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### Research Article

It is widely accepted that the neoplastic B cells from patients with chronic lymphocytic leukemia (CLL) respond poorly to common mitogens. The fungal metabolite cytochalasin B (0.5 micrograms/ml) is a weak mitogen for normal lymphocytes. However, when peripheral blood lymphocytes from 19 patients with CLL of B cell origin (B-CLL) were cultured with 0.5 micrograms cytochalasin B/ml, significant new DNA synthesis ( [14C]thymidine incorporation) occurred in 18. Stimulation indices with cytochalasin B varied widely (range = 1.9-28.2, mean +/- SD = 10.6 +/- 7.5; delta cpm range = 1,157-153,818; n = 26) but in 11 cases exceeded those seen with concanavalin A (Con A), phytohemagglutinin, or pokeweed mitogen. In all 11, the mitogenic response to cytochalasin B exceeded that to pokeweed mitogen, which is believed to be a T cell-dependent B cell mitogen. In three cases, the responses to cytochalasin B were 8.6, 3.5, and 2.3 times greater than those to Con A. As with other mitogens, the DNA synthetic response to cytochalasin B was time and dose dependent. Peak thymidine incorporation occurred at 72-88 h and declined thereafter. Significant mitogenic effects were observed with 0.1-5 micrograms cytochalasin B/ml with a peak at 0.5-2 micrograms/ml. Stimulated DNA synthesis was abolished by 1 mM hydroxyurea. Cells from two patients with B-CLL were separated by rosetting with sheep erythrocytes (E). Depletion of [...]

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# Cytochalasin B is a Potent Mitogen for Chronic Lymphocytic Leukemia Cells In Vitro

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**ABSTRACT** It is widely accepted that the neoplastic B cells from patients with chronic lymphocytic leukemia (CLL) respond poorly to common mitogens. The fungal metabolite cytochalasin B (0.5  $\mu\text{g}/\text{ml}$ ) is a weak mitogen for normal lymphocytes. However, when peripheral blood lymphocytes from 19 patients with CLL of B cell origin (B-CLL) were cultured with 0.5  $\mu\text{g}$  cytochalasin B/ml, significant new DNA synthesis ( $[^{14}\text{C}]$ thymidine incorporation) occurred in 18. Stimulation indices with cytochalasin B varied widely (range = 1.9–28.2, mean  $\pm$  SD =  $10.6 \pm 7.5$ ;  $\Delta\text{cpm}$  range = 1,157–153,818;  $n = 26$ ) but in 11 cases exceeded those seen with concanavalin A (Con A), phytohemagglutinin, or pokeweed mitogen. In all 11, the mitogenic response to cytochalasin B exceeded that to pokeweed mitogen, which is believed to be a T cell-dependent B cell mitogen. In three cases, the responses to cytochalasin B were 8.6, 3.5, and 2.3 times greater than those to Con A. As with other mitogens, the DNA synthetic response to cytochalasin B was time and dose dependent. Peak thymidine incorporation occurred at 72–88 h and declined thereafter. Significant mitogenic effects were observed with 0.1–5  $\mu\text{g}$  cytochalasin B/ml with a peak at 0.5–2  $\mu\text{g}/\text{ml}$ . Stimulated DNA synthesis was abolished by 1 mM hydroxyurea. Cells from two patients with B-CLL were separated by rosetting with sheep erythrocytes (E). Depletion of E-rosette-positive cells from the CLL cell population abolished the response to Con A but did not affect the response to

cytochalasin B. Cytochalasin B is a potent mitogen for B-CLL cells and may be useful in cytogenetic studies of this often indolent neoplasm.

## INTRODUCTION

Chronic lymphocytic leukemia (CLL)<sup>1</sup> is a systemic proliferation of small, mature-appearing, long-lived lymphocytes (1). Except for a small number of patients with well-defined clinical features and T cell antigens on their lymphocytes, most cases of CLL are of B cell origin (2, 3). These CLL cells are characterized by small amounts of monoclonal surface immunoglobulin (SIg) and the presence of membrane HLA-DR framework antigen (Ia-like).

Relatively few CLL cells are actively dividing, and most appear to be resting in the G<sub>0</sub> phase of the cell cycle. Mitogenic plant lectins, which transform normal human peripheral blood lymphocytes and stimulate DNA synthesis, are relatively inactive with CLL cells (4–6). These include concanavalin A (Con A) and phytohemagglutinin (PHA), which are T cell mitogens, and pokeweed mitogen (PWM), which is a T cell-dependent B cell mitogen. More recently, polyclonal B cell mitogens such as lipopolysaccharide (LPS) from *Escherichia coli* or the Epstein-Barr virus have been used to promote mitosis in CLL cells in vitro (7, 8).

During the course of investigations on the potentiating effect of cytochalasin B on the transformation of normal lymphocytes by mevalonic acid (9–12), we

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<sup>1</sup> *Abbreviations used in this paper:* B-CLL, CLL of B-cell origin; CLL, chronic lymphocytic leukemia; Con A, concanavalin A;  $\Delta\text{cpm}$ , difference between  $[2\text{-}^{14}\text{C}]$ thymidine incorporation (counts per minute) of cells in the presence and absence of mitogen; DMSO, dimethyl sulfoxide; E, sheep erythrocytes; LPS, lipopolysaccharide; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; PWM, pokeweed mitogen; SI, stimulation index (cpm mitogen/cpm control); SIg, surface immunoglobulin.

discovered that cytochalasin B by itself is a potent mitogen for malignant lymphocytes from patients with CLL. Cytochalasin B is a fungal macrolide metabolite that has been shown to have profound effects on neutrophil aggregation, degranulation, oxidative metabolism, microfilament formation, and chemotaxis, as well as on the transport of hexoses and hexosamine and possibly nucleosides into cells (13–17). At low concentrations cytochalasin B also enhances the mitogenic response of normal lymphocytes to lectins (18, 19). In this report, we show that cytochalasin B is itself a potent mitogen for CLL cells of B cell origin (B-CLL).

## METHODS

Cytochalasin B and cytochalasin A (Sigma Chemical Co., St. Louis, MO) were each dissolved in dimethyl sulfoxide (DMSO) to a concentration of 3.33 mg/ml and further diluted to a working solution of 100  $\mu$ g/ml in Hanks' balanced salt solution (HBSS), as required. In some experiments to be described, ethanol was used to solubilize the cytochalasin B rather than DMSO. Working solutions of the following mitogens were made with sterile phosphate-buffered saline (PBS) as previously described (20): Con A (Worthington Biochemical Corp., Freehold, NJ) 5 mg/ml; PHA 1 mg/ml; PWM 100  $\mu$ g/ml. LPS W from *E. coli* 055:85 (Difco Laboratories, Detroit, MI) was dissolved in sterile water to 5 mg/ml. Phorbol myristate acetate ester (PMA) (P-L Biochemical, Inc., Milwaukee, WI) was dissolved in DMSO to 20  $\mu$ g/ml (21). [2-<sup>14</sup>C]Thymidine (56 mCi/mmol) was obtained from the Amersham Corp., Arlington Heights, IL. Hydroxyurea was a product of the Boehringer-Mannheim Corp., Indianapolis, IN.

**Isolation of CLL cells.** Heparinized peripheral blood was obtained from patients with CLL and from normal volunteers by venipuncture. Mononuclear cells were isolated by the Ficoll-Hypaque (Lymphoprep; density 1.077 g/ml; Nyegaard and Company, Oslo, Norway) technique as previously described (20). Surface marker studies were performed with fluorescein-conjugated monospecific antisera and various commercial monoclonal antibodies (Ortho Diagnostic Systems, Raritan, NJ).

**Cell fractionation.** Sheep erythrocyte rosette positive (E+) and negative (E-) lymphocyte populations were isolated from Ficoll-Hypaque interface cells (22). E-rosette-positive cells were pelleted through Ficoll-Hypaque, and the lymphocytes were recovered after hypotonic lysis of the erythrocytes. The E-rosette-negative cells were recovered from the saline-Ficoll-Hypaque interface.

**Cell cultures.** Mononuclear cells ( $1.5 \times 10^6$ /ml) were cultured with or without mitogens in tubes with 1.0 ml RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with antibiotics, glutamine, and 12.5% human AB serum (previously heated to 56°C for 30 min). Cultures were incubated at 37°C in humidified, 5% CO<sub>2</sub>. In most experiments, mitogens were added at time zero, and the cultures were harvested 88 h later, 16 h after the addition of 0.2  $\mu$ Ci [2-<sup>14</sup>C]thymidine. Cells were collected on glass fiber filters by suction and washed with PBS containing 10 mM EDTA, followed by trichloroacetic acid, and cold methanol. The amount of isotope incorporated into DNA was measured by scintillation spectrophotometry (model 3375; Packard Instrument Co. Inc., United Technologies, Downers Grove, IL). Tube cultures were performed in triplicate; certain cul-

tures using microculture plates ( $3 \times 10^5$  cells in 0.2 ml vol) were performed in quadruplicate.

**Cytogenetic techniques.** Mononuclear cells ( $10^6$ /ml) were cultured for 1, 2, 4, or 7 d in flasks containing either no mitogen, or 0.1 ml PWM (Gibco Laboratories), or 0.5  $\mu$ g/ml cytochalasin B. Metaphase cells were analyzed by Giemsa staining and quinacrine banding as previously described (23).

## RESULTS

The peripheral leukocyte counts and the percentage of lymphocytes at the time of study of 19 CLL patients are shown in Table I. With the exception of patients C.O. and H.H. (oral prednisone) and K.K. and D.A. (daily chlorambucil), none of these patients received chemotherapy within 2 wk of their initial study. Clinical features and surface marker analyses (SIg+, Ia+) supported the B cell origin of CLL in each of these patients. The predominance of a single surface immunoglobulin light chain (kappa or lambda) demonstrated that the mononuclear cell fraction isolated from each patient studied was enriched in a monoclonal lymphoid population.

**Mitogenic response to cytochalasin B.** Cytochalasin B was a weak stimulator of DNA synthesis in normal lymphocytes (control: mean cpm $\pm$ SD = 1,942 $\pm$ 1,799; lymphocytes + 0.5  $\mu$ g/ml cytochalasin B: 2,385 $\pm$ 1,930;  $P < 0.002$ , paired  $t$  test,  $n = 15$ ) as others have shown (15, 18). When CLL cells from 19 patients with B-CLL were cultured in the presence of 0.5  $\mu$ g/ml cytochalasin B for 72–88 h, the amount of [<sup>14</sup>C]thymidine incorporation into DNA exceeded that observed in control cultures in 18 patients (Table I). Among the 18 responders, the stimulation indices (SI) varied widely (range = 1.9–28.2; mean $\pm$ SD = 10.6 $\pm$ 7.5;  $n = 26$ ) as did the absolute increase in counts per minute ( $\Delta$ cpm range = 1,157–153,818). Two patients were studied three times and four patients were studied twice and a significant mitogenic response to cytochalasin B was observed in each instance. In experiments with lymphocytes from 11 patients, the response to cytochalasin B exceeded that of the same cell suspension to either 50  $\mu$ g/ml Con A (six cases), 10  $\mu$ g/ml PHA (six cases), or 0.5  $\mu$ g/ml PWM (all 11 patients). With cells from eight patients the response to cytochalasin B exceeded that to 100 ng/ml PMA. In only one patient (D.M.) was the incorporation of [<sup>14</sup>C]thymidine into cytochalasin B-treated lymphocytes not significantly greater than into control cells.

**Dose-response.** As has been shown with other lymphocyte mitogens, the mitogenic response of CLL cells to cytochalasin B is dose-dependent and time-dependent. Fig. 1 illustrates the [<sup>14</sup>C]thymidine incorporation (counts per minute) of CLL cells from patient A.H. when cultured in the presence of 0–5  $\mu$ g/ml cy-

TABLE I  
Clinical Data and Response to Mitogens ( $^{14}\text{C}$ Thymidine Incorporation) by CLL Cells

Patient	Leukocyte count	Lymphocytes			Cytochalasin B						
		Ia+	Slg	Control	$\Delta\text{cpm}$	SI	Con A	PHA	PWM	PMA	
		%		<i>cpm</i>	<i>cpm</i>		<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	
A.H.	21,600	65	68	$\mu\gamma\text{-}\kappa$	4,860 $\pm$ 133* 1,496 $\pm$ 202	36,998 $\pm$ 2,110 40,705 $\pm$ 1,772	8.6 28.2	16,360 $\pm$ 940 11,624 $\pm$ 252	13,153 $\pm$ 703 ND†	13,178 $\pm$ 465 ND	52,654 $\pm$ 1,992 ND
M.S.	19,600 6,900	79 74	65	$\delta\text{-}\kappa$	2,132 $\pm$ 207 833 $\pm$ 332	23,428 $\pm$ 1,026 19,754 $\pm$ 3,001	12.0 24.7	89,041 $\pm$ 3,846 14,077 $\pm$ 1,059	80,078 $\pm$ 2,154 ND	21,528 $\pm$ 485 ND	0 ND
D.Bo.	496,000	86	83	$\mu\text{-}\kappa$	1,684 $\pm$ 155 1,978 $\pm$ 101 2,403 $\pm$ 411	26,423 $\pm$ 1,975 46,063 $\pm$ 1,741 41,281 $\pm$ 1,648	16.7 24.3 18.2	7,634 $\pm$ 821 ND 22,901 $\pm$ 966	11,625 $\pm$ 2,599 ND 22,932 $\pm$ 968	3,620 $\pm$ 559 ND 4,094 $\pm$ 142	3,059 $\pm$ 482 ND ND
P.L.	61,900 97,800	74 90	65	$\mu\text{-}\kappa$	852 $\pm$ 157 446 $\pm$ 33	9,205 $\pm$ 141 1,609 $\pm$ 220	11.8 4.6	75,622 $\pm$ 5,032 24,428 $\pm$ 1,824	58,707 $\pm$ 4,614 ND	23,566 $\pm$ 1,213 ND	4,389 $\pm$ 197 ND
K.K.	114,800 26,400	95 86	82	$\mu\gamma\text{-}\kappa$	408 $\pm$ 117 3,073 $\pm$ 237	5,643 $\pm$ 185 12,611 $\pm$ 1,830	14.8 5.1	21,974 $\pm$ 1,809 15,535 $\pm$ 861	36,665 $\pm$ 433 11,741 $\pm$ 301	7,316 $\pm$ 356 10,678 $\pm$ 846	11,876 $\pm$ 216 24,875 $\pm$ 1,755
D.Be.	107,100 83,100 56,800	88 88	88	$\mu\gamma\text{-}\kappa$	1,188 $\pm$ 409 2,793 $\pm$ 124 558 $\pm$ 59	12,333 $\pm$ 3,164 13,508 $\pm$ 735 4,374 $\pm$ 1,463	11.4 5.8 8.8	8,536 $\pm$ 1,491 9,097 $\pm$ 1,225 26,250 $\pm$ 2,220	10,765 $\pm$ 458 ND 27,758 $\pm$ 2,412	4,246 $\pm$ 1,141 ND 5,058 $\pm$ 958	1,346 $\pm$ 133 ND ND
M.T.	199,200	99	94	$\mu\text{-}\kappa$	912 $\pm$ 106	8,200 $\pm$ 654	10.0	36,900 $\pm$ 1,742	30,621 $\pm$ 1,142	5,310 $\pm$ 1,041	11,340 $\pm$ 977
O.O.	62,000	84	ND	$\delta\gamma\text{-}\lambda$	620 $\pm$ 65	5,129 $\pm$ 840	9.3	11,731 $\pm$ 559	21,014 $\pm$ 1,577	5,654 $\pm$ 1,093	5,617 $\pm$ 82
E.B.	57,600	66	ND	ND	2,432 $\pm$ 615	16,752 $\pm$ 1,036	7.9	25,267 $\pm$ 2,215	14,661 $\pm$ 477	4,575 $\pm$ 174	3,675 $\pm$ 1,091
A.S.	53,600	78	100	$\mu\gamma\text{-}\kappa$	22,820 $\pm$ 3,408	153,818 $\pm$ 6,766	7.7	17,889 $\pm$ 2,185	40,892 $\pm$ 7,744	8,485 $\pm$ 2,614	21,299 $\pm$ 3,649
C.O.	35,400	87	ND	ND	1,493 $\pm$ 49	9,649 $\pm$ 611	7.5	18,517 $\pm$ 2,432	14,231 $\pm$ 448	3,981 $\pm$ 639	3,173 $\pm$ 414
H.R.	24,900	78	74	$\mu\text{-}\lambda$	660 $\pm$ 2	3,832 $\pm$ 236	6.8	29,641 $\pm$ 945	25,032 $\pm$ 791	20,111 $\pm$ 1,192	11,208 $\pm$ 524
S.M.	69,700	94	94	$\mu\gamma\text{-}\lambda$	326 $\pm$ 47	1,386 $\pm$ 486	5.3	16,517 $\pm$ 710	19,297 $\pm$ 1,682	9,150 $\pm$ 827	13,194 $\pm$ 1,892
M.M.	21,300	72	ND	ND	401 $\pm$ 155	1,341 $\pm$ 339	4.4	40,702 $\pm$ 4,238	ND	ND	ND
H.H.	88,400	91	88	None	680 $\pm$ 400	1,901 $\pm$ 563	3.8	3,781 $\pm$ 1,482	5,147 $\pm$ 692	0	2,905 $\pm$ 1,054
R.J.	302,000	92	97	$\mu\gamma\text{-}\kappa$	518 $\pm$ 40	1,157 $\pm$ 148	3.2	0	2,602 $\pm$ 374	409 $\pm$ 43	415 $\pm$ 43
V.S.	47,300	77	77	None	850 $\pm$ 125	1,694 $\pm$ 462	3.0	32,865 $\pm$ 2,600	20,718 $\pm$ 591	8,403 $\pm$ 391	42,140 $\pm$ 569
D.A.	75,900	92	50	$\mu\gamma\text{-}\kappa$	2,988 $\pm$ 922	2,725 $\pm$ 879	1.9	30,678 $\pm$ 3,656	65,748 $\pm$ 4,617	14,642 $\pm$ 2,648	38,366 $\pm$ 3,650
D.M.	44,600	61	100	ND	1,208 $\pm$ 115	0	1.0	38,937 $\pm$ 571	26,870 $\pm$ 1,064	10,598 $\pm$ 396	3,401 $\pm$ 129

\* Mean $\pm$ SD.

† ND, not done.

tochalasin B and, for comparison, with 50  $\mu\text{g}/\text{ml}$  Con A. Peak DNA synthesis occurred at 1  $\mu\text{g}/\text{ml}$  cytochalasin B and decreased as the dose increased further. The peak response to cytochalasin B was 4.2 times greater than that to a standard concentration of Con A and 37.2 times greater than that in control cultures. Among all six patients studied in this manner, peak responses to cytochalasin B occurred between 0.5 and 2  $\mu\text{g}/\text{ml}$ . Fig. 2 demonstrates that in patient D.Bo. the response to cytochalasin B was maximal at 0.5–1.0  $\mu\text{g}/\text{ml}$  and exceeded the DNA synthetic responses elic-

ited by all other mitogens over a 20–25-fold dose-response analysis. In this patient, as well as in a second patient (D.Be.), no significant response to LPS (5–100  $\mu\text{g}/\text{ml}$ ) was noted.

*Time-dependence.* In another experiment (Fig. 3), CLL cells from patient A.H. were cultured with and without 0.5  $\mu\text{g}/\text{ml}$  cytochalasin B for 24, 48, 72, and 96 h before addition of 0.2  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]thymidine. Each culture was then harvested 16 h later. Isotope incorporation into DNA increased daily to peak at 72–88 h and then declined over the next 24 h. The initial

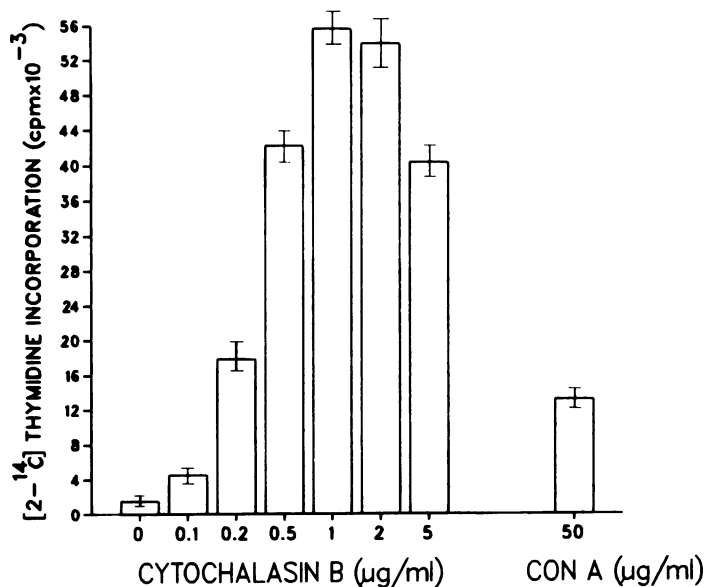


FIGURE 1 DNA synthetic response ( $[^{14}\text{C}]$ thymidine incorporation) of B-CLL cells from patient A.H. to cytochalasin B measured after 88 h culture. The brackets denote  $\pm 1$  SD.

addition of 1 mM hydroxyurea to these cultures abolished the DNA synthesis stimulated by cytochalasin B.

**Effect of DMSO on the response to cytochalasin B.** Our choice of DMSO as solvent for cytochalasin B was dictated by ease of solubility and nonvolatility. The final concentration of DMSO in our cultures was usually 0.016%. In control experiments we showed that DMSO (0.016–0.5%) did not itself increase DNA synthesis in CLL cells (18 patients). Furthermore, cytochalasin B dissolved in ethanol and subsequently diluted to our working concentration in HBSS was as effective in eliciting DNA synthesis in CLL lymphocytes as was DMSO-dissolved (and diluted) cytochalasin B. Again, in these experiments, addition of 1 mM hydroxyurea abolished the DNA synthetic response to cytochalasin B.

**Response to cytochalasin A.** Lymphocytes from three patients whose cells responded well to cytochalasin B were simultaneously cultured for 72–88 h in the presence of 0.1 to 10  $\mu\text{g}/\text{ml}$  cytochalasin A. The stimulated incorporation of  $[^{14}\text{C}]$ thymidine was less than twice that seen in the control cells in each case. Cytochalasin A was minimally stimulatory at 1  $\mu\text{g}/\text{ml}$  and inhibitory at higher concentrations.

**Erythrocyte rosetting experiments.** To character-

ize the cytochalasin B-responsive cell population further, CLL cells from two patients were separated by rosetting with sheep erythrocytes (E). Table II shows the results of experiments with these cells in culture. The experiment was marred by the inability of our rosetting procedure to separate cleanly the various subpopulations. Patient D.Bo., for example, had a peripheral leukocyte count of 427,000; only 2% of these lymphocytes stained positively with OKT3 antibody while 57% were positive for SIg and 99% were positive for HLA-DR (Ia) antigen. Our rosetting procedure enriched the OKT3-positive cells in the E-rosette-positive subpopulation only fivefold, and 32% of the lymphocytes in the E-positive fraction were still SIg-positive.

In both cases (Table II), the unfractionated CLL cells had a significant mitogenic response ( $P < 0.001$ ) to 0.5  $\mu\text{g}/\text{ml}$  cytochalasin B, and these cytochalasin B-responsive cells persisted in the E(-) subpopulation. Of note, the E(-) cells no longer responded to Con A, a T cell mitogen, suggesting that this subpopulation was depleted of T cells. In contrast, the cytochalasin B-responsive cells were either not present (patient A.H.) or relatively reduced (patient D.Bo.) in the lymphocyte subpopulation obtained with sheep erythrocytes (E+), although the experiment with A.H.'s cells

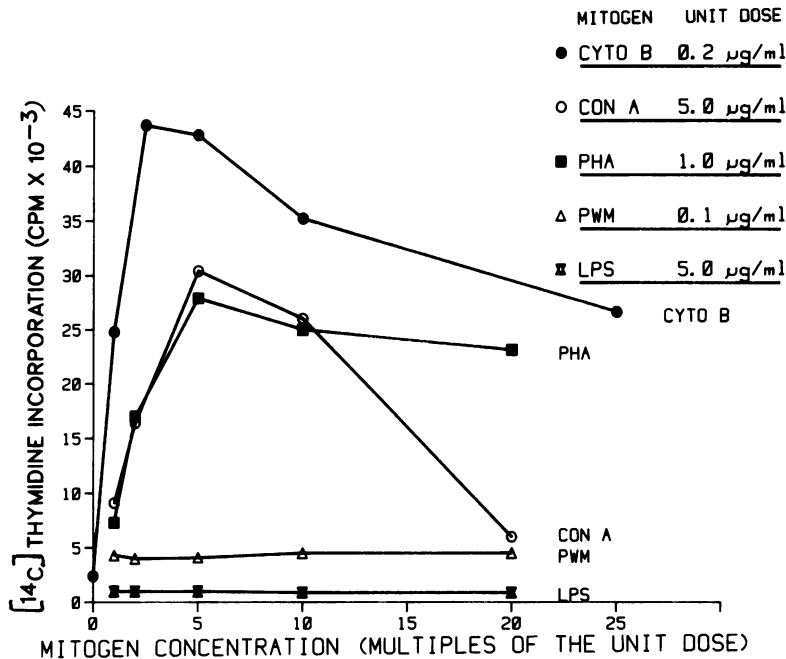


FIGURE 2 DNA synthetic response of B-CLL cells from patient D.Bo. when cultured with cytochalasin B and with other mitogens. [<sup>14</sup>C]Thymidine incorporation was measured from 72 to 88 h culture over a 20–25-fold range of mitogen concentrations. The response to 0.5 µg/ml cytochalasin B was 144% of that to 25 µg/ml Con A ( $P < 0.01$ ). There was no significant difference between the response to 25 µg/ml or to 50 µg/ml Con A in this and in other experiments.

was flawed by the failure of the E(+) cells to respond to Con A as well.

**Cytogenetic analysis of cytochalasin B-stimulated B-CLL cells.** Lymphocyte suspensions from a female B-CLL patient (D.Be.) were cultured for 4 d and analyzed by standard cytogenetic techniques (Giemsa and quinacrine banding) (23). Two of three metaphase cells in an unstimulated culture demonstrated a karyotype of 47,XX,+12 (Table III); the third cell was cytogenetically normal. All 11 metaphase cells in a PWM-stimulated culture had a normal karyotype. In contrast, 12 of the 14 metaphase cells observed in the cytochalasin B-stimulated (0.5 µg/ml) culture had the abnormal karyotype, 47,XX,+12; the other two cells were normal. Trisomy 12 has previously been shown to be the most common clonal chromosomal abnormality in B-CLL (24).

Lymphocytes from a male B-CLL patient (D.Bo.) were analyzed by similar cytogenetic techniques. One of five metaphase cells observed in unstimulated cultures had a karyotype of 45,XY,-19,t(2;14)(p13;q32),t(14;?)(q32;?), and the other cells had a normal 46,XY karyotype. All three metaphase cells in PWM-stimulated cultures had a normal karyotype. Both of the metaphase cells in a 4-d cytochalasin B-

stimulated (0.5 µg/ml) culture had the identical chromosome abnormality described above. Breakpoints at 2p13 and 14q32 have been observed with high frequency in chromosomes of other B cell malignancies (25).

Morphologic evidence of lymphocyte transformation was also observed. Large cells, occasionally binucleate, containing multiple nucleoli were observed in the cytochalasin B-containing cultures but not in unstimulated control cultures.

**Mitogenic response in other lymphoid disorders.** Peripheral blood lymphocytes from a small number of patients with other malignant lymphoid disorders were also studied. Significant DNA synthesis in response to cytochalasin B in culture was observed in cells from one patient with B cell prolymphocytic leukemia (SI = 4.3;  $\Delta\text{cpm} = 1,296 \pm 133$ ), one patient with leukemic large cell lymphoma (SI = 2.8;  $\Delta\text{cpm} = 8,255 \pm 761$ ), and one of two patients with hairy cell leukemia (SI = 2.1;  $\Delta\text{cpm} = 498 \pm 37$ ), but not from two patients with leukemic poorly differentiated lymphocytic lymphoma. A significant but low response to 0.5 µg/ml cytochalasin B (SI = 2.0;  $\Delta\text{cpm} = 603 \pm 26$ ) was observed with cells from one patient with T cell CLL (95% OKT3+, 85% OKT4+, 0% SIg+, 2%

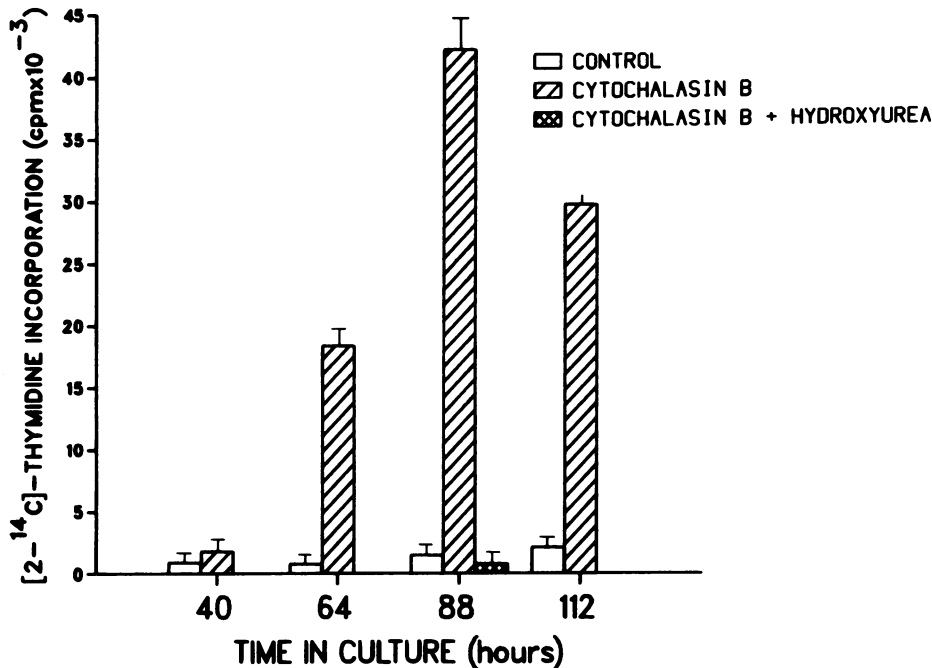


FIGURE 3 DNA synthetic response ( $[^{14}\text{C}]$ thymidine incorporation) of B-CLL cells from patient A.H. when cultured with and without  $0.5 \mu\text{g/ml}$  cytochalasin B for 24, 48, 72, and 96 h before addition of  $0.2 \mu\text{Ci}$   $[^{14}\text{C}]$ thymidine. Cultures were harvested and counted 16 h later. Hydroxyurea ( $1 \text{ mM}$ ) was added together with cytochalasin B to one group of triplicate cultures at time zero. The brackets denote  $\pm 1 \text{ SD}$ .

Ia+, 77% E+); a smaller response ( $\text{SI} = 1.4$ ;  $\Delta\text{cpm} = 68 \pm 31$ ) was seen with a second T-CLL patient (82% OKT3+, 87% OKT4+, 2% SIg+, 6% Ia+, 85% E+).

## DISCUSSION

We have shown that the fungal metabolite cytochalasin B significantly enhances the synthesis of DNA in CLL cells as measured by  $[^{14}\text{C}]$ thymidine incorporation. In common with all other known lymphocyte mitogens, the cytochalasin B effect is dose related and time dependent. In 11 of 19 patients, the response to cytochalasin B exceeded that seen to one or more of the four standard lymphocyte mitogens tested.

The cytochalasin B-responsive cell appears to be the neoplastic B-CLL cell. Surface marker analysis demonstrated that the initial mononuclear cell suspensions were enriched in Ia+ cells with a monoclonal immunoglobulin light chain. Rosetting experiments suggested that the cytochalasin B-responsive cell was retained in the E- subpopulation. Cytogenetic studies identified nonrandom clonal chromosome abnormalities in cytochalasin B-stimulated CLL cells, which were characteristic of B-cell malignancies (25). Although cytochalasin B stimulates DNA synthesis to a

small degree in normal human peripheral blood lymphocytes and in cells from a small number of patients with malignant lymphoid disorders other than CLL, in none of those cases was the magnitude of the response as great as that seen with the malignant lymphocytes from most B-CLL patients.

It is important to emphasize that cytochalasin B-enhanced  $[^{14}\text{C}]$ thymidine incorporation truly reflects DNA synthesis in cycling cells. Others have shown that  $2\text{--}5 \mu\text{g/ml}$  cytochalasin B inhibits the transport of thymidine and other nucleosides into cells (17); thus, the increased  $[^{14}\text{C}]$ thymidine uptake by CLL cells in response to cytochalasin B is unlikely to result from a nonspecific increase in the amount of DNA precursor that enters the cell. The presence of  $1 \text{ mM}$  hydroxyurea in the cultures abolishes the  $[^{14}\text{C}]$ thymidine uptake seen in cytochalasin B-treated cells. Morphologic and cytogenetic evidence of lymphocyte transformation was also observed. Bizarre abnormalities were not observed with chromosome banding studies on stimulated metaphase cells suggesting that cytochalasin B does not induce random chromosome damage.

Cytochalasin B is capable of a variety of effects on cells, some of which depend paradoxically on the cytochalasin B concentration. In addition, most of these

TABLE II  
DNA Synthetic Response to Cytochalasin B of Various Lymphocyte Subpopulations Separated by Rosetting with Sheep Erythrocytes from the Blood of Patients with B-CLL

	Unfractionated	E+	E-
Patient D.Bo.			
Control (cpm)	441±147	82±25	258±30
Con A ( $\Delta$ cpm)	1,657±109	5,981±253	0
Cytochalasin B ( $\Delta$ cpm)	4,088±507	2,936±151	4,710±500
Patient A.H.			
Control (cpm)	632±229	263±227	395±161
Con A ( $\Delta$ cpm)	2,311±370	0	0
Cytochalasin B ( $\Delta$ cpm)	6,892±348	0	6,913±213

Cultures were performed in microtiter plates using  $3 \times 10^5$  cells in 0.2 ml media and 0.5  $\mu$ g/ml cytochalasin B or 50  $\mu$ g/ml Con A. [ $^{14}$ C]Thymidine was added after 72 h and the cultures were harvested and counted 16 h later. All  $\Delta$  counts per minute (except where zero) were significantly different ( $P < 0.001$ ) from their respective control counts per minute.

effects require the continuing presence of cytochalasin B (i.e., the effect is abolished by washing the cells), and many of these effects are markedly altered by the time sequence of addition of cytochalasin B and other experimental substances to the cell culture. For example, at high concentrations (2.5–10  $\mu$ g/ml) cytochalasin B inhibits the chemotactic response of granulocytes, while at low concentrations (0.1–0.5  $\mu$ g/ml) chemotactic responses are enhanced (26). 5  $\mu$ g/ml cytochalasin B inhibits the glucose oxidation burst that occurs when neutrophils are exposed to a nonphagocytizable immunologic stimulus (27). Preaddition of cytochalasin B (0.5–10  $\mu$ g/ml) to neutrophils, followed by addition of a nonparticulate activating substance such as *N*-formyl-methionyl-leucyl-phenylalanine or the activated complement component C5a results in the exocytosis of the contents of both the primary and secondary lysosomal granules associated with an increase of  $Ca^{++}$  ion efflux into the cell (27–30); delay between the time of addition of the neutrophil activator and cytochalasin B results in a rapid loss of both responses (30, 31). The precise mechanism of these

effects, and their relationship to the ability of cytochalasin B to inhibit microfilament function (32), actin polymerization (33), or the transport of metabolites such as glucose or glucosamine across cell membranes (16) are poorly understood. It should also be noted that despite its many effects on granulocyte physiology, cytochalasin B does not suppress the hyperpolarization of the neutrophil membrane, which may be the earliest response neutrophils display when reacting to a soluble or insoluble stimulus.

Cytochalasin B is rapidly and reversibly bound to both high-affinity and low-affinity binding sites by lymphocytes, neutrophils, and erythrocytes (14). Parker et al. (14) have estimated that normal human lymphocytes have  $\sim 10^6$  high-affinity binding sites/cell located in or near the plasma membrane. Because cytochalasin B perturbs calcium ion transport and cyclic AMP metabolism within 2 min of exposure, it is believed that cytochalasin B acts directly on the plasma membrane rather than via a remote effect on intracellular metabolism.

As we and others have shown, cytochalasin B has minimal mitogenic activity on normal lymphocytes in the absence of lectins (15, 18). In the presence of Con A, PHA, or periodate, however, low concentrations of cytochalasin B (0.1–1  $\mu$ g/ml) will enhance the synthesis of DNA, the accumulation of cyclic AMP, the turnover of phosphatidylinositol, the transport of amino acids, and the uptake of calcium in normal human lymphocytes (18). At higher concentrations (5–10  $\mu$ g/ml), cytochalasin B has an inhibitory effect on all these activities. Cytochalasin B does not appear to enhance or inhibit the binding of lectins to lymphocyte

TABLE III  
Cytogenetic Analysis of Mitogen-stimulated B-CLL Cells

Culture	Metaphase cells at 4 d		
	Total	46,XX	47,XX,+12
Control	3	1	2 (67%)
PWM	11	11	0
Cytochalasin B	14	2	12 (86%)



membranes, but cytochalasin B must be added soon after the addition of the lectins to the lymphocyte suspension to have any effect (18, 19).

Of interest, cytochalasin A had little or no stimulatory effect on thymidine incorporation by B-CLL cells, although it is ~10 times more potent than cytochalasin B in potentiating the response of normal lymphocytes to lectins (18). That relatively minor structural alterations are important in the interaction of cytochalasin B with lymphocyte membranes has previously been suggested by Green et al. (18), who found that dihydrocytochalasin B and the  $\gamma$ -lactone derivative of cytochalasin B are inactive in potentiating the response of normal lymphocytes to lectins. Other important differences between cytochalasins A and B with respect to their effects on cellular behavior have been described (34-36).

It has been shown that most or all of the high-affinity binding sites for cytochalasin B in lymphocytes are close to the cell surface (14) and that cytochalasin B alters stimulated membrane phospholipid metabolism, one of the earliest detectable changes in mitogen-activated lymphocytes (37). Cytochalasin B (5-10  $\mu\text{g}/\text{ml}$ ) also inhibits the usual "capping" reaction seen in stimulated lymphocytes, perhaps interacting with actin or myosin within contractile microfilaments (38-40). In addition, cytochalasin B (3-10  $\mu\text{g}/\text{ml}$ ) blocks antigen-dependent binding of normal lymphocytes to macrophages and inhibits the binding of T cells to macrophages even in the absence of specific antigen (41). Therefore, the differential mitogenic effect of cytochalasin B on B-CLL cells vs. normal lymphocytes may involve an interaction with the neoplastic cell membrane of the CLL cells through vestigial receptors normally involved in the immune mechanism.

Whereas the classical mitogens probably stimulate the normal T cell population in patients with CLL, our studies suggest that cytochalasin B stimulates preferentially the leukemic B cell population. Thus, cytochalasin B should be a better agent to use in the search for chromosomal abnormalities in mitogen-stimulated CLL cells than other presently available stimuli. The ability to promote cell cycling in B-CLL cells with cytochalasin B in vitro may have important applications in further investigations of the cytogenetics and cell biology of this often indolent neoplasm.

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