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Failure of B Cells of Chronic Lymphocytic Leukemia in Presenting Soluble and Alloantigens

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B-cell-type chronic lymphocytic leukemia (B-CLL) patients have immunological abnormalities of both B and T lymphocytes. Since T cell defects might depend upon a defective accessory function of neoplastic B lymphocytes, we analyzed the ability of peripheral blood B cells of seven B-CLL patients to stimulate allogenic normal T cells in mixed lymphocyte reaction (MLR) and to present tetanus toxoid (TT) to autologous T cells. In both systems, neoplastic B lymphocytes show a defective antigen-presenting function, which is more evident with disease progression. Such a defect cannot be ascribed to a decreased MHC class II molecule expression nor to an abnormal IL-1β production, but it can be partially accounted for by a low B7 expression. Pretreatment of neoplastic B cells with interleukin-4 (IL-4) restores primary MLR, but has little effect on the response to TT. The effect of IL-4 is not mediated by quantitative modifications of class II and B7 molecule expression or of IL-1β production.

INTRODUCTION

The ability of B cells to serve as accessory cells is well documented. Like macrophages, B cells process and present soluble antigens (1) and are more efficient when antigen uptake is mediated by specific binding to surface immunoglobulins (Ig) (2). Moreover, they may behave as antigen-presenting cells (APC) in primary mixed lymphocyte reactions (MRL) (3). In order to carry out these function, B cells must first be activated, and to this end Epstein–Barr virus (EBV) infection (4), phorbol esters (5), anti-Ig (6) or lipopolysaccaride, and dextran (3, 7) have been used. Accumulating evidence indicates that B lymphocytes may also play a key role in cellular immune responses: the *in vivo* depletion of B cells in anti-μ-treated mice affects several T cell functions such as priming of helper T cells (8) and induction of cytotoxic T lymphocytes (9).

It has been suggested that in B-cell-type chronic lymphocytic leukemia (B-CLL), the malignant B cells might function as APCs (10) and previous studies on murine lymphomas are consistent with this hypothesis (11). Nevertheless, B-CLL B cells display several anomalous features, such as defective capping of surface membrane Ig (12) and a peculiar cytoskeleton organization (13). Moreover, these malignant B-CLL cells present paradoxical dissociation between an activated phenotype and an inability to respond to proliferative stimuli such as those delivered by interleukin-2 (IL-2) and low-molecular-weight B cell growth factor (14). Different accessory-cell-dependent T cell functions are also abnormal in B-CLL, although it is uncertain whether these abnormalities are the cause or an effect of the disease. Reductions in IL-2 production (15), mitogen response (16), helper (17), and cytotoxic (18) activities have been documented.

Since B cells, for their large number and strategic location, might be considered as the most representative APC population in B-CLL patients, we asked whether T cell defects in B-CLL can be ascribed to a defective accessory function of neoplastic B cells. In the present study we show that B-CLL B cells fail to stimulate allogeneic normal T cells in MLR and fail to present tetanus toxoid (TT) to autologous T cells. Pretreatment of B-CLL cells with interleukin-4 (IL-4) restores primary MRL, but has little effect on the response to TT.

MATERIALS AND METHODS

Cell Donors

Heparinized blood samples were collected from seven patients with B-CLL, before treatment was initiated. B-CLL was diagnosed on the basis of clinical (Table 1), morphological, and immunophenotypic features; the patients were staged according to Rai. The percentage of malignant B cells was assessed by immunofluorescence microscopy utilizing rabbit anti-human Ig polyclonal antibodies (Dakopatts, Copenhagen, Denmark), CD19 (B4, Coulter Immunology, Hialeah, FL), and CD5 (Leu 1, Becton-Dickinson, Mountain View, CA) monoclonal antibodies (MoAbs). Since most elderly

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TABLE 1
Clinical Parameters of CLL Patients

Patients	WBC ($\times 10^9$/liter)	Percentage B[a] lymph	Hb (g/dl)	Plts ($\times 10^9$/liter)	Stage[b]
MG	15	72	14.3	253	0
CG	23	83	13.8	321	0
TA	34	91	14.1	279	I
MC	72	92	12.5	305	I
ZG	88	91	13.9	256	I
FA	83	94	10.1	193	III
GD	120	92	9.2	76	IV

[a] Percentage of B cells was evaluated by CD19 staining.
[b] Patients were staged according to Rai.

persons are not immunized against tetanus toxoid, after informed consent, patients were vaccinated by two intramuscular injections of specific antigen (40 IU/dose) (Anatetall, Sclavo, Siena, Italy) administered at 4-week intervals. Blood samples from normal age-matched volunteers and tonsils obtained from children undergoing tonsillectomy were used as controls.

Cell Purification

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll–Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation and washed twice with RPMI 1640 medium supplemented with 5% fetal calf serum (FCS, Gibco, Grand Island, NY). Tonsil samples were teased with blunt forceps and the cell suspensions were washed twice in the same medium. PBMC and tonsil cells were purified into T- and B-cell-enriched fractions by means of rosetting with sheep red blood cells (SRBC). Cell fractions were freed of SRBC by osmotic shock with ammonium chloride (0.87 g/dl) and then washed twice in 5% FCS–RPMI. To remove adherent cells, the cells collected were plated on plastic culture dishes (Falcon, Lincoln Park, NJ) at a concentration of 2×10^6/ml and incubated for 1 hr at 37°C in a 5% CO_2 atmosphere.

The percentage of contaminating cells after rosetting and plastic adhesion was evaluated by immunofluorescence staining with MoAbs specific for CD19, CD3 (Leu4, Becton-Dickinson), and CD11b (Mol, Coulter). In B-CLL samples, analysis of the T cell fraction revealed <2% $CD19^+$ cells and $<1^+$ $CD11b^+$ cells; in the B cell fraction, $CD3^+$ and $CD11b^+$ were 4 and 3% of the purified T cell fraction, while the B cell fraction was contaminated with <2% $CD3^+$ and $CD11b^+$ cells. The vast majority of purified tonsil B cells reacted with CD 19 (>95%), while $CD3^+$ and $CD11b^+$ were <1%.

Mixed Lymphocyte Reaction

Unidirectional standard allogeneic primary MLR were set up with 1.5×10^5 normal T cells and 1.5×10^5 mitomycin-treated (m) neoplastic or normal B cells. Cultures were plated on flat-bottomed microculture plates (Costar, Cambridge, MA) in 0.2 ml of RPMI 1640 with 200 m*M* L-glutamine, 10 U/ml penicillin, 50 mg/ml streptomycin, 25 mM Hepes, and 10% pooled human AB^+ serum; all cultures were performed in triplicate and incubated at 37°C in 5% CO_2 atmosphere for 5 days. Sixteen hours before harvesting, 18 B of [*methyl*-^{3}H]thymidine ([^{3}H]TdR, sp act 74 GBq/mmol; New England Nuclear, Boston, MA) was added to MLR; radionucleotide uptake was determined by liquid scintillation counting. The results were expressed in counts per minute (cpm) as the average value of triplicate cultures. In order to make data more comparable, stimulation index (SI) for each culture was calculated according to the following formula: $SI = cpm(A + B_m) - (B_m)/cpm(A + A_m) - (A_m)$, where $(A + B_m)$ and $(A + A_m)$ are experimental and control combinations or responder (A) and stimulator (A_m, B_m) cells, respectively.

In third-party cultures, 10^5 B-CLL cells were added to control MLR, i.e., normal T cell (responder) and allogeneic mitomycin-treated normal B cells (stimulator), prepared as described above. All of the patients, except MG and GD were bled and their cells were employed on the same day to obtain a more reliable comparison between them. Cells from MG and GD were previously frozen in liquid nitrogen after purification into T and B cell fractions.

Response to Tetanus Toxoid

Mitomycin-treated neoplastic B cells were plated on round-bottomed microculture plates (Costar) at 2×10^4/well with 10^5 autologous T cells in a final volume of 0.2 ml of complete RPMI medium +5% human AB^+ serum. Where specified, unfractionated PBMC from patients were plated at 2×10^5/well. Tetanus toxoid, a generous gift of Dr. Clara Michelangeli (Sclavo, Siena, Italy), was added to each well at a concentration of 10 μg/ml. All cultures were performed in triplicate and incubated at 37°C in 5% CO_2 atmosphere for 7 days. Sixteen hours before harvesting, [^{3}H]TdR was added to each well. Results were expressed in cpm and in SI where SI = cpm(T cells × B cells × TT)/cpm(T cells + TT). Control cultures consisted of normal TT-reactive T cells and mitomycin-treated tonsil or EBV-transformed autologous normal B cells as antigen presenting cells. T cells reactive to TT were obtained, as described by Issekutz *et al.* (4), from cultures of normal PBMC (10^6 cells/ml) repetitively stimulated with 10 μg TT/ml and expanded in the presence of 10 U/ml of recombinant IL-2 (Roche, Nutley, NJ). These TT-reactive T cells were used after 2 weeks for antigen proliferation assays in control cultures.

IL-4 Treatment

B-CLL, normal, or EBV-transformed B cells were cultured at a concentration of 2×10^6/ml in 24-well

plates (Costar) in a final volume of 1 ml of complete medium, in the presence of 1000 U/ml of IL-4 (Genzyme Corp., Boston, MA). The cultures were incubated at 37°C in 5% CO_2 atmosphere. After 24 hr, the cells were collected, washed twice, utilized as APC, and evaluated for class II expression; supernatants were harvested and IL-1β contents were measured.

Expression of Class II MHC Molecules and B7

Flow cytometric analysis of MHC class II molecules and B7 was performed on an Ortho Cytoron instrument (Ortho System, Inc., Raritan, NJ). Expression was quantified by staining with specific MoAbs (Becton-Dickinson). Cell populations were delimited by forward and right scatter on a cytogram, and expression was determined by the fluorescence mean channel value as parameter of fluorescence intensity.

Dosage of IL-1β

Interleukin-1β was measured with a commercially available specific ELISA test (Cistron Biotechnology, Pine Brook, NJ) using two different Abs that recognize the IL-1β molecule. The test was performed according to manufacturer's instructions. Briefly, IL-1β in the samples or standards bound to the anti-IL-1β MoAb was coated on microtiter plates. An anti-IL-1β rabbit antiserum bound to the IL-1β was captured by the MoAb; after washing, a horseradish peroxidase-conjugated anti-rabbit IgG was added and the reaction was detected by a substrate solution. Absorbance was determined at 490 nm. A standard curve was prepared by using different IL-1β concentrations and defined in picograms per milliliter.

RESULTS

MLR Stimulator Ability of B-CLL Cells

Peripheral blood B cells from the seven patients were evaluated for their ability to stimulate allogeneic normal T lymphocytes in a primary MLR. The results are reported in the first column of Table 2. In four cases (MC, ZG, FA, GA), MLR was strongly reduced when compared to the proliferative response obtained with normal control stimulators. B cells from the other three patients (NG, CG, TA) displayed good stimulator activity. If these results are compared with the clinical features of patients reported in Table 1, the number of white blood cells appears inversely proportional to the ability to stimulate in MLR and suggests a relationship between failure in B cell accessory function and tumor progression.

It was shown that B-CLL cells can elaborate soluble factors which down-regulate cell functions (19). The observed impairment of B-CLL B cells in stimulating MLR might thus be ascribed to a suppressor activity exerted on T cells. In order to rule out this possibility, leukemic B cells were added as third party in a normal MRL: as shown in Table 3, no significant effect on the normal MLR response was detectable.

TABLE 2
Proliferative Activity of Normal Peripheral Blood T Cells in Response to Allogeneic CLL B Cells

	Pretreatment of APC			
	None		IL-4[b]	
Patients[a]	cpm	SI[c]	cpm	SI
MG	46,830	21	51,290	23
CG	37,400	17	41,820	19
TA	56,250	25	54,100	24
MC	12,720	6	36,000	15
ZG	9,250	4	33,530	14
FA	21,420	9	54,600	24
GD	16,660	7	42,606	18
Control[d]	67,720	28	64,790	31

[a] Purified B-CLL cells from different patients were used as stimulator of normal allogeneic T cells.
[b] B-CLL or normal B cells were incubated with IL-4 for 24 hr before cultures.
[c] SI, stimulation index, calculated according to the formula reported under Materials and Methods.
[d] Normal B cell fraction was used as stimulator in control cultures.

Presentation of TT by B-CLL Cells

Since it has been shown that the ability to stimulate T cells in MLR is correlated with the capacity to present antigens (20), we asked whether the results obtained in MLR might also be observed in an antigen-driven system. T cells obtained from B-CLL patients after TT immunization were incubated with autologous leukemic B cells and TT. No proliferative activity was detected after 7 days (Table 4, first column), nor after 3 and 5 days of culture (data not shown). Although there is an imbalance of T cell/B cell ratio in peripheral blood of B-CLL patients, also unfraction-

TABLE 3
Effect of Patient B Cells as Third Party Cells on the Proliferative Activity of T Cells in Normal MLR

	Normal MLR[b]	
Third party cells[a]	cpm	SI[c]
MG	70,485	37
CG	57,150	30
TA	47,625	25
MC	59,055	31
ZG	62,865	33
FZ	49,530	26
GD	51,435	27
None	59,055	31

[a] Purified patient leukemic B cells were added to normal MLR.
[b] Normal T cells were incubated with allogeneic normal B cells.
[c] SI, stimulation index, calculated according to the formula reported under Materials and Methods.

TABLE 4
Proliferative Activity of Purified Patient T Cells in Response to TT Presented by Autologous Neoplastic B Cells

	Pretreatment of APC			
	None		IL-4[b]	
Patients[a]	cpm	SI[c]	cpm	SI
MG	1,250	1.1	3,980	3.4
MG-un[d]	2,317	1.5	nd[e]	nd
CG	1,140	1.0	3,648	3.2
TA	1,824	1.2	7,448	4.9
MC	1,224	0.9	3,264	2.4
MC-un	1,752	1.2	nd	nd
ZG	1,170	1.3	2,790	3.1
FA	1,920	1.0	7296	3.8
FA-un	1,443	1.3	nd	nd
GD	2,130	1.5	5964	4.2
EBV-B[f]	11,970	19	14,490	23
Tonsil[f]	8,190	13	10,710	17

[a] Purified patient T cells were incubated with autologous mitomycin-treated B cells from different donors.
[b] B-CLL, EBV-transformed, and tonsil B cells were incubated with IL-4 for 24 hr before cultures.
[c] SI, stimulation index, calculated according to the formula reported under Materials and Methods.
[d] Unfractionated PBMC.
[e] nd, not done.
[f] Normal TT-reactive T cells and autologous EBV-transformed or tonsil B cells were used in control cultures.

ated PBMC from patients MG, MC, and FA were cultivated *in vitro* with TT. Proliferative activity (Table 4) and anti-TT antibody production in the supernatants (data not shown) were evaluated after 7 days, but in none of the cases was a significant antigen-specific stimulation detected. This result can be also related to the low number of T cells that were plated per well using unfractionated PBMC (from 0.6 to 2 × 10^4).

We therefore could not confirm a report showing that B-CLL cells efficiently present PPD and HSV antigens to autologous T lymphocytes (10).

Accessory Molecule Expression and IL-1β Production in B-CLL Cells

The failure observed in TT- and alloantigen-driven T lymphocyte proliferation might be due to a defective expression of MHC class II molecules or other costimulatory molecules involved in antigen recognition. To explore these possibilities, leukemic B cells were stained with anti-HLA-DR and anti-B7 MoAb and then evaluated quantitatively for molecule expression by analyzing the mean channel value of fluorescence intensity. Regarding MHC class II molecules, findings were heterogeneous and in some cases (CG, ZG) comparable to the normal B cell control fraction, and thus, precluded any conclusion regarding a relationship between class II expression and the different capacity to stimulate in MLR and/or in eliciting TT-driven T cell response (Table 5, first column). Analysis of B7 showed a reduced expression of this molecule in most of the patients (Table 5, second column) which was meaningful if compared to normal controls.

In order to provide another explanation to our observations, interleukin-1β production was measured by a specific ELISA method in the culture supernatants of purified patient B cells harvested after 24 hr incubation. In all cases IL-1β was detected (Table 6, first column), although at different levels. Once again, IL-1β production did not correlate with normal or defective APC function. It is unlikely that IL-1β was elaborated by a contaminating monocyte fraction since residual $CD11b^+$ cells were <1% in the B cell fraction (see Materials and Methods).

MLR and Response to TT after IL-4 Pretreatment of B-CLL Cells

It was proposed that IL-4 might provide a competence signal for antigen presentation (21). Thus, B-CLL cells were incubated with 1000 U/ml of IL-4 for 24 hr before using them as APC in MLC and in cultures with TT. IL-4 pretreatment partially restored alloantigen presentation in the four cases (MC, ZG, FA, GD) which displayed a poor stimulatory capacity (Table 2, column 2). Unexpectedly, the same treatment did not modify, or only minimally influenced, the failure observed in the response to TT (Table 4, column 2).

Accessory Molecule Expression and IL-1β Production after IL-4 Pretreatment of B-CLL Cells

The restoration of MLR generation by IL-4 pretreatment of neoplastic APC led us to investigate the effect

TABLE 5
Quantitative MHC Class II and B7 Molecule Expression on B-CLL Cells

	Pretreatment of B-CLL			
	None		IL-4[a]	
Patients	HLA-DR[b]	B7[b]	HLA-DR	B7
MG	57	35	99	32
CG	72	40	104	35
TA	38	28	53	36
MC	31	20	49	25
ZG	80	31	92	30
FA	49	32	81	40
GD	52	27	68	23
EBV-B[c]	87	68	101	nd[d]
Control[e]	67	57	86	61

[a] Purified B cells of different origins were incubated with IL-4 for 24 h before cultures.
[b] Mean channel value of fluorescence intensity on anti-HLA-DR and anti-B7-stained B cells.
[c] EBV-transformed normal B cells.
[d] nd, not done.
[e] Normal B-cell-enriched fraction.

TABLE 6
Interleukin-1β Production in B-CLL Cultures

Patients	Pretreatment of B-CLL cells	
	None	IL-4[a]
	IL-1β (pg/ml)[b]	
MG	90	20
CG	40	35
TA	220	110
MC	130	40
ZG	180	130
FA	70	40
GD	30	nd[c]

[a] Purified leukemic B cells were cultivated for 24 hr in the presence or absence of IL-4.
[b] IL-1β was measured in the supernatants of B-CLL cultures after 24 hr.
[c] nd, not detectable (<20 pg/ml).

of this lymphokine on the accessory-cell-related signals, that is, HLA-DR and B7 molecule expression and IL-1β production.

As shown in Table 5 (column 3), class II antigen expression of B-CLL cells was increased, albeit to a different degree, in all cases after 24-hr incubation with IL-4. Nonetheless, this higher expression could not explain the MLR findings. In four cases (TA, MC, FA, GD), although the amount of class II molecules was increased, it did not substantially differ from the baseline expression detected by B-CLL cells with poor MLR stimulator activity. Interleukin-4 did not show any effect on B7 expression (Table 5, column 4), thus preventing the possibility to draw any conclusion on the role of the reduced levels of this molecule on the surface of neoplastic B cells.

Table 6 (column 2) reports the IL-1β levels detected in the supernatants of B-CLL cultures, after incubation with IL-4 for 24 hr; in all cases a clear reduction in IL-1β production was observed; interestingly, IL-4 exerts the same activity on monocytes (22).

DISCUSSION

Both T and B cells in B-CLL patients show immunological abnormalities. The observations that T cells are defective in IL-2 production (15), mitogen response (16), and in helper activity (17) have not been confirmed by other studies (23–24), which suggest that T cells are not primarily affected. Since depletion of B cells *in vivo* (8–9) and *in vitro* (25) determines similar T cell dysfunctions in experimental murine systems, we investigated whether a primary neoplastic B cell dysfunction could account for the impaired T cell activity. It is well documented that B cells can serve as APC both in MLR and in Ag-driven systems; therefore, we evaluated the ability of neoplastic B cells to present allo- and soluble antigens. In this regard, it has been shown that in the murine acquired immunodeficiency syndrome APCs induce a selective signaling defect in helper T cells which may account for the observed T cell dysfunction (26).

Our data demonstrate that, in patients with progressive disease, B-CLL cell are inefficient stimulators of allogenic normal T lymphocytes in MLR (Table 2, column 1), thus suggesting a relationship between the B cell defect and disease progression. A direct inhibitory effect on T cells may be excluded since addition of B-CLL cells, as a third party to a normal MLC, did not affect proliferation (Table 3).

When B-CLL cells were used as APC in the presence of autologous T lymphocytes and TT, lack of T cell proliferation was observed in all cases, independently of patient stage and/or WBC count (Table 4, column 1). These results should not be attributed to defective TT immunization of patients during disease; in fact, anti-TT antibody titers determined after specific vaccine administration were consistent with efficient priming (data not shown). Since only "professional" APC can turn on virgin T cells (27), the monocyte fraction in B-CLL apparently is not deficient and is probably enough to assure *in vivo* T cell priming; furthermore, neoplastic B cells do not interfere with normal allogeneic reaction *in vitro* (Table 3). In this context, the pathophysiologic relevance of B-CLL B cell impairment in APC function would be of limited importance. Alternatively, serum antibodies detected in our patients might have been raised against T-cell-independent epitopes; this hypothesis would be consistent with absent *in vitro* TT-induced proliferation of unfractionated PBMC, although the low number of responding T cells plated per well cannot reflect the *in vivo* situation.

In order to find an explanation to our observations, we investigated the main events involved in APC–T cell interaction. To deliver the antigen-specific signal to helper T cells, APC must express MHC-encoded class II molecules, which associate with the processed antigen, and the B7 molecule which is the ligand of CD28, the major costimulatory signal receptor for T cells (28). Quantitative class II expression did not vary significantly between different patients, and different expression did not correlate with MLR generation. B7 expression was significantly lower than in normal controls and might account for B-CLL failure in APC function, as suggested by the key role played by B7 in T cell activation (28); it does not correlate, however, with the different behavior in MLC. The normal quantitative class II expression of neoplastic B cells might suggest a qualitative defect. It is thus tempting to speculate that B-CLL cells did not process some antigens or bind some immunogenic peptides due to a putative DNA mutation affecting the genes encoding MHC class II molecules (29). Indeed, melanoma cells exhibit loss of several class II encoding gene fragments (30) and fail to present soluble and alloantigens to autologous T cells (31).

The second type of signal for antigen-induced T cell activation is related to the evidence that, following physical interaction with T cells, APC must provide soluble mediators, such as IL-1. As previously shown by others (32), most of our B-CLL also spontaneously produce IL-1β (Table 6); thus, it seems difficult to attribute APC function defect to a lack in IL-1β production, although we do not know whether the levels detected are sufficient for T cell stimulation.

Since it was proposed that IL-4 plays a role in antigen presentation (21), we questioned whether IL-4 pretreatment of B-CLL cells could induce a competence signal for accessory function. Interleukin-4 restored MLR generation, but minimally affected the response to TT. The different sensitivity of B-CLL cells to IL-4 treatment in MLR and TT systems might be related either to a processing defect or, as proposed above, to an altered affinity of class II molecules for some exogenous antigens. Furthermore, the IL-4-mediated effect cannot be explained by an increase in class II molecules, since no significant difference in their expression was detectable between efficient and inefficient MLR stimulators. Finally, IL-4 does not show any effect on B7 molecule, raising doubts about the meaning of the observed B7 expression reduction in determining B-CLL APC function failure.

In conclusion, our data show that B-CLL cells have a defective antigen-presenting ability, which is more evident as disease progresses, and is only partially restored by *in vitro* addition of IL-4. These findings may provide an insight into the well-known susceptibility of B-CLL patients to infections. Hopefully, further understanding of B-CLL B cell defects will help in developing strategies to restore T cell functions.

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