrane markers of lymphocyte subpopulations. Ca-

TABLE IV

T and B Cells in Subjects 6 and 24 h after Ingestion of 60 mg Prednisone

Hours after prednisone ingestion	Cell type	Subjects		
		M. U.	M. L.	L. R.
0	T late rosettes	74.3±1.2	61±1.0	66.7±1.8
	T early rosettes	9	8.5	7
	AggGRL	33.3	18.5	19.1
	CRL	16±0.6	10.3±0.3	12.3±0.3
6	T late rosettes	59±0.6	50.7±0.3	57.7±0.9
	T early rosettes	7	7	14.5
	AggGRL	56.4	45.9	28.6
	CRL	17.5±0.5	9.5±1.5	13±1.0
24	T late rosettes	67±1.2	65.7±1.7	70.7±0.7
	T early rosettes	12	6	7
	AggGRL	18.0	16.4	19.0
	CRL	7.5±0.5	6±0	12.5±1.0

pacity to form "late" and "early" SRBC rosettes is a marker for T lymphocytes whereas cells binding AggG or possessing complement receptors are considered B lymphocytes (4, 5). Cells forming "late" SRBC rosettes as measured by our method represent most of the T cells in normal peripheral blood (4). The functional significance of lymphocytes forming "early" SRBC rosettes is not known but selective loss of these cells in some patients with malignancies suggests that the early rosettes represented a distinct subpopulation of the T cells (10).

The AggGRL lymphocytes are B cells (5). The proportion is similar to those detected by surface immunoglobulin. The proportion of CRL on the other hand is $\frac{1}{3}$–$\frac{2}{3}$ of the B cell population in the blood, again suggesting that CRL are a subpopulation of the B cells (4).

Our study showed that subjects who received 60 mg prednisone developed both a T and a B lymphopenia (Table V). The T cells were more adversely affected than the B cells, so that that proportion of the T cells in peripheral blood declined after the prednisone whereas the proportion of the B cells was actually elevated (Table III, V). Within the T cell group, both "late" and "early" SRBC rosettes were equally depressed. Within the B cell group, both AggG- and complement-receptor lymphocytes behaved similarly (Table III, V). The lymphopenia and T cell depression at 6 h returned to the original level 24 h after ingestion of the prednisone (Table IV).

This analysis of T and B cell subpopulations rests on the assumption that there is no or minimal overlapping

TABLE V

Changes in Absolute Concn. of T and B Cells after Ingestion of 60 mg Prednisone

Cell types	Time	Lymph. conc./c.mm±SE	% remaining
Late SRBC rosettes	a.m.	2,146±257 (12)	
	p.m.	467±79 (12)	24±4.9
Early SRBC rosettes	a.m.	735±104 (5)	
	p.m.	215±84 (5)	28.9±11.5
AggG cells	a.m.	731±66 (12)	
	p.m.	380±71 (12)	52.2±9.4
CRL	a.m.	331±47 (8)	
	p.m.	143±31 (8)	44.7±8.3

a.m. = 8:00 a.m., presteroid; p.m. = 2:00 p.m., poststeroid. % remaining is expressed as %±SE. Number of subjects examined is in parentheses.

TABLE VI
Effect of Prednisone on Lymphocyte Density Gradient Distribution

Ficoll concn.	60 mg Prednisone (5) Presteroid	Poststeroid
%	%	%
5	0	0
9	0.1±0.1	0
12	0.4±0.3	0.1±0.1
15	2.5±1.8	0.9±0.4
19	51.6±19.3	47.4±17.1
22	32.9±14.5	44.7±16.0
25	12.7±7.4	0.6±3.4

Proportions are expressed as %±SE and the number of subjects examined is in parentheses.

of the T- and B-cell markers in these subjects before and after prednisone challenge. This assumption is probably justified because overlapping of markers was found in only 0–2% of lymphocytes in normal subjects or in chronic lymphocytic leukemia patients by Bentwish, Douglas, and Siegal (11) and Dickler, Siegal, Bentwich, and Kunkel (12) respectively.

Changes in lymphocyte characteristics after corticosteroid challenge were also suggested in an abstract by Fauci and Dale (13). They challenged their subjects with 400 mg of hydrocortisone intravenously and found that the lymphocyte responses to phytohemagglutinin, pokeweed mitogen, concanavalin A, and antigens were all suppressed. However, the responses were suppressed to different degrees. Their data, like ours, show that different lymphocyte subpopulations have different susceptibilities to corticosteroids.

Density gradient distribution. The density gradient distribution measures the density of lymphocytes. With normal lymphocytes density is generally correlated with cell size in that large cells predominate in the low-density layers containing 5–12% Ficoll (6). A striking increase in the proportion of low-density lymphocytes was reported in a patient on long-term thoracic duct drainage, attributed to an increase in the proportion of lymphoblasts (14). In our subjects receiving prednisone, the lack of appearance of high proportion of low density cells indicated that the changes in lymphocyte characteristics described were not complicated by the addition of an entirely different population, at least as judged by this criterion. The occurrence of the latter would make the previous arguments invalid.

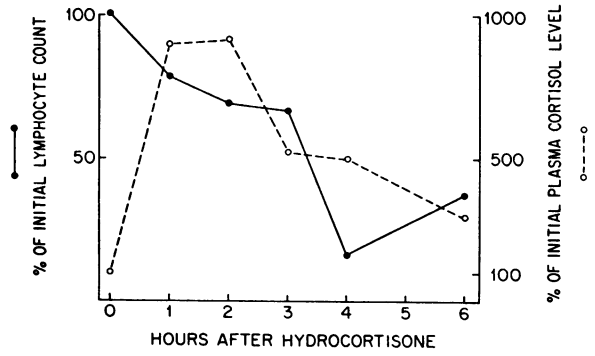

FIGURE 1 Changes in lymphocyte concn. and plasma cortisol levels after an oral dose of 240 mg hydrocortisone.

The mechanism of steroid-induced lymphopenia. Our studies yield no insight into the mechanism of the lymphopenia. The rapid return of lymphocyte count the next day is not a strong argument against direct lympholysis but in vitro studies (2) render this mechanism unlikely. Our data, as summarized in Table VII, are compatible with sequestration of the lymphocytes, either in the bone marrow as in mice (15), or in the lymphoid organs (perhaps because of a transient arrest of lymphocyte recirculation) as in rats (16). In both mice and rats, the predominant effect of corticosteroids is on T cells, and the ultimate consequence is similar to the human situation as demonstrated by us, in that there is a depletion of T relative to B cells in the peripheral blood.

Similar lymphopenia occurred after challenge with hydrocortisone (Fig. 1), suggesting that this effect may be common to most corticosteroids. The relationship between plasma cortisol levels and lymphocyte concn. showed that maximum lymphopenia took place 3 h after plateau cortisol levels had been reached. This lag may represent the time necessary to alter the lymphocyte function. The other possibility is that the lymphocytes were already affected at that instance, but it took 3 h for them to enter the lymphoid organs or bone marrow. Then, as a result of changes in reentry mechanism, they could not return to the blood stream. If so, prednisone challenge may serve as a tool to investigate lymphocyte dynamics in vivo.

The clinical significance of this transient lymphopenia has not yet been explored. In particular, study of sub-

TABLE VII
Summary of Differential Effect of 60 mg of Prednisone on Lymphocyte Subpopulations

Population	Assay	Prednisone effect
T	Late SRBC rosettes	Markedly decreased
	Early SRBC rosettes	" "
B	AggG cells	Decreased
	CRL	"
T and B	Density distribution	No change

jects on chronic, intermittent high-dose corticosteroid therapy is necessary to assess the long-term effects on lymphocyte dynamics and their possible relationship to immunosuppression. Meanwhile, all reports on lymphocyte characteristics of subjects on this group of drugs should be interpreted with caution, since the changes may be time-related.

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