Immunoglobulin Secretory Function of B Cells From Untreated Patients With Chronic Lymphocytic Leukemia and Hypogammaglobulinemia: Role of T Cells

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

The mechanism of the hypogammaglobulinemia in patients with chronic lymphocytic leukemia (CLL) was studied by determining the generation of specific immunoglobulin-secreting cells in response to mitogen and antigen stimulation in culture. Normal peripheral blood B lymphocytes from 18 normal subjects cocultured with equal numbers of autologous T cells generated cells secreting $2.542 \pm 695$ IgG, $2.153 \pm 615$ IgA, and $2.918 \pm 945$ IgM. Normal B lymphocytes cocultured with normal allogeneic T cells generated similar numbers. However, B lymphocytes from patients with chronic lymphocytic leukemia cocultured with T cells from the same patient generated only 0.5% as many cells secreting IgG and 11% and 23% as many secreting IgA and IgM, respectively. The reason for this markedly defective generation of immunoglobulin-secreting cells was investigated by evaluating T-helper, T-suppressor, and B-cell function using B cells from tonsil and T and B cells from peripheral blood of normal and leukemic individuals. T cells from patients with chronic lymphocytic leukemia provided somewhat greater help than did normal T cells to normal peripheral blood B cells and normal help to tonsil B cells, whether stimulated with mitogen or antigen. T cells from patients with chronic lymphocytic leukemia did not demonstrate increased suppressor function compared to normals with B cells from normal peripheral blood. The hypogammaglobulinemia in these patients therefore was associated with a markedly defective generation of immunoglobulin secreting cells, and as there was normal or increased T-cell helper activity without excessive suppressor activity, it seems likely that this was due to an intrinsic B-cell defect.

HYPOGAMMA GLOBULINEMIA is a common accompaniment of chronic lymphocytic leukemia (CLL) and, when present, usually persists throughout the course of the disease. There may be a selective decrease of one of the immunoglobulin classes, though usually the serum levels of all of them are low. While the depressed level of serum immunoglobulin ultimately is due to decreased B-cell function, the mechanism is controversial. The synthesis of immunoglobulin by B cells is regulated by subsets of T cells, which may help or suppress B-cell production of immunoglobulin. Although patients with chronic lymphocytic leukemia have been shown to have an abnormal ratio of suppressor and helper T cells, there is one report to the contrary, and we and others have been unable to demonstrate increased suppressor cell effects in the cell-mediated immune system. Still, it is possible that the low serum immunoglobulin levels in patients with chronic lymphocytic leukemia may be due to decreased helper or increased suppressor cell activity in this disease.

Recently, the mechanism of hypogammaglobulinemia has been elucidated in a number of clinical conditions. In patients with variable hypogammaglobulinemia, defective B-cell function was found in the majority of cases, but in a few patients, there was also lack of T helper cell activity or increased T suppressor cell activity. In the hypogammaglobulinemia accompanying the monoclonal hyperproteinemia of multiple myeloma, increased suppressor cell activity was found. These suppressor cells decreased normal immunoglobulin production. In view of these observations, we studied the mechanism of hypogammaglobulinemia in patients with chronic lymphocytic leukemia, previously untreated. Some investigators have suggested that hypogammaglobulinemia in patients with chronic lymphocytic leukemia was due to defective B cells; others have suggested that it was due to lack of helper T activity and/or increased T suppressor cell activity. The variable findings may have been due to the stimuli used (antigen or mitogen) or the different sources of B cells (peripheral blood or tonsil) and the use of mononuclear cells from patients who had been treated. We therefore determined responses to both antigenic and mitogenic stimulation utilizing both sources of B cells in normal subjects and patients with untreated chronic lymphocytic leukemia.

MATERIALS AND METHODS

Patients

All patients diagnosed as chronic lymphocytic leukemia had persistent lymphocytosis present on at least 2 successive occasions more than 3 mo apart. In the 18 patients studied, this was due to an increase in B cells identified by surface immunoglobulin and by the receptor for the C3 component of complement. None of the patients...
Separation of T Lymphocytes From Peripheral Blood Mononuclear Cells

Thirty to forty milliliters of heparinized venous blood were obtained from patients with chronic lymphocytic leukemia and normal individuals and mononuclear cells were obtained by centrifugation on Ficoll-Hypaque. Mononuclear cells were then washed three times with medium RPMI 1640 (GIBCO, Grand Island, NY), and the T cells were separated by rosetting with sheep erythrocytes and centrifugation on Ficoll-Hypaque as previously described.8,7,2 This procedure was repeated twice. T cells settled to the bottom of the test tube. In normal subjects, between 92% and 95% of the cells that settled out were T cells, while in patients with chronic lymphocytic leukemia, 78%-92% of these cells were T cells. Cells at the interface of Ficoll-Hypaque and medium were primarily non-T cells.

Enrichment of T Helper and Suppressor Cells Using Monoclonal Antibodies

Ten to twenty million T cells suspended in 1 ml of medium RPMI 1640 and 5% fetal calf serum (GIBCO) were placed with 10 μl OKT4 or OKT8 monoclonal antibody for 0.5 hr at 0°C. Then, 300 μl of rabbit complement (Cedarlane batch no. 1108) was added and tubes were placed on a rocker for 1 hr at 37°C. The cells were washed 3 times with RPMI 1640 and fetal calf serum. Aliquots of cells were stained with either fluorescein-conjugated OKT4 or OKT8. Less than 1% of T cells depleted of T4 or T8 subsets stained with their respective fluoresceinated antibodies. The monoclonal antibodies were purchased from Ortho Diagnostic Systems, Inc., Raritan, NJ.

Generation of Immunoglobulin-Producing Cells In Vitro

From peripheral blood mononuclear cells. Various concentrations of T cells, T-cell subsets, and non-T-cells were placed in 5 ml of media containing RPMI 1640, 10% fetal calf serum (GIBCO), 2 mM glutamine, penicillin 10 U/ml, and streptomycin 100 μg/ml.22 The cultures were incubated in 25 sq cm tissue culture flasks (Costar, Cambridge, MA) in a 5% CO2 incubator for 4-7 days at high humidity, after which the cells were washed 3 times with medium RPMI 1640 and suspended in it. Prior studies with pokeweed mitogen (PWM; Sigma, St. Louis, MO) at concentrations of 1, 5, 10, and 20 μg/ml had demonstrated that the optimal number of immunoglobulin-producing cells were obtained at a concentration of 10 μg/ml. Immunoglobulin-producing cells were usually seen on day 4, peaked at day 6, and then declined.

From tonsil cells. Tonsil cells were also used as a source of antibody-producing (B) cells. Single-cell suspensions were teased from tonsils obtained within 1 hr of surgery and cultured as described above. Greater than 95% viability was shown by Trypan blue staining. Various combinations of tonsil cell depleted of T cells and T cells from normal subjects and patients with chronic lymphocytic leukemia were stimulated both with mitogen (10 μg/ml pokeweed mitogen) and antigen (106 sheep erythrocytes/ml). Prior studies with pokeweed mitogen at a concentration of 1, 5, 10, and 20 μg/ml and sheep erythrocytes at a concentration of 0.5 × 109, 1.0 × 109, 2.5 × 109, 5.0 × 109 cells/ml had demonstrated that the maximal number of immunoglobulin-producing cells were obtained at a concentration of 10 μg/ml of pokeweed mitogen and 109 cells/ml of sheep erythrocytes. Optimal time for in vitro culture for tonsils was also 6 days; fewer cells were found on days 5 and 7. Following 5-7 days of culture, the cells were washed twice with

Table 1. Clinical Indices of Untreated Patients With CLL

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age</th>
<th>Absolute Lymphocyte Count (10⁹/Liter)</th>
<th>RAI Stage</th>
<th>G (0.17-2.5 g/Liter)</th>
<th>A (10-15 g/Liter)*</th>
<th>M (10-19 g/Liter)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>80</td>
<td>15,000</td>
<td>0</td>
<td>7.8</td>
<td>1.74</td>
<td>NDDet</td>
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<tr>
<td>2</td>
<td>M</td>
<td>59</td>
<td>23,400</td>
<td>0</td>
<td>6.6</td>
<td>0.72</td>
<td>NDDet</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>40</td>
<td>15,300</td>
<td>0</td>
<td>7.6</td>
<td>0.72</td>
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<tr>
<td>4</td>
<td>F</td>
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<td>0</td>
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</tr>
<tr>
<td>5</td>
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<td>0</td>
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<td>6</td>
<td>F</td>
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<td>0.32</td>
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<td>7</td>
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<td>69</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>81</td>
<td>63,000</td>
<td>II</td>
<td>5.9</td>
<td>0.52</td>
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</tr>
<tr>
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<td>M</td>
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<td>IV</td>
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<td>0.62</td>
<td>NDDet</td>
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<tr>
<td>18</td>
<td>M</td>
<td>62</td>
<td>82,000</td>
<td>IV</td>
<td>5.3</td>
<td>1.08</td>
<td>0.78</td>
</tr>
</tbody>
</table>

NDDet, not detected; ND, not done.

*Normal range.
IMMUNOGLOBULIN SECRETION IN CLL

RPMI 1640 and then plated for demonstration of immunoglobulin-producing cells in the reverse hemolytic plaque assay. Addition of T cells only to tonsil cells resulted in no plaque formation, while addition of PWM and sheep erythrocytes to tonsil cells without T cells resulted in <10% viable cells after 6 days of culture.

Reverse Hemolytic Plaque Assay

Preparation of chromic chloride. Chromic chloride was made up in saline to a concentration of 0.038 M. Every Monday, Wednesday, and Friday for 3 wk prior to use, the pH was adjusted to 5 with 1 M sodium hydroxide.

Coupling of protein A to sheep erythrocytes. Sheep erythrocytes were washed 4 times with normal saline and mixed with equal volumes of protein A (Sigma) in saline at a concentration of 0.5 mg/ml. Chronic chloride, stored for at least 3 wk, was then added in a drop-wise fashion, while continuously mixing the sheep erythrocytes on a vortex mixer. The mixture was then rotated for 1 hr at 37°C, after which the sheep erythrocytes were washed 4 times with saline. Since protein A binds to the Fe portion of immunoglobulin, the binding of protein A to sheep erythrocytes was demonstrated by comparing the binding of 125I-labeled sheep erythrocytes, with and without protein A. Without protein A bound to sheep erythrocytes, 96 ± 11 cpm were obtained. Coupling of protein A to sheep erythrocytes increased binding by approximately 12 times (1,295 ± 141 cpm).

Enumeration of immunoglobulin-producing cells. Immunoglobulin producing cells were identified by the reverse hemolytic plaque assay. Twenty-five microliters of packed sheep erythrocytes that had been complexed with protein A (Sigma), 25 μl antisera to human immunoglobulins with specificity for μ, α or γ heavy chains in a dilution of 1:4 (DAKO), and 25 μl guinea pig complement (Cedarlane) previously absorbed with sheep erythrocytes. One hundred microliters of the mononuclear cell suspension that had been stimulated with pokeweed mitogen or sheep erythrocytes was added, and 0.2 ml of the mixture was then placed in a Petri dish and covered by a cover slip. The Petri dishes were placed in an incubator for 4 hr, and the number of cells secreting specific immunoglobulins was identified by viewing the plates under a microscope and identifying a zone of lysis around individual lymphocytes. Cells from patients and controls were processed simultaneously with triplicate determinations.

RESULTS

Optimal Combinations and Concentrations of Peripheral Blood Mononuclear Cells and Mitogen

Generation of immunoglobulin-producing cells required both T and non-T combinations and stimulation with pokeweed mitogen. No immunoglobulin-producing cells were generated when either T cells alone or non-T-cells alone were stimulated with this mitogen. The optimal number of immunoglobulin-secreting cells was obtained when the T-to-non-T cell ratio was 1:1. Addition of more T cells led to a decrease in immunoglobulin-producing cells. Therefore, the results shown are the numbers of immunoglobulin-producing cells per million cells plated, obtained by incubating 2.5 × 10⁶ T cells and 2.5 × 10⁶ non-T-cells for 6 days with 10 μg/ml pokeweed mitogen.

Effect of Mixed Lymphocyte Reactions on the Number of Immunoglobulin-Producing Cells Generated in Culture

To determine whether mixed lymphocyte reactions would affect the number of immunoglobulin-producing cells generated, cocultures of T cells and non-T-cells from unrelated individuals were compared to autologous T cells and non-T-cells. The results are shown in Fig. 1. For the purpose of statistical comparison, the cells obtained from the blood of 30 healthy individuals were randomly allocated to two groups, each of 15 (group I and group II). The number of immunoglobulin-producing cells resulting from the stimulation of autologous T cells and non-T-cells was determined for each individual in both groups, as shown in Fig. 1, A and B. When T lymphocytes from individuals in one group were cultured with non-T-cells from paired individuals in the other group, the results were essentially unchanged from the autologous responses (Fig. 1, E and F). When T cells from one individual were added to T and non-T combinations from another, such that the T:B ratio was 2:1, the number of immunoglobulin-producing cells was significantly decreased (p < 0.005; Fig. 1C). However, the number of immunoglobulin-producing cells was not decreased if half the number of T cells from each individual were mixed to keep the T:B ratio at 1:1 (Fig. 1D).

Effect of T Cells From Normal Subjects and Patients With CLL on Generation of Immunoglobulin-Secreting Cells by Peripheral Blood B Cells

Since no stimulation or inhibition of immunoglobulin-producing cells was observed when allogeneic combinations were used, we measured immunoglobulin-producing cells with T and non-T combinations from normal individuals and patients with chronic lymphocytic leukemia (Fig. 2). Eighteen untreated patients with chronic lymphocytic leukemia produced cells secreting 12 ± 6 IgG, 255 ± 77 IgA, and 691 ± 441 IgM (Fig. 2A), compared to 2,542 ± 695 IgG, 2,153 ± 615 IgA, and 2,918 ± 945 IgM secreting cells from normal individuals (Fig. 2B). This represented a significant decrease from normal (p < 0.005 IgG and IgA and p < 0.025 IgM). When T cells from normal subjects were cocultured with non-T-cells from patients with chronic lymphocytic leukemia, there was no increase in the number of immunoglobulin-secreting cells (Fig. 2E). Addition of T cells from patients with chronic lymphocytic leukemia to T and non-T cell combinations from normal individuals
resulted in decreased immunoglobulin-producing cells, but the decrease was not significant (Fig. 2C). When the T and non-T cell ratio was kept at 1:1, the immunoglobulin-producing cells did not decrease in number (Fig. 2D). These results were similar to those obtained with T and non-T cells from healthy individuals (Fig. 1C and D), showing that the T cells from patients with chronic lymphocytic leukemia had no abnormal suppressor effect. To demonstrate T helper activity, T cells from patients with chronic lymphocytic leukemia were added to normal non-T-cells prior to culture. This resulted in production of above average numbers of immunoglobulin-producing cells, showing there was no lack of helper T cell effect (Fig. 2F).

**Effect of T-Cell Subsets From Normal Subjects and Patients With CLL on Generation of Immunoglobulin-Secreting Cells by Peripheral Blood B Cells**

Table 2 shows that when $2.5 \times 10^6$ OKT4-enriched cells were added to $2.5 \times 10^6$ non-T-cells and stimulated with PWM for 6 days, $2,179 \pm 871$ IgG, $1,476 \pm 613$ IgA, and $5,545 \pm 2,073$ IgM secreting cells were generated. Addition of $2.5 \times 10^6$ OKT8 cells to $2.5 \times 10^6$ non-T-cells and $5,545 \pm 2,073$ IgM secreting cells were generated.
10^6 unfractionated T cells plus 2.5 × 10^6 non-T-cells suppressed the number of cells secreting immunoglobulin G, A, and M by 62%, 50%, and 72%, respectively.

The effect of normal and CLL T helper and suppressor cell subsets on the generation of immunoglobulin-secreting cells by normal peripheral blood B cells was compared. Seven patients with CLL (3 with stage 0, 1 each with stage II, III, and IV) and 7 normal individuals were studied simultaneously; however, complete sets of data on all patients and normal individuals were not obtained either because of lack of T cells or contamination of some cultures in vitro. Figure 3 shows that the mean values with CLL T helper cells from 6 patients (Fig. 3B) were not significantly different from the mean values with normal T helper cells from 4 individuals (Fig. 3A). Similarly, CLL T suppressor cells added to combinations of normal T and non-T cells in ratios of either 2:1 (Fig. 3D) or 1:1 (Fig. 3F) gave results that were no different from normal individuals (Fig. 3, C and E). Individual values with T helper and suppressor cells obtained from both normal individuals and patients with CLL showed comparable helper or suppressor effects, although the suppressor effect was not marked when mean values were calculated because of considerable variation in the numbers of plaques obtained.

**Effect of T Cells From Normal Subjects and Patients With Chronic Lymphocytic Leukemia on Generation of Immunoglobulin-Secreting Cells by Tonsil B Cells**

In a further attempt to determine whether T cells from patients with chronic lymphocytic leukemia had normal helper cell activity, the ability of T lymphocytes from seven healthy individuals to support the generation of IgM-producing cells from tonsil, in respect to stimulation by mitogen and antigen, was compared to those from seven patients with chronic lymphocytic leukemia. The maximal numbers of immunoglobulin-producing cells were also obtained by stimulation with 10 μg/ml PWM or 10^6 sheep erythrocytes/ml for 6 days in vitro. The optimal T:B ratio for IgM-secreting cells differed between cultures stimu-
related with pokeweed mitogen and sheep erythrocytes. The results at three ratios of T-cell:tonsil-cell are shown in Table 3. The numbers of IgM-producing cells with both mitogenic and antigenic stimulation were not significantly different in patients with chronic lymphocytic leukemia compared to normals, showing that there was no lack of helper T cell activity in patients with chronic lymphocytic leukemia. The numbers of IgM-secreting cells obtained after stimulation with PWM is less than those obtained from peripheral blood, probably because the majority of tonsil cells have already been stimulated in vivo.

**DISCUSSION**

These in vitro studies of cells from 18 patients with untreated chronic lymphocytic leukemia and hypogammaglobulinemia revealed impairment in the leukemic B cell's ability to generate immunoglobulin-secreting cells. In comparison to normals, B cells from the leukemic subjects generated only 0.5% as many IgG, 11% IgA, and 23% IgM secreting cells. While there was a marked overall reduction in production of immunoglobulin-secreting cells, the ability to generate such specific cells was IgM > IgA > IgG. This difference may reside in the ontogeny of B-cell development, since the first capacity achieved is for IgM production, then IgA or IgG, and raises the question of a block in B-cell maturation.

The defect in the generation of immunoglobulin-secreting cells resided primarily within the leukemic B cells themselves, since normal T cells, which provided adequate help to normal allogeneic B cells, did not alter the impaired responses. In fact, the T cells from the patients with B-cell chronic lymphocytic leukemia were able to generate a greater than normal number of immunoglobulin-secreting cells from normal peripheral blood B cells, suggesting that they had enhanced helper activity. However, normal helper activity was demonstrated when the B cells were obtained from tonsils. There was no correlation between the ability to generate immunoglobulin-secreting cells of specific type in vitro, the serum levels of the respective immunoglobulins, and the RAI stage of the disease. Since the source of B cells in these leukemia patients was peripheral blood, whereas in vivo immunoglobulin secretion would occur from plasma cells in marrow, lymph nodes, spleen, and other sites, a correlation would not necessarily be expected.

In not being able to demonstrate a lack of T helper activity nor increased T suppressor activity in patients with chronic lymphocytic leukemia, our work is in agreement with Fauci et al. and Inoshita et al., both of whom used pokeweed mitogen to stimulate peripheral blood B cells. Fauci et al. described innate defects in B cells, and these investigators and Inoshita et al. found normal T-cell function in patients with chronic lymphocytic leukemia, whereas Hersey et al. found B-cell defects in most of their patients, but with a combination of T helper and/or T suppressor defects. However, 5 of Hersey et al.'s 8 tested patients had been treated for their leukemia. Our treated patients do show a combination of defects similar to those described by Hersey et al. (manuscript in preparation), but all of our 18 patients reported here were untreated, and most of them were in the early stage of the disease process. One cannot rule out the possibility that altered T-cell function may be related to later stage of disease process. Semenzato et al. found decreased T helper and increased T suppressor activity utilizing enriched T\(\mu\) cells and T\(\gamma\) cells, as identified by receptors for the Fc portions of IgM and IgG. Recent studies suggest that the Fc receptors may be functionally heterogeneous and do not always correspond to T helper/ suppressor cells as identified by monoclonal antibodies.

In contrast to our findings, Chiorazzi et al. showed lack of T helper activity, and Kay showed both lack of T helper activity and increased T suppressor activity. Chiorazzi et al. found that normal T cells supported anti-sheep erythrocyte antibody production by tonsil cells, while T cells from patients with chronic lymphocytic leukemia did not, resulting in less than 5% of the normal response in all 6 patients tested. The lack of T cell helper activity was not seen when cells were stimulated with pokeweed mitogen. The explanation for this difference with the studies we report is not clear. Stark et al. found that there is a decreased amount of actin in T cells from patients with chronic lymphocytic leukemia, compared to T cells from normal individuals, suggesting that membrane properties of T cells from chronic lymphocytic leukemia patients may differ from T cells from normal individu-

**Table 3. IgM-Producing Cells Generated by Mitogen and Antigen Stimulation of Tonsil Cells (2.5 x 10^6) Cocultured With Varying Concentrations of T Cells From Normal Individuals and From Patients With Chronic Lymphocytic Leukemia**

<table>
<thead>
<tr>
<th>Stimulation by Mitogen (Pokeweed, 10 (\mu)g/ml)</th>
<th>Stimulation by Antigen (Sheep Erythrocytes, 10^9/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal T Cells x 10^5</td>
<td>Normal T Cells x 10^5</td>
</tr>
<tr>
<td>1.25</td>
<td>2.5</td>
</tr>
<tr>
<td>Normal (n = 7)</td>
<td>504 ± 230</td>
</tr>
<tr>
<td>CLL (n = 7)</td>
<td>299 ± 96</td>
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</table>
als. Therefore, in vitro manipulations of T cells, like lysing sheep erythrocytes that had rosetted with T cells with distilled water or 0.87% ammonium chloride, or irradiation of T-cell populations to get rid of radio-sensitive T suppressor cells, may damage CLL T cells more than normal T cells. We routinely use ammonium chloride to lyse sheep erythrocytes that rosette with T cells. The conditions utilized by Chiorazzi et al. were not published. Kay\textsuperscript{15} has shown that isolated T suppressor and nonsuppressor cells from patients with chronic lymphocytic leukemia reduced the proliferative response of normal B cells to pokeweed mitogen stimulation. However, he did not measure immunoglobulin synthesis, so these observations are not directly related to our findings, particularly since a lack of correlation between cell proliferation and immunoglobulin secretion has been reported in normal and CLL lymphocytes.\textsuperscript{12,33}

The role of the monocytes in immunoglobulin production cannot be ignored. Normal monocytes have been reported to either have no effect or decreased immunoglobulin production in response to pokeweed mitogen stimulation.\textsuperscript{34-36} Activated monocytes decrease immunoglobulin production. Patients with chronic lymphocytic leukemia have decreased numbers of monocytes, and their role in immunoglobulin production has not been studied. However, since our data show no inhibitory activity by the non-T-cell mononuclear subpopulation, we would not expect the monocytes to inhibit immunoglobulin production in chronic lymphocytic leukemia, although there could be a lack of accessory cell activity in support of immunoglobulin synthesis.

In patients with chronic lymphocytic leukemia, imbalances of T suppressor/T helper ratios have been reported,\textsuperscript{4,6} with the majority of studies suggesting that T suppressor cells are increased in number, although there is one report to the contrary.\textsuperscript{7} With normal suppressor cell function, one would expect increased suppressor activity. However, we and others have not been able to demonstrate increased suppressor influences either in the cell-mediated\textsuperscript{8,9} or the humoral immune system.\textsuperscript{13,14} These observations suggest that patients with untreated chronic lymphocytic leukemia exhibit normal global T helper and suppressor activity, but may have increased numbers of functionally defective T suppressor cells.

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Immunoglobulin secretory function of B cells from untreated patients with chronic lymphocytic leukemia and hypogammaglobulinemia: role of T cells

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