Immunoregulation of B Lymphocyte Colony Formation by T Cell Subsets in Patients With Chronic Lymphocytic Leukemia

By Louis A. Fernandez, J. Michael MacSween, and G. Ross Langley

Normal B lymphocytes are activated, proliferate, and then differentiate into plasma cells and secrete immunoglobulin (Ig). We have reported that chronic lymphocytic leukemia (CLL) T4 cells help and CLL T8 cells lack suppressor effects on Ig synthesis by normal B cells (Blood 62:767, 1983). We have now explored the earlier phase, proliferation, using B cell colony formation in semisolid media. B lymphocyte colonies from normal individuals and from patients with CLL were grown in 0.3% agarose overlayed with T cells or T cell subsets and the B cell mitogen staphyloccocal protein A. Enriched T cells, OKT4 or OKT8, were obtained either by sheep erythrocyte rosettes or depletion of OKT8 or OKT4 cells by monoclonal antibody or complement, respectively. Twenty thousand B cells from normal subjects yielded 65 ± 9, 64 ± 7, and 19 ± 6 colonies with autologous unFractionated T-, OKT4-, or OKT8-positive cells, respectively. This compared to 29 ± 11, 81 ± 11, and 15 ± 4 colonies from patients with CLL with added autologous unFractionated T-, OKT4-, or OKT8-positive cells. To determine whether the fewer number of colonies in both normal subjects and patients with CLL with OKT8-positive cells was due to suppression or lack of help, the number of OKT4-positive cells was held constant, and OKT8-positive cells were added in increasing numbers. No suppression of colony formation could be demonstrated. Furthermore, the addition of increasing numbers of concanavalin A (Con A)-activated OKT8-positive cells did not suppress colony formation. These results suggest that the CLL T cell subsets behave in a functionally similar manner to normal T cell subsets, namely, (1) that normal and CLL B cell colony growth is helped by OKT4 cells; and (2) in contrast to immunoglobulin secretion by B cells, neither normal nor CLL OKT8 cells, unstimulated or activated by Con A, suppress B cell colony growth.

© 1988 by Grune & Stratton, Inc.

NORMAl B LYMPHOCyTES are activated, proliferate, and then differentiate into plasma cells and secrete immunoglobulin.¹ We have reported that chronic lymphocytic leukemia (CLL) T4 cells help and that CLL T8 cells lack suppressor effects on normal B cell immunoglobulin secretion.² We have now explored the earlier phase, proliferation, using B cell colony formation in semisolid media. In this system small lymphocytes from normal individuals transform into blast cells and form colonies of 50 to 500 cells.³ although some of the cells in normal colonies are mature plasma cells, many remain blast-like, whereas B cell colonies from patients with CLL do not contain mature plasma cells.² the requirement for T cells and their subsets for colony formation from a single clonogenic precursor can be determined in this system.⁴

Normal B cells can be stimulated in liquid culture with a variety of B cell mitogens (pokeweed mitogen [PWM] and lipopolysaccharide [LPS]), and this can be quantitated by incorporation of tritiated thymidine and/or secretion of immunoglobulin.⁵ however, we² and others⁶-¹² have observed that, in response to these B cell mitogens, CLL B cells either do not proliferate or else the fraction of cells that do proliferate is so small that incorporation of tritiated thymidine is not sensitive enough to pick up proliferative activity. In contrast, the B cell colony assay circumvents this problem since both normal and CLL B cell colonies can be grown using similar conditions.⁵,⁶,¹³-¹⁵ T cells and staphylococcal protein A (SPA) are required for colony formation, or alternatively, mitomycin-treated T cells and supernatants obtained from normal T cells stimulated with phytohemagglutinin (PHA) (PHA-TCM). The B cell colonies grown from patients with CLL have the same phenotype as the peripheral blood leukemic cell, and "residual normal B cells" within the leukemic clone do not contribute appreciably to the colony growth.¹⁶ Thus, B cell colony growth in CLL reflects the proliferative potential of the malignant cells. We have therefore used this assay to study some of the characteristics of leukemic B cell proliferation.

MATERIALS AND METHODS

Patients. The studies were performed on 17 patients with untreated CLL, ten males and seven females whose ages ranged from 49 to 83 years. The diagnosis of B cell CLL was based on an absolute lymphocytosis of greater than 10,000 cells/μL on two successive occasions without any known cause, exhibiting infiltration of the bone marrow by morphologically mature lymphocytes. Surface markers showed that the majority of lymphocytes were of the B cell type, as demonstrated by the presence of small amounts of membrane immunoglobulin¹⁷ and receptors for the C3 component of complement.¹⁸ According to the classification of Rai et al,¹ nine were stage 0 (bone marrow and blood lymphocytosis), three were stage I (lymphocytosis with enlarged nodes), four were stage II (lymphocytosis with enlarged spleen), and one was stage III (lymphocytosis and thrombocytopenia). Thus the majority were in early stages of the disease.

Lymphocyte colonies. Mononuclear cells were obtained by centrifuging heparinized venous blood on Ficoll-Hypaque. The cells were then resorted with sheep erythrocytes and centrifuged over Ficoll-Hypaque, and T cells were recovered by lysing the sheep erythrocytes with 0.87% ammonium chloride.²⁰ These T cells, either untreated or treated with mitomycin C, 50 μg/mL, for 30 minutes at 37 °C, were washed three times and used as feeder cells. The mononuclear cells depleted of T cells were allowed to adhere to Petri dishes containing RPMI 1640 and 20% autologous plasma for...
one hour at 37 °C. The nonadherent cells, enriched mainly for B cells, were recovered, washed, and resuspended in RPMI 1640 with 10% fetal calf serum. The enriched B cells from normal individuals and patients with CLL were contaminated with 5% ± 3% and 2% ± 1% T cells, respectively.

B cells were cultured with SPA or PHA-TCM as a mitogenic stimulus. For SPA-induced colonies, B cells were suspended in 0.1 mL of 0.3% agarose containing RPMI 1640, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL kanamycin, and 2.5 μg/mL fungazone. The layer was allowed to harden at room temperature for 30 minutes. It was then overlaid with RPMI containing the aforementioned antibiotics, 24% fetal calf serum, various concentrations of T cells, and SPA. The microtiter plates were covered and placed in a plastic bag. These were then placed in a 5% incubator with a moist atmosphere for five to nine days.

For colonies generated by PHA-TCM, enriched B cells were mixed with mitomycin-treated T cells in various ratios, suspended in 20% PHA-TCM, 8% methyl cellulose, and growth medium and gently mixed on a vortex mixer. Triplicate samples of 0.1-mL aliquots were placed in flat-bottom microtiter plates. The plates were covered and placed in plastic bags and then placed in a 5% incubator with a moist atmosphere for five to nine days. The plates were then examined for B cell colonies, defined as aggregates of greater than 50 cells. These cells were stained for surface immunoglobulins and did not rosette with sheep erythrocytes. Appropriate controls were run for each experiment, namely, T and B cells alone and combinations of T and B cells without mitogens.

PHA-TCM was prepared by stimulating T cells (as obtained in the preceding procedure) with 1% PHA in RPMI 1640 for three days in a 5% CO₂ atmosphere. Supernatants of such cultures from three individuals were pooled and sterilized by passing them through a millipore filter and storing them at -4 °C.

Enrichment of T helper and suppressor cells using monoclonal antibodies. Enriched OKT4 and OKT8 subsets of T cells were obtained as previously described. Briefly, 10 to 20 million cells suspended in 1 mL of RPMI and 5% fetal calf serum (GIBCO, Burlington, Ontario, Canada) were mixed with 10 μL of OKT4 or OKT8 monoclonal antibodies (Ortho Diagnostics, Don Mills, Ontario, Canada) and held for 0.5 hours at 0 °C. Then, 300 μL of rabbit complement (Cedarlane, Hornby, Ontario, Canada, batch 6045) were added, and the tubes were placed on a rotator for one hour at 37 °C. These cells were washed three times with RPMI 1640 containing 5% fetal calf serum. Aliquots of cells were stained with fluorescein-conjugated antibodies to OKT4 or OKT8. Less than 1% of the T cells depleted of T4 or T8 subsets displayed a staining of their respective fluoresceinated antibodies although the OKT4 cells and OKT8 cells were enriched to a mean of 76% and 79%, respectively.

Concanavalin A-activated T8 cells. Five million OKT8 cells were suspended for two hours at 37 °C in 1 mL of RPMI 1640 containing L-glutamine, 2 mmol/L, and concanavalin A (Con A), 10 μg/mL. This concentration was found in our laboratory to be optimal for generating suppressor cells. The cells were then washed three times with medium before use.

Mixed lymphocyte reaction. The mixed lymphocyte reaction (MLR) was performed as described before. Twenty responder cells consisted of T cells, T cell subsets, and Con A-activated T8 cells in different ratios. Fifty thousand responder cells were mixed with an equal number of mitomycin C-treated (50 μg/mL) mononuclear cells from a panel of four donors (stimulator cells). These cells were suspended in RPMI 1640 containing 10% fetal calf serum and placed in microtiter plates for seven days at 37 °C in a 5% CO₂ atmosphere. The evening before harvesting the cells, 0.5 μCi of tritiated thymidine (specific activity, 15.1 mmol/L) was added. The next day the cells were harvested onto glass filters using a Titertek harvester (Flow Laboratories, Mississauga, Ontario, Canada). The filters were then placed in vials containing scintillating fluid and the radioactivity determined in a beta scintillation spectrometer. Statistical analysis was performed using Student’s t test for paired samples and group means.

RESULTS

Appropriate controls showed that B cell colonies were not formed in the absence of mitogens, B cells, or T cells. No colony growth was observed when B cells were cultured either alone (n = 6 normals and 11 patients) or with mitogens without T cells (n = 4 normals and 7 patients). Table 1 shows the number of colonies generated simultaneously from the peripheral blood B cells of ten patients with CLL and normal individuals. These colonies were grown in semisolid media using mitomycin-treated T cells and normal T cell supernatants. There were significantly fewer colonies from B cells of patients with CLL than normal individuals (P < .025). Enrichment of T cells with OKT4-positive cells resulted in more colonies than enrichment with OKT8 cells (normal, P < .05; CLL, P < .025). B cell colony growth was decreased when CLL OKT4 cells were used compared to unfractonated CLL T cells; however, this difference was not significant. When these CLL OKT4 cells were added to normal B cells, 102 ± 22 B cell colonies were obtained, compared to 90 ± 15 with unfractonated CLL T cells, suggesting that CLL OKT4 cells provide normal helper activity for the growth of normal B cell colonies.

Since B cell colonies can be generated using different mitogens, we also grew colonies using SPA as the mitogen (Table 2). The results were similar to those obtained using PHA-TCM, showing fewer colonies from cells of patients with CLL than normal individuals (P < .05) and more colonies with enrichment of T cells by OKT4-positive cells than with enrichment by OKT8 cells (normal, P < .001; CLL, P < .05). The help provided by the CLL OKT4 cells for the leukemic B cell colony growth was increased in comparison with the whole T cell population as the increase was not significant, suggesting that the T4 cells did not have an intrinsic defect in helper activity.

To determine whether the OKT8 cells suppressed colony formation or merely failed to provide adequate help, several experiments were carried out (Tables 3 to 6). When the total number of T cells was kept constant but the numbers of OKT4 and OKT8 varied so that the ratios of T4 to T8 were 1:0, 1:1, 1:2, and 0:1, the number of B cell colonies was decreased both in normal individuals and patients with CLL as the number of T4 cells was decreased and T8 cells

Table 1. Numbers of B Cell Colonies Generated in Semisolid Media Using PHA-TCM as a Mitogen

<table>
<thead>
<tr>
<th>Source of B Lymphocytes</th>
<th>No. B Cell Colonies Generated Using Autologous</th>
<th>T Cells</th>
<th>T4 Cells</th>
<th>T8 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal individuals (n = 10)</td>
<td>149 ± 33*</td>
<td>120 ± 28</td>
<td>90 ± 28</td>
<td></td>
</tr>
<tr>
<td>Patients with CLL (n = 10)</td>
<td>74 ± 18</td>
<td>51 ± 11</td>
<td>20 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SEM.
increased (Table 3). A significant decrease in B cell colonies was seen in normal individuals when the T4:T8 ratio was 1:2 \((P < .05)\) and in patients with CLL when the T4:T8 ratio was 1:1 \((P < .05)\). Similar results were obtained when allogeneic T cells replaced autologous T cells using normal T cells mixed with CLL B cells and vice versa (Table 4).

Since the decrease in number of B cell colonies could be due to suppression by OKT8 cells or to decreased help from fewer numbers of OKT4 cells, we added increasing numbers of OKT8 cells to a constant number of OKT4 cells. As a control, increasing numbers of unfraccionated T cells were added to a constant number of OKT4 cells. As seen in Table 4, there was no significant suppression of B cell colony numbers either in normal individuals or patients with CLL.

To evaluate the possibility that OKT8 cells did not suppress colony formation by virtue of being in an inactive state, they were stimulated with Con A, and the various subpopulations were added to a constant number of OKT4 cells. As seen in Table 5, there was no significant suppression of B cell colony formation either in normal individuals or patients with CLL. When unfractionated, autologous T cells were used to provide help for B cell colonies, fewer colonies were obtained from patients with CLL than normal. A possible explanation for this observation is the known decreased proportion of the T4 subset in the unfractionated T cell population of CLL.23,24

**DISCUSSION**

These studies show that the T cells required for colony formation of cultured B cells from normal individuals can be replaced by the T4 but not the T8 subset of cells. These results are similar to those reported by Whisler et al.,6 although they suggested that the decreasing number of B cell colonies resulting from decreasing ratios of T4:T8 cells was in keeping with suppression by the T8 cells. However, when we maintained the number of T4 cells constant and increased the T8 cells fourfold, there was no suppression of colony formation in either normal individuals or patients with CLL. Other investigators have reported similar data in normal individuals.21 Any further increase in the numbers of T8 cells would take the ratio of T4:T8 out of the range observed in blood of normal individuals. Our interpretation therefore is that an absolute number of T4 cells and T4:B cell ratio is essential for colony formation, and the previous observations suggesting suppression are probably due to a decreased number of helper T cells. In this respect, the effect of normal T8 cells on colony formation differs from their effect on the latter phases of B cell maturation and immunoglobulin secretion, which they suppress.24

Con A activation of the OKT8 subset sufficient to cause a 50% suppression in the MLR did not suppress B cell colony growth either. Similar data were obtained when B cell colonies were grown in methylcellulose using mitomycin-treated T cells and PHA-TCM (data not shown). The normal T cell subnets behaved in a functionally similar manner when they were placed in culture with CLL B cells. When unfraccionated, autologous T cells were used to provide help for B cell colonies, fewer colonies were obtained from patients with CLL than normal. A possible explanation for this observation is the known decreased proportion of the T4 subset in the unfractionated T cell population of CLL.23,24
In general, the functional activity of CLL T cell subsets for CLL or normal B cell colony formation was similar to those described for normal T cells. When methylcellulose and normal PHA-TCM were used, the CLL OKT4 subset was not as effective as unfractionated T cells in generating CLL B cell colonies, suggesting an OKT4 helper defect. However, this was not seen when CLL OKT4 cells were added to normal B cells stimulated with PHA-TCM or to CLL B cells stimulated with SPA. In fact, in the latter system the number of cell colonies generated by OKT4 cells was increased in comparison with unfractionated T cells. The reason for this is not clear; however, in general there was no lack of help in generating B cell colonies by CLL OKT4 cells.

We have shown previously that CLL OKT4 cells provided normal help to normal B cells in secreting immunoglobulin and that, unlike normal T8 cells, CLL OKT8 cells did not suppress this helper activity of the CLL OKT4 cells.2 In the B cell colony assay, however, although both normal and CLL OKT4 cells provide help for colony formation, the OKT8 subset of cells in both cases was unable to suppress B cell colony numbers, and therefore we conclude that both OKT4 and OKT8 cells from patients with CLL function similarly to normal cells in this assay.

Our data suggest that proliferation of CLL B cells to form colonies is not impaired when adequate help is provided. We have also shown that both normal and CLL B cell colony formation is not suppressed by T cells. However, proliferation of B cells may be suppressed by other mechanisms such as immunoglobulin binding to Fc receptors.27 Further experiments are being conducted to delineate the mechanism of suppression of normal B cells and then to apply these to abnormal states such as CLL.

**ACKNOWLEDGMENT**

The authors wish to thank D. Robson and D. Jennex for excellent technical assistance, Dr. O. A. Hayne for referring to us some of the patients included in this study, and Roxy Pelham for secretarial assistance.

**REFERENCES**

4. Ulmer A, Maurer HR: The formation of B-lymphocyte colonies in agar contained in glass capillaries. Immunology 34:919, 1978
20. Fernandez LA, MacSween JM, Langley GR: T cell function

**Table 7. Effect of Con A–Activated OKT8 Cells on Proliferative Responses in MLR Expressed as Counts per Minute**

<table>
<thead>
<tr>
<th>Source of Lymphocytes</th>
<th>MLR Control</th>
<th>MLR Test</th>
<th>Number of OKT8 Cells Added</th>
<th>Number of Con A–Activated OKT8 Cells Added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T/T (T/N)</td>
<td>T/N pool</td>
<td>10,000</td>
<td>25,000</td>
</tr>
<tr>
<td>Normal individual</td>
<td>2,927</td>
<td>45,070</td>
<td>40,616</td>
<td>40,733</td>
</tr>
<tr>
<td>CLL 1</td>
<td>1,832</td>
<td>62,666</td>
<td>73,661</td>
<td>53,911</td>
</tr>
<tr>
<td>CLL 2</td>
<td>ND</td>
<td>4,864</td>
<td>ND</td>
<td>27,154</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Stimulators/responders.

**REFERENCES**

4. Ulmer A, Maurer HR: The formation of B-lymphocyte colonies in agar contained in glass capillaries. Immunology 34:919, 1978
20. Fernandez LA, MacSween JM, Langley GR: T cell function


Immunoregulation of B lymphocyte colony formation by T cell subsets in patients with chronic lymphocytic leukemia

LA Fernandez, JM MacSween and GR Langley