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IMMUNOGLOBULIN SYNTHESIS AND SECRETION BY LEUKEMIC B CELLS FROM PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Abstract—We investigated the ability of purified E-rosette negative largely leukemic B cells from 15 patients with B-cell chronic lymphocytic leukemia (CLL) to synthesize and secrete IgM, IgA and IgG spontaneously or in the presence of purified autologous or allogeneic T4 cells from normal donors, in PWM-induced differentiation system. We observed moderate but significant IgM synthesis and secretion (19.7 ± 8.9 μg/dl, $n = 5$) by leukemic B cells alone in 5 of 15 patients examined. These IgM concentrations were significantly higher ($p < 0.005$) than those produced by purified E-rosette negative cells from normal donors (4.3 ± 4.5 μg/dl; $n = 6$) in the absence of T cells. Purified E-rosette negative leukemic B cells alone from patients with CLL did not produce IgA or IgG. Addition of purified autologous or allogeneic T4 cells from normal donors resulted in significant increase of IgM production by leukemic B cells from certain patients or initiated IgM secretion in others. However, these IgM levels (73.9 ± 56.6 μg/dl) were significantly lower ($p < 0.003$) to those produced by mixtures of T4 cells and B cells form normal donors (211.6 ± 58.0 μg/dl, $n = 6$). Addition of purified autologous or allogeneic T4 cells from normal donors to purified largely leukemic B cells from patients with CLL resulted in production of very small amounts of IgA in 4 of 15 patients (10.6 ± 6.3 μg/dl vs 154.7 ± 35.8 μg/dl produced by T4 and B cells from normal donors; $n = 6$), but did not support IgG synthesis and secretion. Purified T4 cells from certain patients with CLL exhibit defective helper function to immunoglobulin production by E-rosette negative cells from normal donors.

Key words: Chronic lymphocytic leukemia, leukemic B cells, immunoglobulin synthesis and secretion.

INTRODUCTION

CHRONIC lymphocytic leukemia (CLL) is usually a malignancy of B-cell origin, characterized by a monoclonal expansion of leukemic B cells [1–3]. Leukemic B cells in CLL express cell surface immunoglobulin of monoclonal origin, usually of μ- and/or δ-heavy chain and either κ- or λ-light chain, present in lower density than in normal B lymphocytes [4–8]. Leukemic B cells from patients with CLL express receptors for the C3b and C3d complement components [9], Fc receptors for IgG, IgM and IgA [6–11] and form spontaneous rosettes with mouse erythrocytes [12]. Leukemic B cells from these patients express B-cell differentiation antigens, including B1, B2, B4, and others [13, 14]. Also, they respond to interleukin 2 (IL 2) by proliferation and express IL 2 receptors [15, 16].

We investigated the ability of purified E-rosette negative largely leukemic B cells from patients with CLL to synthesize and secrete immunoglobulin spontaneously or in the presence of purified autologous or allogeneic T4-positive cells from normal donors. We observed moderate synthesis and secretion of IgM, but not of IgA or IgG, by leukemic B cells alone from certain patients. Autologous or allogeneic T4 cells from normal donors significantly increased IgM production by leukemic B cells already spontaneously producing IgM. Furthermore, these purified T4 cells were required for IgM synthesis and secretion by another group of patients with CLL, which did not produce IgM spontaneously.

MATERIALS AND METHODS

Patients

Peripheral blood from 15 untreated patients with B-cell chronic lymphocytic leukemia was used in this study, and

Abbreviations: CLL, chronic lymphocytic leukemia; *IgM*, immunoglobulin M; *IgA*, immunoglobulin A; *IgG*, immunoglobulin G; *PWM*, pokeweed mitogen; *AET*, aminoethylisothiouromium; *HBSS*, Hank's balanced salt solution; *FCS*, fetal calf serum; *SRBC*, sheep red blood cells; *FITC*, fluorescein isothiocyanate, *ELISA*, enzyme linked immunoadsorbent assay.

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it was provided by the Hematology/Lymphoma Service of Memorial Hospital. Informed consent was obtained from these patients by the Hematology/Lymphoma Service. Staging of the patients was determined according to Rai classification [34]. Healthy normal donors, free of hepatitis antigen and antibody, provided peripheral blood.

Preparation of monocyte-depleted lymphocytes

Peripheral blood mononuclear leukocytes from normal donors or patients with CLL were prepared by centrifugation on a Ficoll/Hypaque density cushion. The cells were depleted of monocytes by allowing them to ingest iron carbonyl particles followed by centrifugation on a Ficoll/Hypaque density cushion as previously described [28]. Monocyte-depleted peripheral blood lymphocytes were collected from the interface, washed three times with Hank's balanced salt solution (HBSS) and arranged at a concentration of 5×10^6 cells/ml.

Preparation of E-rosette positive and E-rosette negative cells

E-rosette positive cells were prepared from monocyte-depleted lymphocytes by rosetting with 2-aminoethylisothiouronium bromide (AET; Sigma)-treated sheep erythrocytes (SRBC) [16]. Briefly, 3 ml of monocyte-depleted peripheral blood lymphocytes in HBSS (5×10^6/ml) were mixed with equal volume of 1% AET-treated SRBC and 0.6 ml of SRBC-absorbed, heat-inactivated fetal calf serum. The mixture was incubated for 15 min at 37°C, centrifuged at $200 \times g$ for 5 min and incubated at 4°C for 1 h. The cells were gently resuspended, layered on Ficoll/Hypaque density cushion and centrifuged at $400 \times g$ for 20 min at room temperature. E-rosette forming cells were recovered from the pellet after lysis of attached SRBC with treatment with Tris-buffered 0.83% ammonium chloride (pH 7.2) for 5 min at 37°C. The cells were washed three times with HBSS. E-rosette negative cells were recovered from the interface and washed three times with HBSS. Both E-rosette positive and E-rosette negative cells were rosetted for a second time with AET-treated SRBC, as described previously. E-rosette positive cells prepared by this method were more than 95% T lymphocytes, as determined by re-rosetting with SRBC and by analysis with the anti-Leu 5 monoclonal antibody (Becton–Dickinson, Sunnyvale, CA). They were completely devoid of monocytes and contained less than 2% immunoglobulin-bearing cells. E-rosette negative cells from normal donors prepared by this method contained 80–95% immunoglobulin-positive cells, were completely devoid of monocytes and contained less than 1% of E-rosette forming cells. From here on these cells will be referred to as B lymphocytes. E-rosette negative cells from patients with CLL contained more than 95% immunoglobulin-bearing cells and less than 1% E-rosette forming cells. These cells were devoid of monocytes. From here on these cells will be referred to as leukemic B cells.

Preparation of T4 cells

T4 lymphocytes were prepared by treating purified E-rosette forming cells from patients with CLL or normal donors with the OKT8 (Ortho Pharmaceuticals, Raritan, NJ) monoclonal antibody and complement, as previously described [27]. Briefly, $4–6 \times 10^6$ E-rosette positive cells were treated with the OKT8 monoclonal antibody for 1 h at 4°C. The cells were washed once with RPMI-1640 containing 10% heat-inactivated fetal calf serum and incubated with 6% rabbit complement (Pel Freeze, Little Rock, AR) for 1 hr at 37°C. The cells were carefully layered on top of a 3-ml cushion of fetal calf and centrifuged at $400 \times g$ for 20 min and washed three times with RPMI-1640, supplemented with 10% fetal calf serum. This treatment was sufficient for lysis of all T8 cells, as determined by immunofluorescence analysis using the OKT8 monoclonal antibody after removal of dead cells. More than 90% of the viable cells of these preparations were T4-positive as determined by immunofluorescence, using the OKT4 monoclonal antibody, and contained less than 1% of T8-positive cells. From here on these cells will be referred to as T4 cells.

Cell surface immunofluorescence

Cell surface immunofluorescence was determined by a standard procedure, as previously described [29]. FITC-conjugated $F(ab')_2$ fragments of goat antiserum specific for either δ, γ or μ human heavy chain or for either κ or λ light chain were obtained from Kallestad (Chaska, MN). OKT3, OKT4 and OKT8 monoclonal antibodies were obtained from Ortho.

Differentiation of B cells to immunoglobulin synthesizing and producing plasma cells

Purified B cells (5×10^5 per well) from patients with CLL or normal donors were cultured alone or with autologous or allogeneic purified T4 cells (0.5×10^5 per well) in round-bottomed 96-well microtiter plates (Nunc, Copenhagen, Denmark), in RPMI-1640 supplemented with 10% fetal calf serum 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. PWM (10 μg/ml; Gibco) were added to the cultures of T and B cells from the beginning [18]. After 7 days at 37°C, in a humidified incubator with 5% CO_2, the plates were centrifuged at $200 \times g$ for 5 min and supernatants were carefully removed for immunoglobulin determination by ELISA.

Determination of IgG, IgA and IgM by heavy-chain specific enzyme-linked immunoabsorbent assay (ELISA)

These determinations were carried out by a modification of the ELISA method described by Engvall and Perlmann [23]. Rabbit anti-human immunoglobulin antibody (2 μg/ml) heavy chain specific (μ or γ or α) (Accurate Chemicals, Hickville, NY) was added into 96-well, flat-bottomed microtest III flexible plates (Falcon 3912), in coating buffer (0.1 M Na_2CO_3, 0.26 M $NaHCO_3$, pH 9.6) and incubated for 3 h at 37°C. The plates were incubated for an additional 18 h at 4°C and could be stored at this temperature for up to two weeks. Immediately before use, the plates were washed three times with PBS containing 0.02% Tween 20. Several dilutions of the unknown immunoglobulin-containing supernatants were prepared in 0.02% Tween 20 and transferred (in triplicate) to the wells. The plates were incubated for 5 h at room temperature, the supernatants were removed and the plates were washed four times with PBS containing 0.02% Tween 20. Appropriate dilutions of alkaline phosphatase conjugated rabbit-anti-human immunoglobulin, μ-chain specific (Bionetics, Kesington, MD), or α-chain or γ-chain specific (AMF Immunoreagents, Austin, TX) were added and the plates were incubated for 16 h at room temperature. Unbound conjugates was removed by washing the plates four times and *p*-nitrophenyl-phosphate (1 mg/ml, Sigma) was added to the plates in 1 M diethanolamine–HCl buffer, pH 9.8, containing 0.5×10^{-3} M $MgCl_2$. The released *p*-nitrophenolate was

TABLE 1. CHARACTERISTICS OF PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

Patients	Stage*	WBC cells × 10^3/mm^3	lymphocytes (%)	T4† (%)	T8† (%)	T4/T8 ratio	Sm Ig‡	Serum immunoglobulin (mg/dl)‖ IgM	IgA	IgG
1	II	184.0	96	44	43	1.02	Mλ+§	56	62	68
2	III	539.0	99	34	31	1.09	Mκ+	420	110	1540
3	III	8.5	80	64	49	1.30	Mκ±	86	45	480
4	II	50.9	82	41	49	0.84	Mλ+	19	80	1080
5	III	49.0	85	33	38	0.87	Mκ+	24	37	780
6	I	58.0	65	48	49	0.97	Mλ+	ND	ND	ND
7	0	24.8	91	54	48	1.14	Mλ+	184	70	740
8	I	50.0	96	32	41	0.78	Mκ+	ND	ND	ND
9	III	138.0	84	40	42	0.95	Mκ+	41	69	670
10	II	60.3	ND	53	42	1.26	M	15	26	125
11	III	182.0	98	50	53	0.94	Mκ+	13	35	662
12	I	37.0	69	57	49	1.16	Mλ±	38	115	540
13	IV	541.0	95	53	45	1.20	Mλ+	ND	ND	ND
14	0	33.0	81	49	69	0.71	Mκ+	46	112	783
15	III/IV	114.0	91	51	53	0.96	M	ND	ND	ND
Patients (mean ± S.D.)	137.9 ± 172.0	86.6 ± 10.6	46.9 ± 9.3	46.7 ± 8.5	1.1 ± 0.2					
Normal controls, n=15 (mean ± S.D.)	6.7 ± 1.9	35.2 ± 8.9	63.8 ± 5.2	36.1 ± 4.3	1.8 ± 0.1					

* Determined according to Rai *et al.* (1975).
† Determined on purified E rosette-forming cells.
‡ Surface immunoglobulin was determined by immunofluorescence.
§ Strong clonal excess; ± light clonal excess.
‖ Normal range for adults: IgM, 60–280 mg/dl; IgA, 90–450 mg/dl; IgG, 800–1800 mg/dl.
ND; not done.

TABLE 2. IMMUNOGLOBULIN SYNTHESIS AND SECRETION BY PURIFIED LARGELY LEUKEMIC B CELLS FROM PATIENTS WITH CLL*

Patients	Immunoglobulin (μg/dl)		
	IgM	IgA	IgG
1	0	0	0
2	16.4 ± 0.6	0	ND
3	12.3 ± 1.2	ND	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	34.6 ± 1.2	6.0 ± 1.0	ND
9	14.6 ± 7.4	0	0
10	0	0	0
11	0	0	0
12	0	0	0
13	0	0	0
14	0	0	0
15	20.7 ± 1.0	0	0
Normal donors, $n=6$ (E-rosette negative cells)	4.3 ± 4.5	9.5 ± 10.5	14.6 ± 21.3

* Highly purified E-rosette negative cells from patients with CLL or normal donors were cultured for seven days at 37°C. IgM, IgA and IgG were determined in the supernatants by heavy-chain specific ELISA.

measured at 405 nm using an automatic Titertek ELISA reader. Standard curves were constructed using purified IgM, IgA or IgG immunoglobulins (Kallestad, Chaska, MN). Suitable range for immunoglobulin determination by this method was 0.1–8.0 μg/dl for IgG and 0.5–10 μg/dl for IgM and IgA.

Determination of serum immunoglobulin levels

Serum IgM, IgG and IgA levels were determined by radial immunodiffusion as described by Mancini *et al.* [24].

RESULTS

Fifteen untreated patients with B-cell CLL were used in this study. The characteristics of these patients are shown in Table 1. None of the patients had IgM "M" band in the serum. Immunoglobulin synthesis and secretion by highly purified E-rosette negative largely leukemic B cells from patients with CLL is shown in Table 2. These cells contained less than 1% of E-rosette forming cells. Significant synthesis and secretion of IgM by E-rosette negative largely leukemic B cells alone was observed in 5 of 15 patients with CLL (Nos. 2, 3, 8, 9 and 15). IgM levels (19.7 ± 8.9 μg/dl) produced by leukemic B cells from these five patients were significantly higher ($p < 0.005$) than that produced by purified E-rosette negative cells from normal donors (4.3 ± 4.5 μg/dl; $n = 6$), in the absence of T cells. Purified largely leukemic B cells alone, from patients with CLL, did not produce IgG and IgA, with the possible exception of patient No. 8, where small amounts of IgA (6 μg/dl) were produced. Purified E-rosette negative cells from normal donors (containing less than 1% E-rosette positive cells) produced small amounts of IgA and IgG, in the absence of T cells (Table 2).

IgM synthesis and secretion, by largely leukemic B cells from patients with CLL was significantly increased by the addition of purified allogeneic T4 cells from normal donors in five (Nos. 2, 7, 8, 14 and 15) of 15 patients examined in the PWM-induced differentiation system (Fig. 1). However, IgM production (73.9 ± 56.6 μg/ml) by these leukemic B cells in the presence of T4 cells from normal donors was significantly lower to that produced by mixtures of T4 cells and B cells from normal donors (211.6 ± 58.0 μg/dl; $p < 0.003$). Significant production of IgM by leukemic B cells alone in the absence of T4 cells, was observed in three of these five patients (No. 2, 8 and 15). This production was significantly increased by the addition of T4 cells from normal donors. Largely leukemic B cells from the other two patients (Nos 7 and 14) produced IgM only in the presence of T4 cells from normal donors.

Addition of purified autologous T4 cells to leukemic B cells resulted in production of significant levels of IgM in 3 out of 15 patients (Nos 2, 8 and 15). Addition of autologous or allogeneic T4 cells to leukemic B cells from two other patients (Nos 3 and 9) with CLL, that produced alone small amounts of IgM, had no effect (Fig. 1).

Purified T4 cells from three (Nos 9, 10 and 11) of six patients with CLL were defective in providing helper function to IgM, IgA and IgG production by purified E-rosette negative cells from normal donors (Figs 1–3), suggesting that a helper T cell defect to the differentiation of B cells to immunoglobulin synthesizing

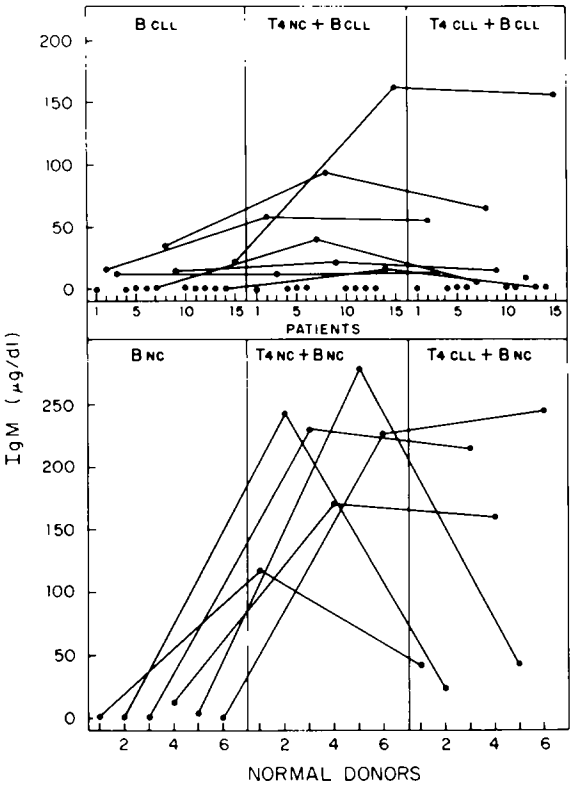

FIG. 1. IgM synthesis and secretion by purified E-rosette negative largely leukemic B cells from patients with chronic lymphocytic leukemia, in the presence of autologous T4 cells or allogeneic T4 cells from normal donors, in the PWM-induced differentiation system. IgM synthesis and secretion by mixtures of T4 cells and B cells from normal donors or T4 cells from patients with CLL and B cells from normal donors are also shown for comparison purposes. The cultures were set up as described in Materials and Methods and the concentration of IgM produced was determined by μ-chain specific ELISA.

and secreting plasma cells is present in certain patients with CLL [5, 20].

Addition of purified T4 cells from normal donors to purified largely leukemic B cells from patients with CLL resulted in production of very small amounts of IgA (10.6 ± 6.3 μg/dl) in 4 (Nos 2, 7, 14 and 15) of 15 patients (Fig. 2), whereas mixtures of T4 and B cells from normal donors produced significant amounts of IgA (154.7 ± 35.8 μg/dl). It is possible that these small amounts of IgA are produced by non-malignant B lymphocytes present in these preparations. However, leukemic B cells from all these four patients produced significant amounts of IgM in the presence of T4 cells from normal donors. Addition of autologous T4 cells to leukemic B cells from patients with CLL supported the production of very small amounts of IgA in three patients (Nos 2, 5 and 14) (Fig. 2).

Addition of purified autologous T4 cells from patients with CLL or allogeneic T4 cells from normal donors to largely leukemic preparations of B cells did not result in synthesis and secretion of IgG, with the exception of patient No. 14, where small amounts were produced (Fig. 3). However, purified T4 cells from only 3 of 6 patients with CLL were able to support IgG synthesis and secretion by purified E-rosette negative cells from normal donors, to an extent similar to that observed with T4 cells from normal donors (Fig. 3). T4 cells from the remaining 3 patients with CLL were defective in supporting IgG synthesis and secretion by B cells from normal donors.

DISCUSSION

We investigated the ability of leukemic B cells from patients with CLL to produce immunoglobulin spontaneously or to mature into immunoglobulin producing cells, in the presence of autologous or allogeneic T4 cells from normal donors and PWM. We observed moderate but significant spontaneous IgM synthesis and secretion by largely leukemic B cells alone in 30% of the patients examined. Leukemic B cells from these patients appear to be more mature than those of the others. Gordon *et al.* [13] reported spontaneous IgM production in 5 of 14 patients. Stevenson *et al.* [27] reported that lymphocytes from patients with CLL without detectable paraprotein, exported small amounts of

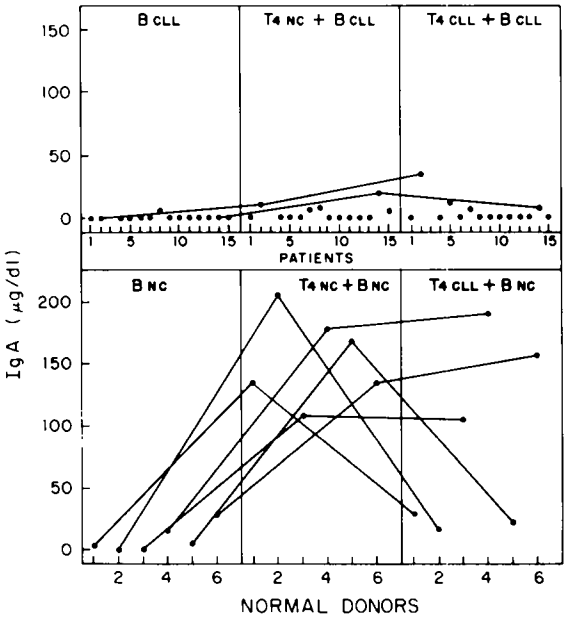

FIG. 2. IgA synthesis and secretion by purified E-rosette negative largely leukemic B cells from patients with chronic lymphocytic leukemia, in the presence of autologous T4 cells or allogeneic T4 cells from normal donors, in the PWM-induced differentiation system. IgA synthesis and secretion by mixtures of T4 cells and B cells from normal donors or T4 cells from patients with CLL and B cells from normal donors are also shown for comparison purposes. The cultures were set up as described in Materials and Methods and the concentration of IgA produced was determined by α-chain specific ELISA.

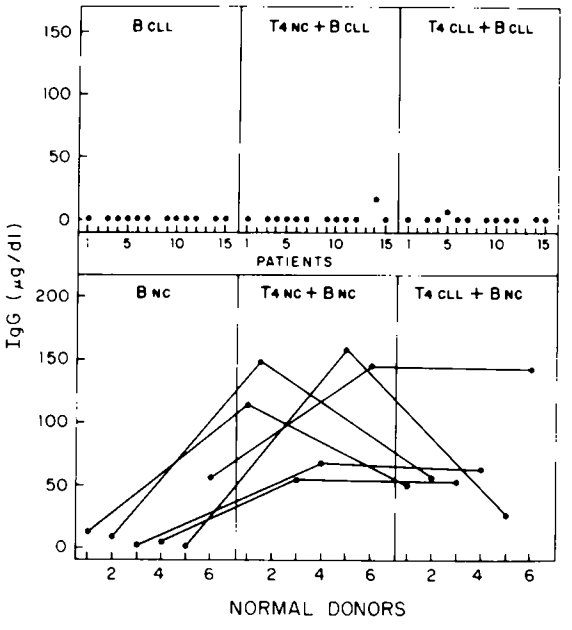

FIG. 3. IgG synthesis and secretion by purified E-rosette negative largely leukemic B cells from patients with chronic lymphocytic leukemia, in the presence of autologous T4 cells or allogeneic T4 cells from normal donors, in the PWM-induced differentiation system. IgG synthesis and secretion by mixtures of T4 cells and B cells from normal donors or T4 cells from patients with CLL and B cells from normal donors are also shown for comparison purposes. The cultures were set up as described in Materials and Methods and the concentration of IgG produced was determined by γ-chain specific ELISA.

idiotypic IgM and in certain cases IgD. Immunoglobulin production by leukemic B cells is significantly increased by phorbol esters [28].

Addition of purified T4 cells to largely leukemic B cells resulted in significant increase of IgM production in certain patients. Addition of these T4 cells also resulted in production of very small amounts of IgA in 4 of 15 patients (which also produced significant amounts of IgM), but did not support production of IgG. Whether or not these small amounts of IgA were produced by non-malignant B lymphocytes present in small proportions in these E-rosette negative cell preparations or by leukemic B cells remains to be determined. However, the fact that IgG is not produced implies that leukemic B cells are responsible for production of IgM and possibly IgA.

Fu *et al.* [29] and Saiki *et al.* [30] reported that leukemic B cells from three patients with CLL were able to differentiate into plasma cells in the presence of autologous or allogeneic T cells. Other investigators [31–33] reported immunoglobulin production by leukemic B cells by polyclonal activators (PHA, PWM, EBV, TPA) in the presence of T cells. In contrast, Han *et al.* [34] communicated that leukemic B cells from 20 patients with CLL did not differentiate into plasma cells in the presence of helper T cells or after removal of suppressor T lymphocytes.

Purified T4 cells from 3 of 6 patients with CLL exhibited defective helper function for the maturation of B lymphocytes to immunoglobulin producing cells. Furthermore, purified T4 cells from patients with CLL were defective in supporting IgM synthesis and secretion by autologous largely leukemic B cells, when compared to T4 cells from normal donors. T lymphocytes from patients with B-cell CLL exhibit several abnormal functions and phenotypic characteristics [11, 25, 26, 35–39]. These include defective helper function of purified T cells [25, 26] to immunoglobulin production. Defective T-cell helper function may be responsible in part for the generation of the hypogammaglobulinemia, which is commonly observed in patients with CLL.

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