

Mechanisms of Lymphocyte Activation

THE ROLE OF SUPPRESSOR CELLS IN THE PROLIFERATIVE RESPONSES OF CHRONIC LYMPHATIC LEUKEMIA LYMPHOCYTES

GUY B. FAGUET, *Departments of Medicine and Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902*

ABSTRACT Binding of ^{125}I -leukoagglutinin (LPHA) to lymphocyte membrane receptors at equilibrium generated similar curvilinear Scatchard plots in 20 patients with bursa-derived (B)-cell-type chronic lymphatic leukemia (CLL) and 15 controls. If biphasic plots are assumed, the two linear components show markedly diminished receptor capacity (15 and 137 ng/ 10^6 lymphocytes) in CLL as compared to controls (60 and 668 ng). In contrast, affinity was similar in patients ($1.0 \times 10^8 \text{ M}^{-1}$ and $2.1 \times 10^6 \text{ M}^{-1}$) and controls ($1.8 \times 10^8 \text{ M}^{-1}$ and $1.5 \times 10^6 \text{ M}^{-1}$). Highly purified B cells from patients and controls generated binding data comparable to that obtained from the mixed lymphocyte (ML) suspensions from which they originated. Maximal DNA synthesis of highly purified, normal, thymus-derived (T) and B cells in response to LPHA stimulation was comparable to that of ML (mitotic index [MI] 19.9, 20.1, and 23.4, respectively), though B-cell responses were slightly delayed. In CLL the markedly decreased and delayed DNA synthesis by ML (MI 2.3), and by highly purified T (MI 1.6) and B (MI 1.9) cells seemed out of proportion to their decreased receptor capacity for LPHA. The impaired mitogenic responses of leukemic cells from five patients were not enhanced when cocultured with normal lymphocytes. In contrast, cells from eight patients inhibited cocultured normal lymphocyte responses to LPHA by 94.3%. Sera from these patients and supernates from their cultured cells did not mediate this suppressor effect. These observations indicate that the decreased DNA synthesis observed in CLL is not an attribute of B cells and does not represent the expected response of a few residual normal T lymphocytes, but rather reflects impaired responses by all CLL cells. The defect does not relate to the density or function of membrane receptors for LPHA, to the presence of inhibitors in these patients' sera, or to depletion of helper T cells.

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Our data strongly suggest that one mechanism for the immunoincompetence observed in CLL reflects excessive suppressor-cell activity.

INTRODUCTION

The decreased (1) and delayed (2) response of chronic lymphatic leukemia (CLL)¹ cells to phytohemagglutinin has been attributed to a few normal thymus-derived (T) cells diluted by a majority of unresponsive monoclonal bursa-derived (B) lymphocytes (3), or alternatively has been viewed as the expected response of the predominant B cell (4). However, several other mechanisms must be entertained: (a) a quantitative imbalance in cells that regulate immune responses such as an excess of suppressor cells or a depletion of helper cells (5–7); (b) a deficiency of humoral factors necessary for the expression of immune competence (8); (c) the presence in CLL plasma of inhibitory factors which hinder cell proliferation (9); (d) because normal B lymphocytes possess binding capacity for leucoagglutinin (LPHA) comparable to that of normal T cells (10, 11) and are reactive to LPHA (11), the demonstration of decreased receptors for erythroagglutinin (12), and other lectins (13) on CLL cells suggests a physical basis for the abnormal mitogenic responses observed in CLL. Because the binding of LPHA to normal lymphocyte receptors and the resulting cell activation have been well characterized (14–16), this lectin is particularly suited to ascertain whether the impaired mitogenic responses of neoplastic lymphocytes might reflect defective membrane events such as decreased receptor capacity or affinity for the lectin (17). In the present studies we have examined the proliferative responses of neoplastic lymphocytes and have at-

¹ *Abbreviations used in this paper:* CLL, chronic lymphatic leukemia; FCS, fetal calf serum; Ka, receptor affinity; LPHA, leucoagglutinin; ME medium, Eagle's Minimum Essential Medium; MI, mitotic index; ML, mixed lymphocytes; MLR, mixed lymphocyte reactions; Ro, receptor capacity.

tempted to elucidate whether these cells' functional impairment relates to altered receptor density or affinity for LPHA, the presence of inhibitory serum factors, depletion of functional helper cells or excess numbers of suppressor cells, or whether the metabolic abnormality observed reflects a defect of effector-cell function inherent to the neoplastic cell.

METHODS

Selection of donors. 20 patients with CLL ranging from 37 to 66 yr of age with stages III and IV disease (18) and a mean absolute lymphocyte count of $58.8 \pm 19.3 \times 10^3/\mu\text{l}$ constituted our patient population. No patient received any chemotherapy within 3 wk of study. 15 healthy volunteers ranging from 22 to 49 yr of age with a mean absolute lymphocyte count of $2,014 \pm 146 \times 10^3/\mu\text{l}$ and who were taking no medications were used as controls.

PHA purification and iodination. Bactophytohemagglutinin (Difco Laboratories, Detroit, Mich.) was purified according to a modification of the column chromatography method of Weber (19) to homogenous LPHA as previously described (15). This mitogenic fraction was utilized for both metabolic and receptor studies. For receptor studies, LPHA was iodinated (reductant-free ^{125}I , 17 Ci/mM sp act, ICN, Chemical & Radioisotope Div., Life Sciences Group, Irvine, Calif.) by the chloramine-T method of Hunter (20) and adjusted to a final specific activity of 150,000 cpm/ μg LPHA-protein in 0.5% bovine serum albumin (albumin, Grand Island Biological Co., Grand Island, N. Y.). Details of the LPHA purification and iodination procedures have been published elsewhere (15).

Cell characterization by immune markers. T- and B-lymphocyte subpopulations were assessed by the E-rosette and immunofluorescence techniques, respectively. Spontaneous rosette formation with sheep erythrocytes (E-rosette) was evaluated according to a modification (21) of the technique of Jondal et al. (22). The presence of membrane immunoglobulins was investigated by direct immunofluorescence (23) as follows. Two million cells were incubated at room temperature for 60 min with 0.1 ml of fluorescein-conjugated antisera specific for each heavy and light chain, and with polyvalent antiserum (Meloy Laboratories, Inc., Richmond, Va.). After washing the cells twice with Eagle's Minimum Essential Medium (ME medium, Grand Island Biological Co.), one drop of cell suspension was placed on a slide, covered, and examined with a Leitz fluorescent microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with an Osram HBD 200 mercury arc lamp (Osram, Inc., Berlin, West Germany) and with an interference primary filter fluorescein isothiocyanate 495. The specificity of the conjugates was ascertained by double diffusion and by staining fixed marrow specimens from patients with myeloma of known immunoglobulin class and type.

Lymphocyte isolation and purification procedures. Mixed lymphocytes (ML), virtually free of granulocytes, platelets, and erythrocytes, were prepared according to previously described methods (24). Monocytes represented <1 and 0% of the total as determined by morphology (supravital and Wright stains) and by phagocytosis of carbonyl iron particles (SF-special, GAF Corp., New York), respectively. In addition, purified T and B cells from some patients and controls were obtained by repeatedly submitting ML to Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Piscataway, N. J.; Hypaque, Winthrop Laboratories, Sterling Drug Co., New York) gradients after E-rosette formation as follows: 1 ml of

sheep erythrocytes ($4 \times 10^8/\text{ml}$) was added to 1 ml of ML ($5 \times 10^7/\text{ml}$) and heat-inactivated fetal calf serum (FCS, Grand Island Biological Co.). This mixture was centrifuged at 200 g for 5 min and incubated at room temperature for 60 min. Cells from two or three such incubations were pooled, gently re-suspended, layered over 4 ml of Ficoll-Hypaque, and centrifuged at 400 g for 40 min. The cell button containing T cells and sheep erythrocytes was washed twice in ME medium, gently agitated, and layered over a Ficoll-Hypaque gradient to break up the rosettes and remove the sheep erythrocytes, respectively. The population at the interface, rich in B cells, was reprocessed through the rosette-gradient cycle once or twice. These procedures yielded a recovery of 40–60% B cells and 70–80% T cells in controls but somewhat less in patients. The final T-cell population in patients and controls was >98% pure and contained <1% contaminating B cells. The final B-cell suspensions from patients and controls were >98% and >97% pure, respectively, with <1% T-cell contamination. All lymphocyte suspensions were washed three times in ME medium before use.

Lymphocyte cultures. The LPHA-induced metabolic responses of ML and purified T- and B-cell subsets from patients and controls were ascertained by DNA synthesis measurements (25). Unless otherwise specified, 10^6 lymphocytes were cultured in a 2-ml vol of ME medium, with 20% FCS, antibiotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml mycostatin), and the appropriate concentrations of LPHA. To ascertain the full spectrum of lymphocyte responses to LPHA stimulation, lectin concentrations ranging from 0.1 to 200 $\mu\text{g}/\text{culture}$ were used. For all other studies, cells were exposed to 20 $\mu\text{g}/\text{culture}$ which induced maximal or near maximal responses from controls and CLL cells, respectively (as will be shown). After incubation for up to 7 d (to be specified for each set of experiments) at 37°C, DNA was pulse labeled (4 h) with 0.5 μCi of tritiated thymidine (New England Nuclear, Boston, Mass.; 6.7 Ci/mmol sp act), precipitated twice with 10% trichloroacetic acid, washed twice with 95% methanol, dissolved in 0.3 ml of Nuclear Chicago solubilizer and counted in a toluene-based scintillation fluid. cpm were measured at ambient temperature in an LS-150 Beckman spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) with counting efficiency of $45.0 \pm 0.9\%$, calculated according to the external standardization method (26). All cultures were done in triplicate plastic culture tubes (Falcon Plastics, Div. BioQuest, Oxnard, Calif.). Results are expressed either as mean \pm SEM dpm or as mean \pm SEM mitotic index (MI) (dpm LPHA-stimulated culture/dpm unstimulated culture).

Characterization of LPHA binding to lymphocyte receptors. The methodology involved in the evaluation of the binding of LPHA to specific lymphocyte membrane receptors has been described in detail elsewhere (15, 16). In brief, aliquots of 10^6 lymphocytes suspended in 0.1 ml of ME medium containing 0.1% bovine serum albumin (ME Medium/albumin) were incubated in duplicate plastic culture tubes with ^{125}I -LPHA concentrations ranging from 5 to 200 μg (0.1–200 μg in some cases) containing in 0.4 ml of phosphate-buffered saline with 0.1% albumin (NaCl-Pi/albumin). Incubation tubes were presoaked in 0.5% albumin in NaCl-Pi adjusted to pH 7.2 to reduce nonspecific binding. After 60-min incubation at 22°C, the reaction was abruptly terminated by temperature jump (addition of 10 ml of NaCl-Pi/albumin at 4°C). Unbound ^{125}I -LPHA was removed by washing four times with cold (4°C) ME medium containing 0.1% albumin, cells were extracted and collected on 0.5% albumin-pres soaked 0.45- μm filters (Millipore Corp., Bedford, Mass.), and cell-bound radioactivity was assessed in an Autowell gamma counter (Picker Corp., Charlotte, N. C.). The nonspecific binding component determined from replicates containing

no cells was subtracted from the total counts to obtain receptor-specific data.

RESULTS

Lymphocyte subpopulations. An average of $21.8 \pm 0.9\%$ of normal lymphocyte bore membrane immunoglobulin. Characteristically, heavy and light chains were variously represented in each individual. In contrast, $86.3 \pm 2.8\%$ of CLL lymphocytes were B cells of monoclonal origin. The surface immunoglobulin identified was $\mu\kappa$ in 18 patients, $\mu\lambda$ in 1, and $\gamma\lambda$ in 1. Stained lymphocytes demonstrated the characteristic punctuate fluorescence discretely distributed but occasionally polarized in a crescent formation. The intensity of the fluorescence was subnormal in patients with CLL. However, reproducibility was excellent regardless of the cell source and the type or percentage of the immunoglobulin identified. Although $62.0 \pm 2.8\%$ of normal circulating lymphocytes formed E-rosettes, only $5.4 \pm 1.0\%$ of CLL lymphocytes were T cells (range of 1–12%).

Measurements of LPHA binding to lymphocyte receptors. Specificity in the binding of ^{125}I -LPHA to lymphocyte membrane receptors has previously been reported (15). Binding to leukemic lymphocytes was time and temperature dependent, as was binding to normal cells (15). Binding equilibrium was reached after 60 min incubation at 22°C , twice the equilibrium binding time to normal lymphocytes (15). Furthermore, the magnitude of ^{125}I -LPHA binding to leukemic cells was markedly subnormal. Binding capacity and affinity by leukemic and normal lymphocytes were assessed by Scatchard analysis (27). Data obtained from a 40-fold ^{125}I -LPHA concentration spectrum (5–200 μg) demonstrated straight forward second order kinetics. However, using a 2,000-fold concentration range (0.1–200 μg), the linearity of the Scatchard plot gave way to complex binding curves in both patients and controls (Fig. 1). As noted in the literature (16, 28), the complexity of receptor-ligand interactions is only brought forth when studied over a wide spectrum of ligand concentrations, thus explaining the straight-forward single component plots obtained with narrow concentrations of erythroagglutinin (12) and LPHA (11). After exclusion of alternate models (15, 29, 30), curvilinear Scatchard plots of upward concavity may be attributed to (a) multiple classes of binding sites, each with different but fixed affinity on the same or different cells; (b) a single class of receptors with variable affinity inversely proportional to receptor occupancy; or (c) multiple classes of interacting binding sites. Our equilibrium data reflect complex receptor-LPHA interactions of all LPHA-binding lymphocytes rather than the existence of distinct orders of receptors on different lymphocyte subsets. Support for this view comes

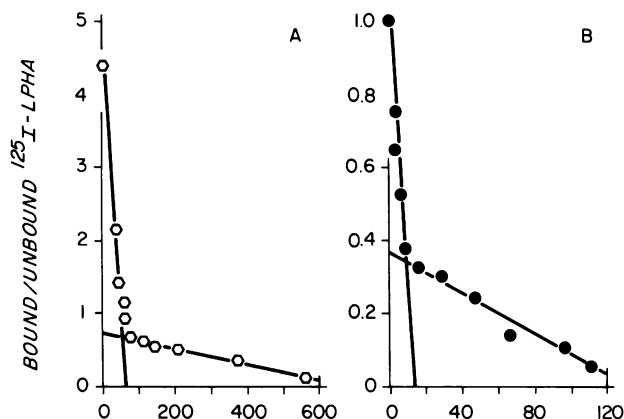


FIGURE 1 Scatchard plots of receptor-specific binding of ^{125}I -LPHA to normal and CLL lymphocytes. Bound/unbound ^{125}I -LPHA ($\times 100$) is plotted as a function of nanograms of bound ^{125}I -LPHA/ 10^6 cells. Plot A was generated by normal lymphocytes; plot B emerged from CLL cells. If biphasic plots are assumed, the first components in controls ($n = 12$) and patients with CLL ($n = 5$) show receptor capacities of 60 and 15 ng ^{125}I -LPHA/ 10^6 lymphocytes ($P < 0.01$) with affinities of 1.8 and $1.0 \times 10^8 \text{ M}^{-1}$ ($P > 0.05$) and correlation coefficients >0.98 ($P < 0.001$) and >0.93 ($P < 0.001$), respectively. The second components yield capacities in controls ($n = 15$) and patients ($n = 20$) of 668 and 137 ng ^{125}I -LPHA/ 10^6 lymphocytes ($P < 0.01$) with affinities of 1.5 and $2.1 \times 10^6 \text{ M}^{-1}$ ($P > 0.05$) and correlation coefficients >0.94 ($P < 0.001$) and >0.98 ($P < 0.001$), respectively.

from characteristic curvilinear Scatchard plots generated (a) by ML from patients and controls (Fig. 1); (b) by highly purified B cells from controls and patients with B-cell CLL (not shown); and (c) by highly purified T cells isolated from controls and from a patient with T-cell-type CLL (not shown). Detailed studies on receptor-LPHA dissociation kinetics suggest that the binding complexity reflects site-site interactions resulting in occupancy-dependent receptor affinity (16), thus precluding accurate calculations of lymphocyte binding capacity (R_0) for LPHA and of physically meaningful affinity constants (K_a). However, if a biphasic plot is assumed, R_0 and K_a can be calculated from the intercept on the abscissa and from the slope of each component, respectively. These values (Table I) indicate that although the density of membrane receptors for ^{125}I -LPHA on leukemic cells was 20–25% of normal, affinity for the ligand was normal. Purified B cells from patients and controls generated binding data comparable to that obtained from the ML from which they originated (Table I). The decreased receptor capacity for LPHA and other mitogens (12, 13) reflects structural membrane abnormalities of these cells rather than their subnormal size (31) because it appears out of proportion to the small difference in cell size. Furthermore, other membrane abnormalities on CLL cells are well documented (32, 33).

TABLE I
Normal and Leukemic Lymphocyte Receptors for ^{125}I -LPHA*

Receptor capacity/affinity	Mixed lymphocytes		B Lymphocytes	
	Normal	CLL	Normal	CLL
Ro_1 , ng/ 10^6 cells	$60 \pm 13 \ddagger$	15 ± 6 (<0.01)	45 ± 5	8 ± 1 (<0.01)
Ka_1 , $\times 10^8 \text{ M}^{-1}$	$1.8 \pm 0.6 \ddagger$	1.0 ± 0.4 (NS)	1.6 ± 0.4	1.1 ± 0.4 (NS)
Ro_2 , ng/ 10^6 cells	$668 \pm 86 \ddagger$	137 ± 20 (<0.01)	540 ± 64	104 ± 60 (<0.01)
Ka_2 , $\times 10^8 \text{ M}^{-1}$	$1.5 \pm 0.2 \ddagger$	2.1 ± 0.7 (NS)	1.9 ± 0.6	2.4 ± 0.7 (NS)

* Ro and Ka derived from the intercept on the abscissa and from the slope of each component of Scatchard plots (see text) are expressed as mean \pm SEM.

‡ Within each donor group, the differences observed in Ro_1 , Ka_1 , Ro_2 , and Ka_2 between cell populations were NS.

LPHA stimulation as a function of lymphocyte subpopulations and incubation time. These experiments were undertaken to ascertain the extent to which LPHA stimulated highly purified T and B cells. Such information is pertinent to the study of CLL by virtue of conflicting data from normal B-cell responsiveness to PHA (11, 34–36) and the suggestion that the impaired and delayed PHA responses by CLL cells might reflect a B-cell attribute (4).

The time-course of lymphocyte stimulation by 20 μg LPHA/ 10^6 lymphocytes is shown in Fig. 2. The kinetics of DNA synthesis by normal and leukemic ML,

highly purified T and B lymphocytes are depicted as the upper and lower curves of panels A, B, and C, respectively. Several points are apparent. First, normal B cells responded to LPHA as well as did normal ML and T cells, though their peak response occurred on the 4th rather than the 3rd d. Second, normal T- and B-cell responses at 6 and 7 d were less than that of ML. Third, responses by ML, T, and B leukemic cells were delayed and markedly impaired when compared to their respective normal counterpart.

LPHA stimulation as a function of lectin concentrations. DNA synthesis in response to 2.5–200 μg of LPHA/ 10^6 lymphocytes (0.1–200 μg in five patients and five controls) was ascertained at the time of peak response (after stimulation of normal and CLL lymphocytes for 3 and 6 d, respectively). A characteristic normal lymphocyte response profile is shown in Fig. 3 (upper curve). Significant cell activation occurred in response to 8.5 ng of receptor-bound ^{125}I -LPHA/ 10^6 lymphocytes. Escalation of LPHA concentrations magnified the DNA synthesis response reaching a maximum (MI 23.4 ± 5.4) when 120.8 ng of ^{125}I -LPHA was receptor-bound, beyond which the lectin becomes progressively lymphotoxic (17). In contrast, DNA synthesis by CLL cells (Fig. 3, lower curve) was markedly impaired. When analyzed in each individual patient, DNA synthesis was consistently subnormal and bore no relationship to the size of the T-cell subset. The abnormal CLL response profile is characterized by cell insensitivity to the lectin (17), judged by the greatly increased (>fivefold) amounts of cell-bound lectin needed to induce a significant response, and by the impaired or absent metabolic responses to the subnormal amounts of receptor-bound LPHA. Indeed, when calculated as a function of cell-bound ^{125}I -LPHA, DNA synthesis by CLL cells was only a fraction of normal. This suggests that the decreased density of LPHA receptors on leukemic lymphocytes might not satisfactorily explain these cells' impaired responses to the lectin.

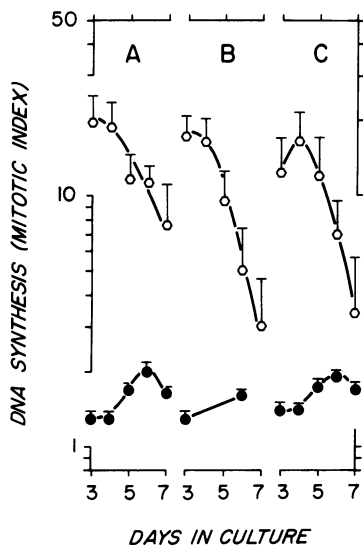


FIGURE 2 Time-course of lymphocyte responsiveness to LPHA. DNA synthesis by normal mixed lymphocytes ($n = 15$) and by highly purified normal T ($n = 7$) and B ($n = 4$) cells are depicted as the upper curves of panels A, B, and C, respectively. Lower curves represent mitogenic responses by leukemic mixed lymphocytes ($n = 20$) and by leukemic T ($n = 3$) and B ($n = 7$) cells. Results are expressed as mean \pm SEM MI and are plotted as a function of time.

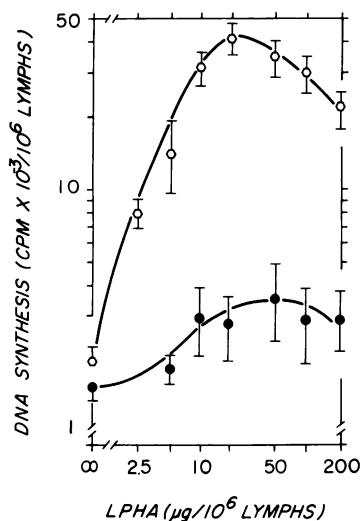


FIGURE 3 Lymphocyte responsiveness to LPHA stimulation. DNA synthesis from normal ($n = 15$) and leukemic ($n = 20$) mixed lymphocytes are shown as the upper and lower curves, respectively. Results expressed as mean \pm SEM dpm/ 10^6 lymphocytes are plotted as a function of the stimulating lectin concentrations.

Cocultured experiments. Another explanation for the abnormal CLL response might relate to alterations in cells or humoral factors that regulate immune reactions. To determine whether the hyporesponsiveness to LPHA in CLL was a result of an intrinsic defect of effector cell function or whether it could derive from a lack of helper cells or from an excess of suppressor cells (5–7) we examined the LPHA-induced DNA synthesis by leukemic and normal lymphocytes cocultured with normal and leukemic cells, respectively.

DNA synthesis by CLL cells cocultured with normal lymphocytes. Leukemic cells (0.8×10^6) and normal lymphocytes (0.2×10^6) were cultured separately and together in the presence and absence of $20 \mu\text{g}$ of LPHA for 6 d (Table II). All other culture conditions were kept constant. If the mitogenic hyporesponsiveness of CLL cells reflects a lack of helper cells, addition of a small number of normal lymphocytes containing the putative helper cells would be expected to amplify the DNA synthesis response of effector CLL cells. The adequacy of the leukemic:normal cell ratio used is suggested by several reports (37, 38). The small numbers of normal lymphocytes used was also selected to minimize mixed lymphocyte reactions (MLR) and to reduce these cells' direct contribution to total DNA synthesized by the cocultured cells. The adequacy of the incubation time is based on our Fig. 2. Indeed, whether the kinetics of an enhanced response by leukemic effector cells "reconstituted" with normal helper cells follows a normal or leukemic pattern (peak response at 3 or 6 d, respectively), such enhancement would be ap-

parent by day 6. The impaired leukemic cell responses were not corrected by the addition of normal lymphocytes, thus excluding defective helper function in CLL. Indeed, as shown in Table II, spontaneous and LPHA-induced DNA synthesis by cocultured cells were not significantly different than that of leukemic cells cultured separately. Significant MLR was ruled out by these results and by appropriate controls (not shown) in which mitomycin-C-treated ($25 \mu\text{g/ml}$) stimulating leukemic or normal cells were cocultured with responding normal or leukemic cells, respectively, in the absence of LPHA.

DNA synthesis by normal lymphocytes cocultured with leukemic cells. Normal lymphocytes (0.8×10^6) and leukemic cells (0.2×10^6) were cultured separately and together in the presence and absence of $20 \mu\text{g}$ of LPHA for 3 d. All other culture conditions were kept constant. Such cell mixtures would not be expected to generate appreciable MLR after 3-d cultures (39). Furthermore, absence of MLR was documented in control experiments (not shown) using mitomycin-C-treated cells, as described above. As shown in Table III, CLL cells inhibited normal lymphocyte responses to LPHA by 94.3% ($P < 0.001$). This effect was observed for each of eight CLL patients studied (range of inhibition, 83–100%). Moreover, spontaneous DNA synthesis by normal lymphocytes was inhibited by 75% ($P < 0.001$) and was induced by cells from all patients (range of inhibition, 68–87%). To determine whether this suppressor effect was mediated by humoral factors secreted or released by leukemic cells, normal lymphocytes were cultured in 20% leukemic serum from three of the eight patients shown to have suppressor activity, and in cell-free supernates obtained from leukemic cells cultured 48 h with and without $20 \mu\text{g}$ LPHA. Selection of 48-h supernates was based on the premise that if suppression observed in cocultures at 72 h is humorally mediated, suppressor activity should be

TABLE II
DNA Synthesis by CLL Cells Cocultured with Normal Lymphocytes*

DNA synthesis	Normal cells† (n = 5)	CLL cells† (n = 5)	CLL cells plus normal cells‡ (n = 5)
			dpm/culture
Spontaneous	491 \pm 45	495 \pm 34	516 \pm 44
LPHA-induced	951 \pm 147	707 \pm 81	863 \pm 104

* Expressed as mean \pm SEM.

† Normal cells (0.2×10^6), CLL cells (0.8×10^6) cultured separately.

‡ CLL cells (0.8×10^6) cocultured with normal cells (0.2×10^6).

^{||} Significance of difference between CLL cells and CLL cells plus normal cells was NS.

TABLE III
*DNA Synthesis by Normal Lymphocytes Cocultured with CLL Cells**

DNA synthesis	CLL cells§ (n = 8)	Normal cells§ (n = 8)	Normal cells plus†			
			CLL cells (n = 8)	CLL supernates (n = 3)	CLL sera (n = 3)	Normal sera (n = 3)
			<i>dpm/culture</i>			
Spontaneous	639±90	2,189±136	549±57	2,011±85	2,056±144	1,978±157
LPHA-induced	693±92	22,160±855	1,585±338	17,524±1,583	18,277±2,171	28,383±1,668

* Expressed as mean±SEM.

† Normal cells (0.8×10^6) cocultured with CLL cells (0.2×10^6), or cultured in CLL supernates; CLL sera; and normal sera.

§ CLL cells (0.2×10^6), normal cells (0.8×10^6) cultured separately.

^{||} Significance of differences between normal cells and normal cells plus CLL cells was <0.001 , between normal cells and normal cells plus either CLL supernates, CLL sera, and normal sera was NS.

present in 48-h supernates. Normal cells cultured in 20% FCS and in 20% autologous serum served as controls. These experiments demonstrated (Table III) that the marked suppressor effect of leukemic cells on spontaneous and LPHA-induced DNA synthesis by normal lymphocytes was not mediated by humoral factors. Indeed, the mild inhibitory effect that leukemic sera and supernates appeared to exert on normal lymphocyte responses was not significant ($P > 0.05$) and was seen in only two of the three patients studied. To determine the origin of CLL suppressor cells, i.e., whether T or B cells are responsible for the suppression, normal lymphocytes (0.8×10^6) were cocultured as described above with highly purified T, B, or with mixed cells (0.2×10^6) from three patients with suppressor activity. The marked suppressor effect (71%) of mixed cells on normal lymphocyte responses to LPHA was also exerted by highly purified CLL T (65%) and by highly purified CLL B (68%) cells. The degree of suppression by the various cell populations was comparable for each patient studied.

DISCUSSION

The decreased proliferative responses of leukemic lymphocytes to LPHA can be attributed to: (a) In vitro cell death. This is excluded, however, by the comparable cell viability observed in patients and controls; (b) A small fraction of residual normal T cells as the only proliferating cell (3). Although a few diluted T cells contribute to the overall response observed, they are not the only proliferative cells, because there was no discernible correlation between DNA synthesis and the size of the T-cell subpopulation in our group of patients. Furthermore, highly purified T lymphocytes from three patients with B-cell-type CLL showed no greater responses to LPHA than their B-cell counterparts. In addition, a 1:10 dilution of PHA-responsive cells with mitomycin-C-treated autologous lympho-

cytes or with autologous acute myeloblastic leukemic cells failed to delay maximal responses to PHA (36); (c) Hyporesponsive leukemic B cells reflecting their nonthymic origin rather than their neoplastic nature. This view (4) is based on the reported absolute or relative insensitivity of normal B lymphocytes to soluble mitogens (34, 36). However, in this and other studies (11, 35) normal B cells were as responsive to LPHA stimulation as were T cells and ML from which they originated. Moreover, we observed markedly impaired DNA synthesis by highly purified T cells isolated from patients with B-cell CLL and from one patient with T-cell CLL; (d) A functional or quantitative defect of leukemic receptors for LPHA. However, leukemic receptor affinity for LPHA was normal and the decreased LPHA receptor density on leukemic cells might not satisfactorily explain their diminished proliferative responses to the lectin. Indeed, equivalent amounts of receptor-bound LPHA induced greatly diminished responses from CLL cells, as compared to normal lymphocytes. Furthermore, little response to LPHA (MI 7.3 in response to $20 \mu\text{g}$ LPHA) was observed in a patient with T-cell-type CLL (with 87% T and 2% B cells) in spite of normal receptor capacity and affinity for the lectin; (e) The presence of humoral mitogenic inhibitors or absence of lymphokines necessary to amplify and accelerate the mitogenic response. However, our failure to demonstrate mitogenic inhibitors in patients' sera and in supernates of CLL cell cultures, and the production of lymphokines by CLL cells despite their poor PHA responses (8) argue against these possibilities; (f) The presence of suppressor cells or the absence of helper cells. Our studies show that mitogenic responses of cells from five randomly selected patients with CLL were not enhanced when cocultured with normal lymphocytes. In contrast, cells from eight random patients markedly inhibited cocultured normal lymphocyte responses to LPHA. This suppressor effect was not mediated by humoral factors

in patients' sera or in cell-free supernates, suggesting either very labile soluble suppressor factors or the need for direct suppressor-target cell interactions. The distribution of lymphocyte subsets in our patient population and the virtual absence of monocytes from our cell suspension suggest that suppressor cells in CLL might be of nonthymic origin. However, we could not link suppressor activity to presence of membrane immunoglobulins or E-rosette receptors. This suggests at least three possibilities: (a) the presence of CLL T and CLL B suppressor cells with similar nonspecific suppressor activity, an unlikely possibility; (b) contamination of our T- and B-cell suspensions by an undetectable, small number of highly functional suppressor cells of homogeneous origin; (c) alternatively, suppressor CLL cells may be homogeneous in origin but may exhibit heterogeneous surface determinants. Membrane abnormalities (perhaps related to neoplastic transformation) shown in this and other reports (12, 32, 33), suggest that the true origin of CLL cells may not be conclusively ascertained by the presence or absence of surface markers. It could be argued that the decreased receptor density for LPHA on leukemic cells and their poor mitogenic response to the lectin might suggest shedding of LPHA receptors or LPHA blocking factors from the CLL cell surface into the supernate, thus explaining the decreased mitogenic responses by cocultured normal lymphocytes. However, the suppressor effect exerted by CLL cells on normal lymphocytes was not mediated by CLL supernates and was also observed in the absence of LPHA. In addition, the markedly decreased mitogenic responses by CLL cells was out of proportion to their decreased receptor capacity for LPHA.

Suppressor cells have been described in a number of congenital and acquired immunodeficiencies in animal models and in the human (6, 7, 37, 38). These cells appear to play a primary as well as a secondary pathogenic role in the development of certain diseases (6). Our studies did not examine whether the development of suppressor cells in CLL represents a primary pathogenic mechanism or whether leukemic lymphocytes exhibit intrinsic cell defects reflecting the neoplastic transformation with secondary acquisition of suppressor cells. However, whether primary or secondary, through nonspecific inhibition (7, 40) of various target tissues, suppressor cells may exert a significant role in the pathogenesis of several complications of CLL. Thus, blocked of polyclonal B-cell maturation by suppressor cells similar to that reported in experimental (41) and human multiple myeloma (6) may also account for the hypogammaglobulinemia frequently present in patients with CLL. Of interest is the recent report that severe anemia in a case of T-cell-type CLL may be a result of interactions between neoplastic T cells and erythroid precursors (42). The biologic role of suppres-

or cells may be the inhibition of tumor cell proliferation. However, suppressor cells of tumor bearing hosts may inhibit in vivo immune responses to the tumor by interfering with in vivo expansion of tumor-reactive T cells in a manner similar to the nonspecific inhibition of in vitro LPHA-induced lymphoproliferation shown in this report.

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REFERENCES

1. Rubin, A. D., K. Havermann, and W. Dameshek. 1968. Other studies of the proliferative abnormality of the blood lymphocyte. *Blood*. **33**: 313-328.
2. Havermann, K., and A. D. Rubin. 1968. Delayed response of chronic lymphocytic leukemia lymphocytes to phytohemagglutinin in vitro. *Proc. Soc. Exp. Biol. Med.* **127**: 668-671.
3. Wybran, J., S. Chantler, and H. H. Fudenberg. 1973. Isolation of normal T cells in chronic lymphatic leukemia. *Lancet*. **I**: 126-129.
4. Aisenberg, A. C., and K. J. Bloch. 1972. Immunoglobulins on the surface of neoplastic lymphocytes. *N. Engl. J. Med.* **287**: 272-276.
5. Katz, D. H., and B. Benacerraf. 1972. The regulatory influence of activated T cells on B cell responses to antigen. *Adv. Immunol.* **15**: 1-94.
6. Waldmann, T. A., R. M. Blaese, S. Broden, and R. S. Krakauer. 1978. Disorders of suppressor immunoregulatory cells in the pathogenesis of immunodeficiency and autoimmunity. *Ann. Intern. Med.* **88**: 226-238.
7. Gorczynski, R. M. 1974. Immunity to murine sarcoma virus-induced tumors. II. Suppression of T cell-mediated immunity by cells from progressor animals. *J. Immunol.* **112**: 1826-1838.
8. Kasakura, S. 1975. MLC stimulatory capacity and production of a blastogenic factor in patients with chronic lymphatic leukemia and Hodgkin's Disease. *Blood*. **45**: 823-832.
9. Arpels, C., and C. M. Southam. 1969. Cytotoxicity of sera from healthy persons and cancer patients. *Int. J. Cancer*. **4**: 548-559.
10. Greaves, M. G., S. Bauminger, and G. Janossy. 1972. Lymphocyte activation. III. Binding sites for phytomitogens on lymphocyte subpopulations. *Clin. Exp. Immunol.* **10**: 537-554.
11. Boldt, D. H., R. P. MacDermott, and E. P. Jorolan. 1975. Interaction of plant lectins with purified human lymphocyte populations: binding characteristics and kinetics of proliferation. *J. Immunol.* **114**: 1532-1536.
12. Kornfeld, S. 1969. Decreased phytohemagglutinin receptor sites in chronic lymphocytic leukemia. *Biochim. Biophys. Acta.* **192**: 542-545.
13. Novogrodsky, A., M. Biniaminov, B. Ramot, and E. Katchalski. 1972. Binding of Concanavalin-A to rat, normal human and chronic lymphatic leukemia lymphocytes. *Blood*. **40**: 311-316.
14. Faguet, G. B. 1978. Cellular immunity in sarcoidosis. Evidence for an intrinsic defect of effector cell function. *Am. Rev. Respir. Dis.* **118**: 89-96.
15. Faguet, G. B. 1977. Mechanisms of lymphocyte activation.

- I. Binding kinetics of phytohemagglutinin (PHA) to human lymphocytes. *J. Biol. Chem.* **252**: 2095-2100.
16. Faguet, G. B. 1976. Mechanisms of lymphocyte activation. II. Negative cooperativity in the binding of phytohemagglutinin (PHA) to its lymphocyte receptors. In *Leukocyte Membrane Determinants Regulating Immune Reactivity*. V. P. Eijssvoogel, D. Ross, and W. P. Zeijl-maker, editors. Academic Press, New York. 65-72.
 17. Faguet, G. B. 1976. Mechanisms of lymphocyte activation. III: effect of ligand concentration and induction time. In *The Reticuloendothelial System in Health and Disease*. S. M. Reichard, M. R. Escobar, and H. Friedman, editors. Plenum Press, New York. **73A**: 329-337.
 18. Rai, K. R., A. Sawitsky, E. P. Cronkite, A. D. Chanana, R. N. Levy, and B. S. Pasternack. 1975. Clinical staging of chronic lymphatic leukemia. *Blood*. **46**: 219-234.
 19. Weber, T. H. 1969. Isolation and characterization of lymphocyte stimulating leucoagglutinin from red kidney beans. *J. Clin. Lab. Invest.* **14**(Suppl. III): 1-80.
 20. Hunter, W. M. The preparation of radioiodinated proteins of high activity, their reaction with antibody in vitro: the radioimmunoassay. In *Handbook of Experimental Immunology*. Blackwell Publishing Co., London. 606.
 21. Brouet, J. D., G. Flandrin, and M. Seligmann. 1973. Indications of the thymus-derived nature of the proliferating cells in six patients with Sezary syndrome. *N. Engl. J. Med.* **289**: 341-344.
 22. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. *J. Exp. Med.* **136**: 207-215.
 23. Pernis, B., M. Serrarini, L. Forni, and L. Amante. 1971. Immunoglobulins on lymphocyte membranes. In *Progress in Immunology*. B. Amos, editor. Academic Press, New York. 95-106.
 24. Faguet, G. B. 1974. Lymphocyte purification: an improved method, qualitative and quantitative evaluation. *Bio-medicine (Paris)*. **21**: 153-157.
 25. Faguet, G. B. 1975. Quantitation of immunocompetence in Hodgkin's Disease. *J. Clin. Invest.* **56**: 951-957.
 26. Cavanaugh, R. 1970. Statistical considerations in external standardization. In *The Current Status of Liquid Scintillation Counting*. E. D. Bransome, Jr., editor. Grune and Stratton, Inc., New York. 293-304.
 27. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**: 660-672.
 28. Krug, U., M. D. Hollenberg, and P. Cuatrecasas. 1973. Changes in the binding of Concanavalin-A and wheat germ agglutinin to human lymphocytes during in vitro transformation. *Biochem. Biophys. Res. Commun.* **52**: 305-312.
 29. DeMeyts, P. 1976. Cooperative properties of hormone receptors in cell membranes. *J. Supramol. Struct.* **4**: 241-258.
 30. Rodbard, D. 1973. Mathematics of hormone-receptor interactions. I. Basic principles. *Adv. Exp. Med. Biol.* **36**: 289-326.
 31. Schrek, R. 1972. Ultrastructure of blood lymphocytes from chronic lymphocytic and lymphosarcoma cell leukemia. *J. Natl. Canc. Inst.* **48**: 51-64.
 32. Hakomori, S. C., C. Treather, and A. Andrews. 1968. Organizational difference of cell surface "hematoside" in normal and virally transformed cells. *Biochem. Biophys. Res. Commun.* **33**: 563-568.
 33. Brody, J. I., and L. H. Beizer. 1965. Alteration of blood group antigens in leukemic lymphocytes. *J. Clin. Invest.* **44**: 1582-1589.
 34. Geha, R. S., F. S. Rosen, and E. Merler. 1974. Unresponsiveness of human B lymphocytes to phytohemagglutinin. *Nature (Lond.)*. **248**: 426-428.
 35. Phillips, B., and W. M. Rotte. 1973. Evidence for transformation of human B lymphocytes by PHA. *Nat. New Biol.* **241**: 254-256.
 36. Schultz, E. G., S. Davis, and A. D. Rubin. 1976. Further characterization of the circulating cell in chronic lymphocytic leukemia. **48**: 223-234.
 37. Kontiainen, S., and M. Feldmann. 1976. Suppressor cell induction in vitro I. Kinetics of induction of antigen-specific suppressor cells. *Eur. J. Immunol.* **6**: 296-301.
 38. Kirchner, H., A. V. Muchmore, T. M. Chused, H. T. Holden, and R. B. Herberman. Inhibition of proliferation of lymphoma cells and T lymphocytes by suppressor cells from spleen of tumor-bearing mice. *J. Immunol.* **114**: 206-210.
 39. Twomey, J. J., O. Sharkey, Jr., J. A. Brown, A. H. Laughter, and P. H. Jordan, Jr. 1970. Cellular requirements for the mitotic response in allogeneic mixed leukocyte cultures. *J. Immunol.* **104**: 845-853.
 40. Eggers, A. E., and J. R. Wunderlich. 1975. Suppressor cells in tumor-bearing mice capable of nonspecific blocking of in vitro immunization against transplant antigens. *J. Immunol.* **114**: 1554-1556.
 41. Krakauer, R. S., W. Strober, and T. A. Waldmann. 1977. Hypogammaglobulinemia in experimental myeloma: the role of suppressor factors from mononuclear phagocytes. *J. Immunol.* **118**: 1385-1390.
 42. Hoffman, R., S. Kopel, N. Dainiak, L. Solomon, S. D. Hsu, E. D. Zanjani, and R. Papac. 1978. Chronic lymphocytic leukemia: presence in bone marrow and peripheral blood of T-cells that suppress erythropoiesis in-vitro. *Clin. Res.* **26**: 436A. (Abstr.)