Prednisolone Levels in Plasma and Leukemia Cells During Therapy of Chronic Lymphocytic Leukemia

N. S. PANESAR *, C. C. BIRD *x, B. E. ROBERTS ‡, and J. A. CHILD ‡

Received March 26, 1982, from the **Pathology Department, University of Leeds, Leeds LS2 9JT, England*, and the ‡*Hematology Department, The General Infirmary, Leeds LS1 3EX, England.* Accepted for publication November 12, 1982.

Abstract □ The capacity of chronic lymphocytic leukemia cells to concentrate prednisolone has been evaluated *in vivo* and *in vitro* employing a radioimmunoassay for prednisolone. No evidence to suggest that leukemia cells are capable of selectively concentrating glucocorticoid was found. The relative uptake of glucocorticoid by leukemia cells was found to be greater *in vitro* than *in vivo*. This may be attributable to differences in steroid bioavailability to cells. Substantially lower levels than previously reported of free prednisolone were found in plasma of patients receiving oral treatment with prednisolone. This may result from the failure in previous studies to remove derivatives of prednisolone and other steroids prior to assay for hormones.

Keyphrases □ Prednisolone—levels in plasma and leukemia cells during therapy of chronic lymphocytic leukemia, glucocorticoid □ Lymphocytic leukemia—prednisolone levels in plasma and leukemia cells during therapy, chronic, glucocorticoid □ Glucocorticoids—prednisolone levels in plasma and leukemia cells during therapy of chronic lymphocytic leukemia

Glucocorticoid hormones, usually in combination with other chemotherapeutic agents, are used extensively for treatment of human leukemias and lymphomas. Clinical trials have clearly shown the efficacy of including glucocorticoid in treatment schedules, although the therapeutic mode of action of these steroids is still poorly understood (1). In the main, therapeutic effects have been attributed to the direct cytolethal and/or cytostatic action of the steroid on malignant cells, although glucocorticoids also have a wide variety of other effects on cells and tissues throughout the body (1).

In previous studies with a large series of human lymphoid cell lines (2–6) and freshly isolated leukemia cells from patients with chronic lymphocytic and hairy-cell leukemia (7), it was found that human lymphoid cells are remarkably resistant to the cytolethal and cytostatic effects of glucocorticoids *in vitro* despite the presence of substantial levels of cytoplasmic glucocorticoid receptors and evidence of uptake and binding of glucocorticoid by target cells. In nearly every case lymphoid cells required exposure to suprapharmacological concentration ($>10^{-6}$ M) of steroid for prolonged periods (>48–72 h) before significant cytolethal or cytostatic responses resulted. Since in therapeutic practice glucocorticoids are administered as repeated daily doses of low concentrations of steroid ($<10^{-6}$ M), it seems unlikely that leukemia cells *in vivo* are ever exposed to the glucocorticoid concentrations required to induce cytolethal responses *in vitro*. However, virtually nothing is known about target cell concentrations of glucocorticoids in leukemia patients during therapy and whether or not leukemia cells are capable of concentrating glucocorticoid to an extent that *in vitro* conditions may be simulated.

The results of such an investigation in which the concentrations of free prednisolone in plasma and leukemia cells of a small series of patients with chronic lymphocytic leukemia was measured at various time intervals after administration of a single oral therapeutic dose of prednisolone are reported herein. The results are compared with the *in vitro* uptake of prednisolone by freshly isolated leukemia cells. A radioimmunoassay (RIA) developed specifically to measure prednisolone levels also indicates that previous estimates of free prednisolone in plasma during therapy may be grossly inaccurate.

EXPERIMENTAL

Materials—[^{3}H]Prednisolone (25–50 Ci/mmol)[1] was purified by TLC before use. The xylene-based scintillation fluid contained *p*-bis-2-(5-phenyloxazole)[2] (0.02% w/v), 2,5-diphenyloxazole[3] (0.3% w/v), and isooctylphenoxypolyethoxyethanol[3] (25% v/v). Organic solvents[4] of analytical grade were used as supplied. Pharmaceutical [^{3}H]prednisolone (5 μCi/80 mg)[5] was prepared commercially and used as supplied.

Patients—Ethical restrictions required the complete study to be undertaken with 11 patients with hematologically confirmed chronic lymphocytic leukemia. Six patients (subjects 1–6) were used for the *in vivo* study and five patients (subjects 7–11) were used for the *in vitro* study.

In Vivo *Study*—Subjects 1–4 were given a single oral dose (45 mg/m^2) of prednisolone. In two other patients (subjects 5 and 6) the oral dose of steroid also included 5 μCi of [^{3}H]prednisolone. Blood samples (20–25 mL) were removed from the cubital vein at intervals up to 24 h. Leukemic lymphocytes were separated from other peripheral blood cells in a density gradient[6] (8). Plasma was drawn off without agitating the cell layer and stored at −20°C until assayed. Leukemia cells were washed twice in tissue culture medium[7] containing 10% heat-inactivated fetal calf serum[8], then counted before being stored as a pellet at −20°C. Normally cells were frozen within 1 hr of sample collection.

In Vitro *Study*—Leukemic lymphocytes were separated from the peripheral blood of five previously untreated patients (subjects 7–11) on the density gradient and washed twice with sterile tissue culture medium containing 10% heat-inactivated fetal calf serum. Approximately 25×10^6 viable eells were incubated at 37°C with a saturating concentration of [^{3}H]prednisolone ($7.22 \pm 0.062 \times 10^{-8}$ M) in 2 mL of growth medium. Cells were incubated for 1, 30, 60, and 120 min after which they were immediately centrifuged at 300×*g* for 1 min at 4°C. The pellet was washed with 3 × 2-ml aliquots of ice-cold medium and recovered by centrifugation (300×*g* for 1 min). The whole washing procedure took ~15 min to complete. After the third wash the pellet was resuspended in 2 mL of fresh medium, and 2 × 0.5-mL aliquots, each containing 6.25×10^6 viable cells, were added to 8 mL of scintillation fluid and assayed for radioactivity in a liquid scintillation counter[9] (efficiency ~50%). The remaining fraction was centrifuged, and a 0.5-mL aliquot was removed to determine the radioactivity of the supernatant. The latter was subtracted from the former to determine the activity in 6.25×10^6 viable cells. The intracellular concentration was calculated by assuming an average cell volume of 4.22×10^{-13} L (cell diameter 7.5 nm).

Radioimmunoassay for Prednisolone—Antiserum to prednisolone 21-hemisuccinate conjugated to bovine serum albumin (9) was raised in

[1] Amersham International, Amersham, Bucks, England.
[2] Nuclear Enterprises Ltd., Edinburgh, Scotland.
[3] Sigma London Chemical Co., Poole, Dorset, England.
[4] B.D.H. Ltd.; Poole, Dorset, England.
[5] Upjohn Ltd., Crawley, Sussex, England.
[6] Ficoll, Pharmacia Fine Chemicals AB, Uppsala, Sweden; Triosil, Nyegaard & Co., Oslo, Norway.
[7] RPMI 1640, Flow Laboratories, Irvine, Ayrshire, Scotland.
[8] Gibco Co., Paisley, Scotland.
[9] Nuclear Chicago Isocap 300—G.D. Searle, High Wycombe, Bucks, England.

0022-3549/84/0100-0066$01.00/0

Table I—Plasma and Intracellular Concentrations of Prednisolone in Chronic Lymphocytic Leukemia Patients Following a Single Oral Dose of Prednisolone

Subject		Prednisolone Concentration, M					
		1 h	3 h	6 h	9 h	12 h	24 h
1	Plasma	7.94×10^{-9}	3.33×10^{-7}	1.53×10^{-7}	1.11×10^{-7}	ND[b]	ND
	Intracellular[a]	0.66×10^{-9}	0.90×10^{-9}	0.81×10^{-8}	0.53×10^{-8}	ND	ND
2	Plasma	4.83×10^{-7}	5.05×10^{-7}	2.69×10^{-7}	1.75×10^{-7}	9.16×10^{-8}	1.42×10^{-8}
	Intracellular[a]	1.05×10^{-8}	2.26×10^{-8}	1.18×10^{-8}	ND	2.50×10^{-9}	ND
3	Plasma	7.67×10^{-7}	5.04×10^{-7}	2.21×10^{-7}	1.65×10^{-7}	ND	ND
	Intracellular[a]	1.83×10^{-8}	1.08×10^{-8}	5.46×10^{-9}	3.88×10^{-9}	ND	ND
4	Plasma	7.53×10^{-7}	3.83×10^{-7}	2.06×10^{-7}	1.34×10^{-7}	ND	ND
	Intracellular[a]	1.06×10^{-8}	1.15×10^{-8}	7.30×10^{-9}	3.90×10^{-9}	ND	ND

[a] Determined assuming average cell volume 4.22×10^{-13} L (cell diameter 7 nm). [b] ND = not determined.

Table II—Total and Free Prednisolone Concentrations in Plasma of Chronic Lymphocytic Leukemia Patients Receiving a Single Oral Dose of Radioactive Prednisolone, 5 μCi

Subject		Prednisolone Concentration, M					
		1 h	3 h	6 h	9 h	12 h	24 h
5	Total Steroid[a]	3.90×10^{-6}	3.30×10^{-6}	2.30×10^{-6}	1.8×10^{-6}	1.40×10^{-6}	6.40×10^{-7}
	Free Steroid[b]	8.40×10^{-7}	ND[c]	1.87×10^{-7}	1.09×10^{-7}	3.67×10^{-8}	1.14×10^{-8}
6	Total Steroid[a]	3.90×10^{-6}	2.50×10^{-6}	1.80×10^{-6}	1.40×10^{-6}	1.00×10^{-6}	2.50×10^{-7}
	Free Steroid[b]	7.73×10^{-7}	5.1×10^{-7}	2.18×10^{-7}	1.03×10^{-7}	6.89×10^{-7}	8.30×10^{-9}

[a] Free prednisolone plus metabolites as determined by measuring total radioactivity. [b] Unmetabolized prednisolone assayed by column chromatography. [c] ND = not determined.

New Zealand White rabbits according to the method of Exley *et al.* (10). To plasma or cellular extracts (each cell pellet was resuspended, made up to 5 mL with water, and homogenized) was added ~26,000 dpm of [^{3}H]prednisolone to monitor recovery. These were extracted with dichloromethane, and the extracts were evaporated under a steady stream of nitrogen. The residue was reconstituted in 1 mL of benzene–ethanol (95:5) and loaded on a hydroxypropylated cross-linked dextran chromatography column[10] (10 × 0.8 cm) equilibrated in the above solvent system. The column was flushed with 55 mL of the eluant (benzene–ethanol, 95:5). The fraction that contained hydrocortisone was discarded. The column was further eluted with 30 mL of eluant, and the fraction was collected. This fraction contained virtually all (>99%) of the labeled prednisolone loaded to monitor recovery; there were no breakdown products. Aliquots (0.1 or 0.25 mL) of eluate were evaporated in triplicate for radioimmunoassay. The reference standard tubes containing 0, 17.3, 34.7, 69.4, 138.3, and 277.7 fmol of prednisolone were balanced with 0.1 or 0.25 ml of blank column eluate, and the organic phase was evaporated in a vacuum oven at 30°C. Phosphate buffer (0.1 mL, 0.01 M, pH 7.4) containing ~10,000 dpm of [^{3}H]prednisolone was added to each tube, and the tubes were thoroughly mixed. Antiserum solution (0.1 ml, diluted to 1:4000 with 0.1% gelatin and 0.01% menthiolate in phosphate buffer) was added to each tube, the contents mixed and incubated at 4°C for a minimum of 3 h. Dextran-coated charcoal solution (0.5 mL, 0.025% dextran and 0.25% charcoal in phosphate buffer) was added to each tube, and the solution was agitated and allowed to incubate for a further 10 min at 4°C. The tubes were centrifuged at 1000×*g* for 10 min at 4°C, the supernatant was decanted into a scintillation vial containing 8 mL of scintillation fluid, and the radioactivity was determined. A standard curve was constructed from reference standards and the results for test samples were read directly. The results were corrected for losses and expressed finally as molar concentrations. The intra- and interassay coefficients of variation were <4 and 8%, respectively.

RESULTS

***In Vivo* Study**—Figure 1 shows the mean plasma concentration of free (unmetabolized) prednisolone during the 24-h following a single oral dose of the glucocorticoid. A rapid rise in plasma concentration of free steroid occurred during the first 1–3 h followed by a rapid but progressively declining rate of fall in steroid level until barely detectable levels were reached by 24 h. Table I shows concurrent plasma and intracellular leukemia cell concentrations of free prednisolone during the 24-h period after an oral dose of nonradioactive steroid. As apparent from the results, there is nothing to suggest that leukemia cells are capable of selectively concentrating prednisolone. In Table II, total (unmetabolized and metabolized) and free (unmetabolized) concentrations of prednisolone in plasma of leukemia patients receiving radioactive steroid are shown. It is evident that prednisolone is rapidly metabolized *in vivo*, and 1 h after administration <25% of the total prednisolone remaining in plasma constitutes unmetabolized steroid.

***In Vitro* Study**—The intracellular concentration of prednisolone in leukemia cells as a function of time in the presence of constant extracellular steroid levels is shown in Table III. During the first hour progressive uptake of steroid occurred, but by 2 h the level had declined. In other experiments with cultured human lymphoid cell lines it was found that a similar rise and fall in intracellular steroid levels occurred over the same time interval (2). In no instance did intracellular concentrations of prednisolone reach or exceed extracellular levels.

DISCUSSION

These studies represent the first attempt to measure plasma and target cell concentrations of steroid concurrently in leukemia patients receiving therapeutic doses of glucocorticoid. The comparative *in vivo* and *in vitro* capacity of leukemia cells to concentrate glucocorticoid in the presence of known extracellular steroid levels has also been studied. It is evident that chronic lymphocytic leukemia cells do not possess the capacity for selectively concentrating glucocorticoids *in vivo* or *in vitro*, and steroid levels previously shown *in vitro* (5, 7) to be necessary for inducing cytolethal or cytostatic responses were not achieved in the plasma of leukemia patients receiving therapeutic doses of prednisolone.

The possible reasons for the apparent discrepancy between *in vitro* and *in vivo* glucocorticoid sensitivity of human lymphoid cells have been

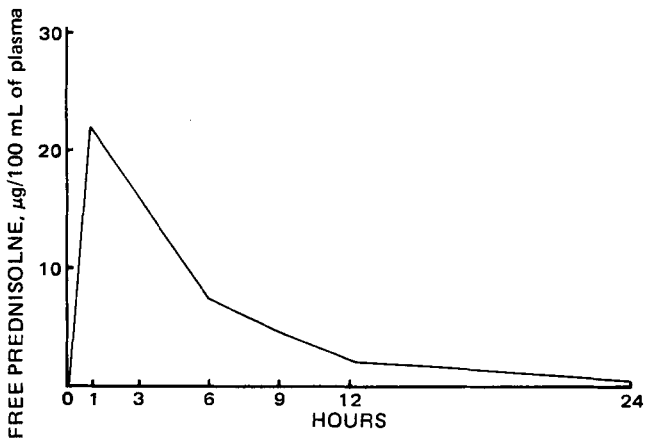

Figure 1—*Mean concentration of free prednisolone in plasma of chronic lymphocytic leukemia patients at various time intervals after a single oral dose of prednisolone.*

[10] Sephadex LH-20—Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Table III—Intracellular and Extracellular Concentrations of Prednisolone in Cultured Chronic Lymphocytic Leukemia Cells

Subject	Extracellular Concentration, M	Intracellular Concentration, M[a]			
		1 min	30 min	60 min	90 min
7	8.1×10^{-8}	1.42×10^{-8}	2.72×10^{-8}	3.02×10^{-8}	2.72×10^{-8}
8	7.0×10^{-8}	1.27×10^{-8}	1.54×10^{-8}	1.53×10^{-8}	1.46×10^{-8}
9	6.8×10^{-8}	1.92×10^{-8}	3.44×10^{-8}	3.42×10^{-8}	3.10×10^{-8}
10	7.2×10^{-8}	1.52×10^{-8}	4.06×10^{-8}	3.83×10^{-8}	3.83×10^{-8}
11	7.0×10^{-8}	1.82×10^{-8}	3.37×10^{-8}	4.36×10^{-8}	4.06×10^{-8}
Mean ± *SD*	$7.22 \pm 0.26 \times 10^{-8}$	$1.59 \pm 0.08 \times 10^{-8}$	$3.03 \pm 0.92 \times 10^{-8}$	$3.23 \pm 1.52 \times 10^{-8}$	$3.03 \pm 1.07 \times 10^{-8}$

[a] Determined assuming average cell volume 4.22×10^{-13} L (cell diameter = 7.5 nm).

Table IV—Comparative Mean Plasma Concentration (μg/100 mL) [a] of Prednisolone Following Oral Steroid Administration

Sampling Time, h	*Colburn* and Buller (11)[b]	*DiSanto* and DeSante (12)[b]	Sullivan *et al.* (13)[b]	Present Study[c]
0	0	0	0	0
1.0	76.1	70.0	115.0	87.5
3.0	56.2	80.7	84.0	65.6
6.0	31.8	49.4	43.5	45.6
12.0	7.1	15.3	10.0	27.5
24.0	0	2.1	1.8	9.3

[a] Calculated as equivalent to a 50-mg oral dose of glucocorticoid. [b] Free (unmetabolized) steroid. [c] Total (unmetabolized and metabolized) steroid.

discussed in detail elsewhere (1). They cannot be attributed simply to differences in cytoplasmic receptor expression or the failure of target cells to take up and bind steroid (2–4, 7). If the therapeutic responses to glucocorticoids are mediated through direct cytolethal and/or cytostatic actions, it is evident the mechanisms involved *in vitro* and *in vivo* differ fundamentally since glucocorticoid concentrations required to induce these effects are of different orders of magnitude. Alternatively, *in vivo* responses to glucocorticoids may be mediated at least in part by indirect means by virtue of some synergistic action when used in combination with other chemotherapeutic agents or through some more generalized tissue or cellular response. Further studies to explore these possibilities are urgently required.

It is also evident from the findings reported here that the intracellular concentration of prednisolone in circulating leukemic lymphocytes was always <4% of that in concurrently removed (extracellular) plasma samples. By contrast, the relative intracellular concentration of steroid achieved *in vitro* was 4–15 times greater than that *in vivo* although in no instance did this reach or exceed extracellular concentrations. The relatively lower uptake of steroid *in vivo* may be accounted for by differences in availability of the glucocorticoid to target cells for a variety of reasons. First, although corticosteroid-binding globulin (transcortin) in plasma has a much lower affinity for prednisolone than hydrocortisone, free prednisolone will be sequestered to some extent *in vivo* by this steroid-binding protein. Since *in vitro* incubations were performed in medium containing heat-inactivated serum no transcortin was present to impede the uptake of steroid. In other experiments it has been found that cells incubated in medium containing non-heat-inactivated serum (thereby containing intact transcortin) show comparatively lower levels of steroid uptake. Second, prednisolone would be sequestered to some extent *in vivo* by adipose tissue with the production of a depot effect and reduced levels of circulating steroid. Third, in contrast to *in vitro* conditions, these results show that glucocorticoids are rapidly metabolized *in vivo* to produce water-soluble derivatives (sulfates and glucuronides), thus reducing the level of free steroid available for uptake by target cells. Finally, leukemic lymphocytes *in vivo* normally compete with other cells and tissues for unbound steroid in contrast to *in vitro* situations where leukemia cells have unrestricted access to the steroid.

In contrast to other workers (11–13), substantially lower concentrations of free prednisolone have been found in plasma following oral administration of the steroid. However, it is apparent from examination of results that the total (unmetabolized and metabolized) prednisolone levels in this study are roughly equivalent to what others have regarded as free (unmetabolized) glucocorticoid (Table IV). This study has shown that prednisolone is rapidly metabolized *in vivo*, and 1 h after oral administration <25% of prednisolone in plasma remains as free (unmetabolized) steroid. It must be assumed, therefore, that any discrepancies in findings between this and other studies are the result of differences in methods employed for assaying steroid levels. In the radioimmunoassay described here samples are purified by chromatography prior to assay to eliminate other cross-reacting steroids. Other workers have failed to remove such metabolites, and it would appear that most previous estimates of plasma concentrations of prednisolone during therapy are grossly inaccurate. Further studies are required to establish the true pharmacokinetics of prednisolone during steroid therapy.

REFERENCES

(1) C. C. Bird, in "7th Tenovus Workshop: Glucocorticoid Action and Leukemia," Alpha Omega, Cardiff, 1979, pp 123–139.

(2) I. D. Barrett, N. S. Panesar, C. C. Bird, A. C. Abbott, H. M. Burrow, and C. M. Steel, *Diagn. Histopathol.*, **4**, 189 (1981).

(3) C. C. Bird, A. W. Waddell, A. M. G. Robertson, A. R. Currie, C. M. Steel, and J. Evans, *Br. J. Cancer*, **32**, 700 (1975).

(4) C. C. Bird, A. M. G. Robertson, J. Read, and A. R. Currie, *J. Pathol.*, **123**, 145 (1977).

(5) H. M. Burrow, C. C. Bird, J. V. Warren, C. M. Steel, I. D. Barrett, and N. S. Panesar, *Diagn. Histopathol.*, **4**, 175 (1981).

(6) A. M. G. Robertson, C. C. Bird, A. W. Waddell, and A. R. Currie, *J. Pathol.*, **126**, 181 (1978).

(7) I. D. Barrett, N. S. Panesar, H. M. Burrow, C. C. Bird, J. C. Cawley, J. A. Child, and B. E. Roberts, *Clin. Lab. Haematol.*, **4**, 285 (1982).

(8) A. Boyum, *Scand. J. Clin. Lab. Invest., Suppl. 97*, **21**, 77 (1968).

(9) B. F. Erlander, F. Borek, S. M. Beiser, and S. Lieberman, *J. Biol. Chem.*, **228**, 713 (1957).

(10) D. Exley, M. W. Johnson, and P. D. G. Dean, *Steroids*, **18**, 605 (1971).

(11) W. A. Colburn and R. H. Buller, *Steroids*, **21**, 833 (1973).

(12) A. R. DiSanto and K. A. DeSante, *J. Pharm. Sci.*, **64**, 109 (1975).

(13) T. J. Sullivan, R. G. Stoll, E. Sakmar, D. C. Blair, and J. G. Wagner, *J. Pharmacokinet. Biopharm.*, **2**, 29 (1974).

ACKNOWLEDGMENTS

Supported by a grant to C. C. Bird from Yorkshire Cancer Research Campaign.