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Research Article

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Virtually total inhibition of protein synthesis with cycloheximide was found to decrease the accumulation of AIB in cells from four patients which had high rates of AIB transport, but had no effect on transport in cells from four patients which accumulated AIB more slowly. These results indicate that active transport depends, in part, upon the presence of labile protein with a turnover rate which varies among different cell populations.

Treatment with 10 μM cortisol for 240 min in vitro reduced the initial rate of AIB-3-^{14}C accumulation (V_o) by 43.4±4.1% (SE) (range, 9-66%) in cells from 16 patients. The degree of inhibition did not vary appreciably over a 9 month period in four of five patients. The […]

Alpha-Aminoisobutyric Acid Transport in Human Leukemic Lymphocytes: In Vitro Characteristics and Inhibition by Cortisol and Cycloheximide

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ABSTRACT We have studied the transport of alpha-aminoisobutyric acid (AIB)-3-^{14}C and its response to cortisol and cycloheximide in vitro in blood lymphocytes from untreated patients with chronic lymphocytic leukemia. The accumulation of AIB-3-^{14}C increased in a linear fashion for 60 min, and reached an apparent steady state in 120 min. The initial rate of AIB accumulation (V_o) varied from 1.1 to 10.2 μmoles/kg cell H_2O per min in cells from 16 different patients; however, V_o was reproducible in cells from five of six patients which were studied repeatedly over 1–9 months, and correlated positively with the lymphocyte count ($r = 0.51$, $P = < 0.01$).

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Treatment with 10 μM cortisol for 240 min in vitro reduced the initial rate of AIB-3-^{14}C accumulation (V_o) by 43.4±4.1% (SE) (range, 9–66%) in cells from 16 patients. The degree of inhibition did not vary appreciably over a 9 month period in four of five patients. The effect of cortisol was proportional to its starting concentration, and developed at low concentrations (0.1–1.0 μM). Cortisol appears to decrease AIB accumulation by inhibiting active uptake, since it neither enhanced the exodus of AIB, nor inhibited apparently nonsaturable transport. Inhibition was noncompetitive in type, suggesting that cortisol decreases the total capacity of the active transport mechanism.

Cortisol inhibited AIB transport indirectly by a process which involved de novo protein synthesis, since inhibition (*a*) appeared only after 60 min of treatment, (*b*) was present in treated cells which were subsequently incubated for 60 min in cortisol-free medium, and (*c*) failed to develop during simultaneous blockade of protein synthesis with cycloheximide, even when cycloheximide itself did not decrease AIB transport.

INTRODUCTION

Although the manifestations of chronic lymphocytic leukemia vary greatly from patient to patient, cellular factors which contribute to this variation are poorly understood. A mounting body of evidence indicates that variations in the behavior of many types of mammalian cells are accompanied by significant alterations in plasma membrane function. Transport of certain free amino acids increases during periods of cell proliferation and heightened cell activity, and diminishes progressively with aging and with decreases in rates of growth (1–9). These observations suggest that an investigation of plasma membrane function might yield important information about the biological behavior of leukemic lymphocytes. In addition, the toxic effect of adrenal glucocorticoids on leukemic lymphocytes (10) which appears to underlie their therapeutic effectiveness in leukemic patients, may stem in part from modifications in plasma membrane function. Glucocorticoids have been found to impair the transport of a variety of substances, including free

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amino acids, in lymphoid cells obtained from experimental animals (11–22).

In the present study, we examine the characteristics of amino acid transport in human leukemic lymphocytes, and its variation in cell populations from different patients, and from the same patient at different times, using the nonmetabolizable amino acid alpha-aminoisobutyric acid (AIB).[1] We also examine the effect of cortisol on AIB transport in order to determine the degree to which cortisol inhibits transport processes in human leukemic lymphocytes, and to explore the mechanism of this inhibition.

METHODS

Lymphocyte isolation. Venous blood was obtained from untreated subjects with chronic lymphocytic leukemia and the blood was anticoagulated with sodium heparin (14 U/ml). White cell and differential counts were performed and the leukocytes isolated after sedimentation of the red cells. 4 vol. of blood was added to 1 vol of 4.0% polyvinylpyrrolidone (PVP) in Hanks's balanced salt solution (HBSS) and incubated at room temperature for 90 min. The supernate was removed and centrifuged for 5 min at 950 *g* in a Sorvall RC-3 General Purpose Centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at 2°C. The cells were suspended in HBSS, centrifuged at 950 *g* and resuspended in HBSS at a final concentration of $1.0–1.2 \times 10^8$ lymphocytes/ml. Red cell contamination was less than 8% as determined by counting 300 cells with a phase contrast microscope. Over 95% of leukocytes were lymphocytes.

Incubation conditions for AIB uptake. Lymphocyte suspensions were added in 0.4 ml portions to flasks containing 2 ml of HBSS with 0.05% albumin (four times recrystallized), or 2 ml of 20% (v/v) autologous or isologous plasma in HBSS, yielding a final concentration of $1.7–2.0 \times 10^7$ lymphocytes/ml. Isologous plasma was obtained from healthy donors of red cell type AB. Cell suspensions were incubated at 37°C in a Dubnoff incubator and shaken at 10 cycle/min in air. Cortisol was added as a solution in absolute ethanol so that the final ethanol concentration was 0.04% (v/v). Untreated cell suspensions received an identical amount of ethanol. AIB, cortisol, and cycloheximide were added at times and in concentrations warranted by each experiment. At termination, the contents of each flask were transferred to cooled 2-ml conical centrifuge tubes (Kimax, Owens-Illinois, Inc., Toledo, Ohio) and sedimented at 950 *g* for 45 sec at 2°C. The supernate was decanted immediately, the side of each tube was dried, and the surface of the pellet was blotted with a thin strip of filter paper. The cell pellet was digested by incubation overnight in a National incubator (National Appliance Co., Portland, Oreg.) at 37°C in 0.5 ml of 0.3 M potassium hydroxide (KOH). $\frac{1}{10}$ ml of KOH digest was added to 10 ml of Bray's solution (23) and its total radioactivity determined in a Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downer's Grove, Ill.). The degree of quenching was estimated by external standardization and disintegrations per minute (dpm) calculated. The protein content of the cell pellet KOH digest was measured by the method of Lowry, Rosebrough, Farr, and Randall (24).

Persistence of the cortisol effect. To examine the persistence of the cortisol effect, lymphocytes were incubated for 3 hr in the presence or absence of steroid. The cells were centrifuged at 950 *g* at 25°C in 2-ml conical tubes (Kimax, Owens-Illinois, Inc., Toledo, Ohio), and resuspended in steroid-free medium, and incubated at 37°C for 60 min at which time AIB uptake was measured.

Incorporation of leucine-1-^{14}C into protein. The inhibitory effect of cycloheximide on protein synthesis was verified by measuring the incorporation of leucine-1-^{14}C into protein. Lymphocytes were incubated in the presence and absence of cycloheximide, 100 μg/ml, for 240 min. Leucine-1-^{14}C was added for the last 2 hr of incubation. Protein was precipitated with 2 ml of 10% trichloroacetic acid (TCA) at 0°C. The resulting precipitate was centrifuged at 950 *g* for 5 min, and washed once with 2 ml of 10% TCA and once with 2 ml of diethyl ether. The TCA-ether insoluble fraction was dissolved overnight at 37°C in 0.5 ml of 0.3 M KOH. $\frac{1}{10}$ ml of the digest and 0.1 ml of the TCA soluble fraction were added to 10 ml of Bray's solution (23) and their radioactivities determined.

Estimation of intracellular water (ICW). Replicate treated and untreated cell suspensions were incubated with sucrose-^{14}C in place of AIB-3-^{14}C and centrifuged in tared 2-ml conical tubes. The wet weight of the pellet was determined and the cell pellet dried at 80°C for 18 hr. Total pellet water was calculated by subtracting the dry pellet weight from its wet weight. The dry pellet was digested in 0.5 ml of 0.3 M KOH and the radioactivity of 0.1 ml of the digest measured in 10 ml of Bray's solution (23). Extracellular trapped water (ECW) was calculated by dividing total pellet radioactivity by the radioactivity per 0.1 ml of the incubation medium. ICW (total pellet water minus ECW) was 65–75% of the total cell weight. Neither cortisol nor cycloheximide altered the ECW or ICW after 1–4 hr of exposure.

Calculation of intracellular amino acid content and expression of transport data. The intracellular accumulation of labeled amino acid per milliliter of ICW (AIB_i) was calculated from the formula:

$$AIB_i = \frac{R_t - (AIB_o \times V_e)}{V_t - V_e},$$

where R_t equals the net radioactivity of the cell pellet, AIB_o the radioactivity per milliliter of incubation medium, V_e the volume of ECW in milliliters, and V_t the total pellet water in milliliters. Results are expressed either as the distribution ratio AIB_i/AIB_o (A_i/A_o) where AIB_i is radioactivity per milliliter of cell water, and AIB_o the radioactivity per milliliter of incubation medium or as V_o, $\mu moles \cdot kg^{-1}$ cell $H_2O \cdot min^{-1}$ (μmoles/kg cell H_2O per min) calculated from the formula: $V_o = AIB_i$/specific activity AIB-3-^{14}C per time of label in minutes. The contribution of apparently nonsaturable transport to the accumulation of AIB was estimated according to the method of Akedo and Christensen (25) and kinetic data were adjusted to represent saturable transport.

Materials. Cortisol of the highest purity was purchased from Calbiochem, Los Angeles, Calif. AIB-3-^{14}C (1 mCi/mmole), leucine-1-^{14}C (4 mCi/mmole), sucrose-UL-^{14}C (5 mCi/mmole) were obtained from New England Nuclear

[1] *Abbreviations used in this paper:* AIB, alpha-aminoisobutyric acid; ECW, extracellular trapped water; FTR, fractional turnover rate; HBSS, Hanks's balanced salt solution; ICW, intracellular water; PVP, polyvinylpyrrolidone; V_o, initial rate of AIB accumulation.

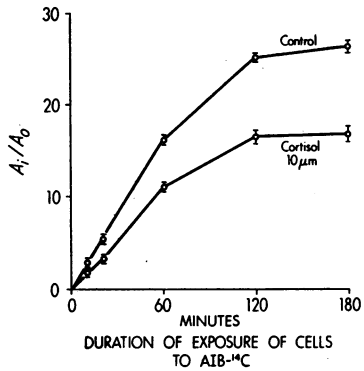

FIGURE 1 Time-course of AIB accumulation in cortisol-treated and untreated leukemic lymphocytes. Cells from patient W. F. were incubated for 240 min in the presence or absence of 10 μM cortisol. AIB-3-^{14}C was then added to each flask so that its concentration in the medium was 10 μM, and the incubation was continued for an additional 10–180 min. The data represent distribution ratios of AIB-3-^{14}C (A_i/A_o) at each time point. Longer periods of labeling (e.g. 180 min) were necessarily associated with longer periods of exposure (e.g. 420 min) to cortisol. These experimental conditions would not be expected to cause a greater degree of inhibition than that seen in cells which were treated for a shorter period of time, since cortisol inhibition appears to be maximal in 3–4 hr (see Fig. 5).

Corp., Boston, Mass., and cycloheximide from Sigma Chemical Co., St. Louis, Mo.

Statistical methods. Means, variances, correlation coefficients, coefficients of variation, and regression lines were calculated by standard formulas.

RESULTS

Initial rates and equilibrium values of AIB transport in untreated and cortisol-treated leukemic lymphocytes. The accumulation of AIB by leukemic lymphocytes increased in linear fashion for the first 60 min of exposure to AIB-3-^{14}C and reached an apparent steady state in 120 min (Fig. 1). In order to compare AIB transport in leukemic lymphocytes from different patients, we examined initial rates of AIB accumulation (V_o), which were calculated from the amount of AIB-3-^{14}C accumulated during the first 20 min of exposure to AIB, after a preliminary incubation for 4 hr without added AIB. These studies revealed marked variation in the magnitude of V_o between cell populations from 16 patients (Table I), but little variation among quadruplicate samples from the same cell population (Table I legend). The source of plasma added to the incubation medium did not influence the rate of AIB accumulation. V_o was virtually constant in cells from the same patient incubated in medium containing isologous plasma from each of several different sources, or in autologous plasma (data not shown). There was a significant positive correlation ($r = 0.51$, $P < 0.01$) between V_o and the lymphocyte count (Fig. 2) indicating that about 25% ($r^2 = 0.25$) of the variation in AIB accumulation or in lymphocyte count is due to variation in the other.

TABLE I

Initial Rates of AIB Accumulation (V_0) by Cortisol-Treated and Untreated Lymphocytes from Patients with Chronic Lymphocytic Leukemia

Subject	White cell count	V_o Control	V_o Cortisol	Cortisol inhibition
	× 10³/μl	μmoles/kg cell H_2O per min		%
F. Ho.	240	10.2	4.9	52
L. K.	900	6.4	5.1	20
A. M.	35	6.1	3.0	51
B. R.	365	6.1	3.7	39
F. Ha.	131	4.9	1.7	65
V. S.	240	4.8	3.1	35
L. S.	208	4.4	3.0	32
A. B.	98	4.1	1.4	66
M. M.	172	3.7	1.8	51
G. B.	160	3.3	1.4	58
W. F.	87	2.7	1.7	37
G. L.	46	2.3	1.4	39
F. A.	31	2.3	1.2	48
K. C.	21	1.6	1.1	31
E. F.	90	1.3	0.5	62
H. S.	36	1.1	1.0	09
Mean ±SE		4.1±0.6	2.3±0.3	43.4±4.1

Lymphocytes from each patient were incubated at 37°C for a total of 260 min in the presence or absence of 10 μM cortisol. AIB-3-^{14}C was added to each cell suspension 20 min before termination in a concentration of 10 μM. Each value for V_o represents the mean of four separate cell suspensions which were incubated and prepared simultaneously. The average coefficient of variation for these quadruplicate observations was 11.4%.

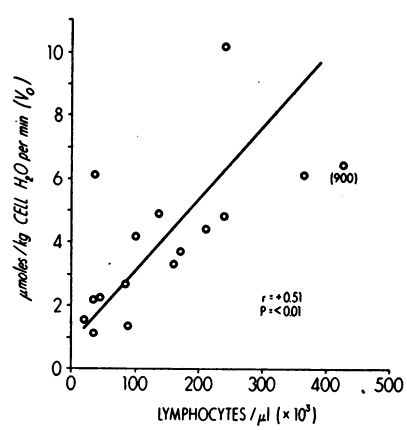

FIGURE 2 Correlation of V_o with blood lymphocyte count in 16 patients with chronic lymphocytic leukemia. Individual values are also given in Table I.

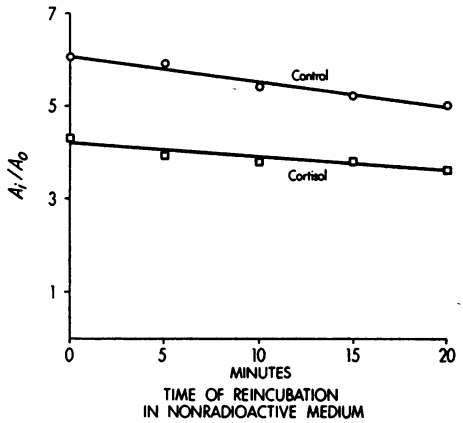

FIGURE 3 Effect of cortisol on the exodus of AIB-3-^{14}C. Cells from patient A. B. were distributed into 50-ml Erlenmeyer flasks containing 25 ml of incubation medium, with or without cortisol, 10 μM. After incubation for 180 min, AIB-3-^{14}C was added to each cell suspension so that its concentration in the medium was 3.3 μM, and the incubation continued for an additional 120 min. The reduced concentration of AIB was used so as to obtain steady-state cellular AIB concentrations which approximated those seen after 20 min of exposure to AIB-3-^{14}C. Cells from each flask were harvested by centrifugation, and resuspended in AIB-free medium at 37°C with or without cortisol according to their treatment during the initial period of incubation. The resuspended cells were transferred to new 50-ml flasks, and incubated for 20 min. 2-ml portions of each cell suspension were removed at the start of the incubation, and every 5 min thereafter, and the cellular concentration of AIB-3-^{14}C was estimated as described under Methods. The data are given as distribution ratios of AIB-3-^{14}C (A_i/A_o) at each point in time.

Treatment with 10 μM cortisol for 4 hr reduced the initial rate of AIB accumulation (V_o) in cells from 16 patients by a mean of 43.4±4.1% (SE). A statistically significant ($P < 0.05$) reduction appeared in cells from 15 of 16 patients (Table I). Cortisol was found to inhibit V_o and the apparent steady-state accumulation of AIB to the same degree (Fig. 1).

The fact that cortisol decreased the initial rate of accumulation suggests that it inhibited the uptake of AIB, since exodus would not be expected to modify net AIB accumulation appreciably during this period. This interpretation was substantiated by the failure of cortisol to enhance the exodus of AIB from cells which had been exposed to AIB-3-^{14}C for 180 min before transfer to a medium free of AIB (Fig. 3). The fractional turnover rate (FTR) of AIB-3-^{14}C was calculated by the formula $(\ln(AIB)_{t_1} - \ln(AIB)_{t_2})/(t_2 - t_1)$, where t_1 = zero time and t_2 = 20 min. The FTR of AIB from cortisol-treated cells was less (0.0069) than that of control cells (0.0099); however, this difference was accounted for by the higher gradient for exodus in control cells. Initial pool size (concentration) of AIB was 48% greater and FTR 43% greater in control as compared with cortisol-treated cells. These data indicate that the inhibition of AIB accumulation by cortisol at apparent steady state is not explained by an acceleration of exodus.

Reproducibility of AIB accumulation and of sensitivity to cortisol. Since the magnitude of V_o and cortisol inhibition varied over a wide range in different populations of leukemic cells, we studied cells from the same patients at different times over a 9 month period to determine the reproducibility of amino acid accumulation and cortisol sensitivity. Little variation in V_o was seen on the numerous occasions when an individual patient was studied on successive days or weeks, suggesting that the differences in V_o between patients did not stem from experimental variation. There was a small to moderate degree of variation in V_o in cells from five of six patients (W. F., G. B., M. M., V. S., and L. K.) restudied at longer intervals (Table II), and marked variation in one patient (A. B.). The degree of cortisol inhibition did not vary appreciably in cells from five patients studied at different times (G. B., V. S., M. M., A. B., and L. K.) and varied greatly in one patient (W. F.).

Effects of cortisol on the kinetic parameters of active AIB transport and on transport which is apparently non-

TABLE II

Reproducibility of AIB Accumulation and Cortisol (10 μM) Sensitivity in Six Patients

Patient	Date	Control	Cortisol	Cortisol inhibition
		μmoles/kg cell H_2O per min		%
W. F.	9–70	2.7±0.1	1.7±0.1	37
	1–71	2.9±0.1	1.3±0.1	55
	3–71	3.1±0.2	2.4±0.1	23
	5–71	2.5±0.1	1.7±0.1	32
	6–71	2.9±0.2	—	—
G. B.	11–70	3.3±0.3	1.4±0.1	58
	2–71	3.2±0.4	1.5±0.05	53
	6–71	4.3±0.4	1.7±0.3	60
M. M.	11–70	3.7±0.4	1.8±0.2	51
	12–70	3.2±0.3	1.9±0.4	41
	5–71	2.9±0.1	1.5±0.1	48
V. S.	1–71	4.8±0.2	3.1±0.2	35
	1–71	4.9±0.3	3.3±0.06	33
	6–71	3.7±0.2	—	—
A. B.	11–70	4.1±0.8	1.4±0.1	66
	6–71	1.5±0.1	0.6±0.04	60
L. K.	10–70	6.4±0.3	5.1±0.2	20
	11–71	6.9±0.2	6.1±0.2	12

Lymphocytes were treated as described in Table I. Each value represents the mean ±SE of quadruplicate determinations of V_0 (micromoles/kilogram cell H_2O per minute).

saturable. To determine more precisely the nature of cortisol action on AIB uptake, we studied the kinetic parameters of active transport by leukemic lymphocytes. Graphic representation of V_o according to the method of Lineweaver and Burke, at AIB concentrations ranging from 10 to 500 μM, indicated the presence of a single transport process having an apparent K_m of 0.48 mM and a V_{max} of 200 μmoles/kg cell H_2O per min (Fig. 4). Treatment with 10 μM cortisol for 4 hr markedly decreased the V_{max} but failed to alter the apparent K_m appreciably, suggesting, under conventional assumptions, a decrease in the total capacity of the transport mechanism for AIB rather than a decreased affinity of AIB for the cells. We performed additional experiments 2 wk later with cells from the same patient, using a higher concentration range of AIB (500 μM to 5 mM), and represented the results in an alternate graphic form, V_o vs. V_o/C_s, which has been found by Dowd and Riggs to give statistically more reliable estimates for apparent K_m and V_{max} (26) (not shown). These experiments yielded kinetic parameters (V_{max} = control, 183 μmoles/kg cell H_2O per min; cortisol 82 μmoles/kg cell H_2O per min; K_m = control, 0.62 μM; cortisol, 0.51 μM) which were similar to those obtained with the Lineweaver and Burke representation, and therefore support the interpretation that cortisol inhibits the total capacity for AIB transport. The similarity of the kinetic parameters observed at 10–500 μM AIB and at 500 μM to 5 mM AIB is consistent with the presence of a single active mechanism for AIB transport.

To determine whether cortisol inhibits nonsaturable as well as saturable uptake of AIB, cortisol-treated and untreated lymphocytes were labeled with a concentration of AIB (20 mM) at which apparent nonsaturable uptake accounts for 80% of AIB accumulation over the 20 min of study (see Fig. 4 for V_{max}). Cortisol had no effect on the accumulation of AIB under these conditions (Table III) suggesting that its action is limited to the saturable mechanism for AIB transport.

Time-course, dose-response relationships, and persistence of cortisol effect. Glucocorticoids are known to inhibit the uptake of free amino acids in rat thymic lymphocytes in vitro (19–22). This inhibition appears to arise indirectly, since it develops at low steroid concentrations, is delayed in onset, depends on continued protein synthesis, and persists after treated cells are transferred to steroid-free medium (19–22). To determine whether cortisol inhibits AIB uptake in human leukemic lymphocytes by a similar indirect mechanism, we examined the time of onset and disappearance of cortisol inhibition and its dose-response relationships. No statistically significant effect was produced by 1 hr of treatment in any of the four patients studied (Fig. 5). Statistically significant inhibition was evident at 2 hr in the one patient studied

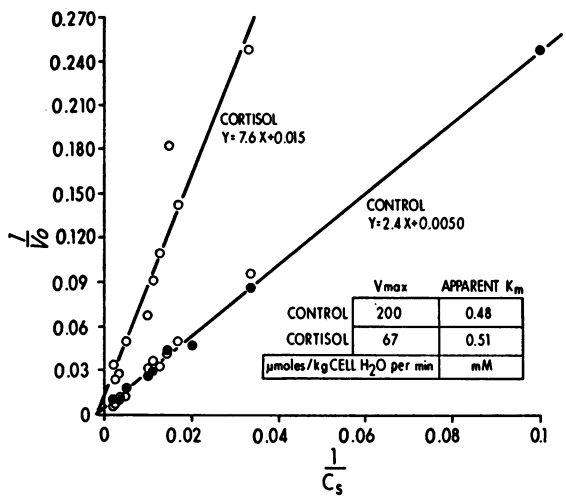

FIGURE 4 Lineweaver-Burk representations of initial rates of AIB-3-^{14}C accumulation (in this case expressed as micromoles/kilogram cell H_2O per minute) at concentrations of AIB (C_s) ranging from 10 to 500 μM. Lymphocytes from patient M. M. were incubated for 260 min in the presence or absence of cortisol, 10 μM, and were exposed to AIB-3-^{14}C during the final 20 min of this period. Various AIB concentrations were achieved by adding an appropriate amount of nonradioactive AIB and a constant amount of AIB-3-^{14}C to the cell suspensions. The experimentally observed points were found to satisfy the assumption of linearity required for linear regression analysis which was used to obtain equations defining the two lines. The data for untreated cells are the composite of two separate experiments performed at a 30 day interval. These experiments produced virtually identical kinetic parameters for AIB-3-^{14}C accumulation.

at that time and at 3 hr in all patients. In order to determine whether established cortisol inhibition persisted in the absence of steroid, we examined AIB uptake in lymphocytes which had been treated for 180 min and then transferred to steroid-free medium. Marked inhibition of AIB accumulation persisted for 60 min after

TABLE III

Effect of Cortisol (10 μM) on the Accumulation of AIB at High and at Low AIB Concentrations

10 μM AIB		20 mM AIB	
Control	Cortisol	Control	Cortisol
μmoles/kg cell H_2O per min			
4.3±0.3	1.7±0.2	1100±25	1050±26

Lymphocytes from patient G. B. were treated and incubated with AIB-3-^{14}C as described in Table I. AIB-3-^{14}C was diluted with nonradioactive AIB before addition in order to achieve a concentration of 20 mM. Each value represents the mean ±SE of quadruplicate determinations of V_0 (micromoles/kilogram cell H_2O per minute).

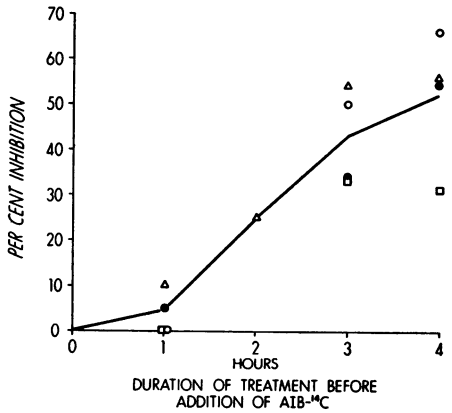

FIGURE 5 Time-course of cortisol inhibition of AIB transport. Lymphocytes were obtained from four patients (○, A. B.; △, W. F.; ●, G. B.; □, V. S.) and incubated in the presence or absence of cortisol (10 μM) for 1, 2, 3, or 4 hr. The cell suspensions received AIB-3-^{14}C 20 min before the termination of each treatment period. For convenience of presentation, data are presented as per cent inhibition. Periods of treatment longer than 4 hr caused no greater inhibition. Absolute values for V_0 at 4 hr of treatment appear in Table II.

transfer in lymphocytes from two patients (54 and 47%, respectively). In studies with two separate patients, the degree of inhibition of AIB uptake was directly related to the starting concentration of cortisol (Fig. 6). Moreover, as little as 0.1 μM cortisol was inhibitory in cells from one patient (A. B.).

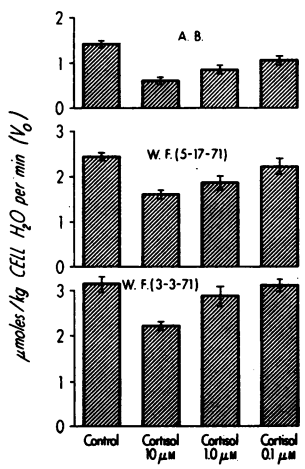

FIGURE 6 Degree of inhibition of AIB transport by cortisol in concentrations ranging from 0.1 to 10 μM. Lymphocytes were treated as indicated in the figure for 260 min, including a final 20 min period of labeling with 10 μM AIB-3-^{14}C. All three concentrations of cortisol inhibited AIB accumulation to a statistically significant degree ($P < 0.01$) in cells from patient A. B., whereas only 10 μM cortisol produced a statistically significant effect ($P < 0.01$) in cells from patient W. F.

Effects of cycloheximide on AIB transport and its inhibition by cortisol. Cycloheximide, a potent inhibitor of protein synthesis in lymphoid cells (*27*), has been shown to decrease the uptake of AIB in rat thymic lymphocytes (*22*), suggesting that labile protein may be involved in the transport of low molecular weight substrates across the lymphocyte plasma membrane. Hence, cortisol could decrease AIB transport in leukemic lymphocytes by inhibiting the replenishment of such a labile transport protein. Alternatively, cortisol could act by a mechanism which requires *de novo* protein synthesis (e.g. by inducing the synthesis of a protein which inhibits transport). To differentiate between these possibilities, lymphocytes were incubated in the presence of cycloheximide, cortisol, or both. Treatment with cycloheximide for 4 hr inhibited the transport of AIB to a statistically significant degree in cells from only four of eight patients (Table IV). Studies performed in parallel (data not shown) revealed that cycloheximide inhibited the incorporation of leucine-1-^{14}C into protein by more than 95% whether or not it decreased AIB transport. The same results were obtained when one cycloheximide-sensitive and one cycloheximide-resistant cell population were studied again (Table IV). It is of particular interest that the four cell populations which responded to cycloheximide with decreased AIB accumulation had higher rates of AIB transport than any of the nonresponsive populations. Moreover, cycloheximide inhibited AIB accumulation to the greatest degree in cells from

TABLE IV
Effect of Cortisol and Cycloheximide on the Accumulation of AIB

Subject	Control	Cortisol, 10 μM	Cycloheximide, 360 μM	Cortisol + cycloheximide
Sensitive to cycloheximide				
1. F. Ho.	10.2	4.9	4.4	—
2. V. S.	4.8	3.1	3.6	3.4
3. F. Ha.	4.9	1.7	3.2	3.6
4. L. S.	4.4	3.0	3.4	3.3
Mean ±SE	6.1±1.4	3.2±0.7	3.7±0.3	3.4±0.1
Resistant to cycloheximide				
5. G. B.	3.3	1.4	3.6	3.6
6. M. M.	2.9	1.5	2.8	3.0
7. F. A.	2.3	1.2	2.0	1.9
8. W. F.	2.5	1.7	2.8	2.8
Mean ±SE	2.7±0.3	1.5±0.1	2.8±0.3	2.8±0.4
Reexamination (R)				
2R V. S.	3.7		2.9	
8R W. F.	2.9		3.2	

Lymphocytes were treated as indicated for a total of 260 min, and received AIB-3-^{14}C 20 min before termination. Each value represents the mean of quadruplicate estimations of V_0. Variances for quadruplicates are not shown. Differences between control and cortisol values (patients 1–8) are all statistically significant ($P < 0.05$). Differences between control and cycloheximide values are statistically significant ($P < 0.05$) only in cells from patients 1–4.

patient F. Ho., which transported AIB more than twice as rapidly as cells from other patients. Cycloheximide prevented the appearance of cortisol inhibition in cells from all eight patients when they were treated with both agents simultaneously (Table IV). The fact that virtually complete blockade of protein synthesis failed to inhibit AIB uptake in some cases, yet prevented cortisol inhibition in all cases is most suggestive of a mechanism in which cortisol acts by promoting the synthesis of new protein.

DISCUSSION

The presence of intrinsic differences in the active mechanism for AIB transport best explains the observed variation in V_o among different leukemic lymphocyte populations. Experimental variation, and differences in the extracellular concentrations of other free amino acids and in the rate of protein synthesis do not appear to account for these differences, since V_o was quite reproducible in cells from the same patient at different times and incubated in plasma from different sources, and was not altered by cycloheximide in some cell populations. The fact that V_o could be correlated with the blood lymphocyte count suggests that transport of free amino acids might determine the ability of lymphocytes to proliferate or survive. This possibility is supported by the previous demonstration that an acceleration of AIB transport precedes the proliferation of human lymphocytes induced by phytohemagglutinin in vitro (8).

The ability of cycloheximide to inhibit AIB transport in some lymphocyte populations provides indirect evidence for the participation of protein with a short half-life in the transport system, as has been proposed in other tissues (e.g. rat diaphragm, thymic lymphocytes, embryonic chick bone [17, 28–31]). Although inhibition of protein synthesis could have decreased transport by disrupting cell function generally, or by increasing extracellular concentrations of competitive free amino acids, the failure of cycloheximide to modify the transport of AIB in half of the cell populations studied despite near total inhibition of protein synthesis argues against these possibilities.

The nature of this putative transport protein is unknown. However, the finding that the magnitude of V_o was greater in cycloheximide-sensitive than in cycloheximide-resistant cells suggests that it may be of considerable functional significance. Differences in its rate of turnover could determine the overall rate of amino acid transport. Alternatively, the active transport of AIB might be mediated by two separate mechanisms, one of which depends upon such a protein, but is not present in all leukemic lymphocyte populations. Furthermore, the magnitude of V_o, which is related to the mass of circulating lymphocytes as well as cycloheximide sensitivity, might be a function of cell age. In studies with rat diaphragm in vitro, Elsas, MacDonell, and Rosenberg have shown that the rate of amino acid transport and the sensitivity of transport to inhibitors of protein synthesis are inversely related to the age of the study animals (9).

The experimental results indicate that cortisol inhibits active AIB transport indirectly, and by a process which requires *de novo* protein synthesis. Inhibition appeared slowly and at low concentrations, persisted after transfer of treated cells to cortisol-free medium, and was not seen during simultaneous inhibition of protein synthesis. The observation that virtually complete inhibition of protein synthesis with cycloheximide did not in itself decrease AIB transport in cells from four of eight patients, but nullified the effect of cortisol in each case, strongly favors the hypothesis that synthesis of new protein is involved in inhibition of amino acid transport. Kinetic studies in cells from one patient suggest that this protein may regulate the total capacity (V_{max}) of the transport system, rather than the affinity (apparent K_m) of the system for AIB. However, these kinetics must be confirmed in studies with additional patients because of the wide variation between patients in rates of AIB transport and cortisol sensitivity.

Cortisol has been found to exert many effects on the metabolism of animal lymphocytes, including inhibition of transport processes (11–22), and of macromolecule synthesis (19, 20, 22, 32, 33) and these effects appear to arise indirectly. Studies of Mosher, Young, and Munck (34) and Munck (35) indicate that an initial binding of glucocorticoids to specific nuclear receptors may trigger the *de novo* synthesis of RNA and protein, and that this protein (or proteins) mediates in an unknown fashion the subsequent inhibitory effects of the hormone.

The inhibitory effect of cortisol on AIB transport in human leukemic lymphocytes may not be related entirely to its glucocorticoid activity, since nonglucocorticoids in high concentration (e.g. 10 μM progesterone) have been found to exert a similar effect in animal lymphocytes (19). This would imply that glucocorticoid-specific receptors are not involved in this action of cortisol. However, the response of human lymphocytes to cortisol resembled the glucocorticoid-specific responses of thymic lymphocytes in two ways: sensitivity to low concentrations of hormone (0.1 μM and 1 μM), and a requirement for *de novo* protein synthesis. Additional studies with lower cortisol concentrations and with other steroids will be necessary in order to establish the specificity of cortisol action in the human cells.

The wide but reproducible variation in the degree to which cortisol inhibited AIB transport in leukemic lymphocytes from different patients was noteworthy. Each cell population may possess a characteristic sensi-

tivity to cortisol, since, in most instances, the effectiveness of cortisol on a given cell population did not change with time. Many factors could participate in determining this sensitivity. One important possibility is that cortisol sensitivity may depend on the number or affinity of cellular receptors. Indeed, mouse lymphosarcoma cells which are resistant to the metabolic effects of glucocorticoids appear to bind less glucocorticoid than hormone-sensitive lymphosarcoma cells (36, 37).

Glucocorticoids produce a late (3–4 hr) decrease in the incorporation of amino acids and nucleosides into protein and nucleic acids in human leukemic lymphocytes in vitro (38, 39). Since a shorter period of treatment suffices to inhibit the uptake of free amino acids, these changes in incorporation may result from impaired transport of precursors rather than from direct inhibition of macromolecule synthesis, and thereby represent an alteration in plasma membrane function induced by cortisol.

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