INDUCTION OF IN VITRO DIFFERENTIATION AND IMMUNOGLOBULIN SYNTHESIS OF HUMAN LEUKEMIC B LYMPHOCYTES*

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Chronic lymphocytic leukemia (CLL) in man has been proven to be mainly a disease due to clonal proliferation of B lymphocytes. Although immunofluorescent studies indicate that the majority of the leukemic lymphocytes from patients with CLL bear surface immunoglobulin (Ig), it has been shown that very little intracellular Ig was demonstrable by this technique (2–4). It appears that a differentiation block is present in the majority of CLL cases. However, this block has been proven to be incomplete in certain patients (5, 6).

In a previous investigation involving a patient with CLL and a monoclonal IgM band (5), an anti-idiotypic antiserum also identified a small number of the cells to be plasma cells. Thus the IgM on the membrane of the lymphocytes, the intracellular IgM in the plasma cells, and the monoclonal IgM protein in the serum were idiotypically identical. It appeared that a small number of the leukemic lymphocytes matured by a process of differentiation in vivo to plasma cells which synthesized the serum monoclonal IgM.

The present studies indicate that leukemic B cells in these cases could be induced to differentiate to plasma cells in vitro at an accelerated rate with the provision of helper T cells. A T-cell helper function defect was demonstrated in these patients which may have played a role in the limited differentiation in vivo.

Materials and Methods

Isolation of Lymphocytes. Mononuclear cells were isolated by Ficoll-Hypaque gradients (Pharmacia Fine Chemicals, Piscataway, N. J.) from the peripheral blood of normal individuals and patients with CLL. Tonsillar mononuclear cells were isolated as described previously (7). Spontaneous rosette formation between human lymphocytes and sheep erythrocytes (SRBC) was performed with neuraminidase-treated SRBC (En). The E rosette-forming cells were separated from the nonrosette-forming cells by Ficoll-Hypaque gradient centrifugation. The rosette-forming cell fraction was purified further by a second density gradient centrifugation. Repeated gradient centrifugation was performed until no significant cells were observed at the

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Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; Con A, concanavalin A; PFC, plaque-forming cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SRBC, sheep erythrocytes.
interphase. The E rosette-forming cells were recovered after lysis of SRBC by a Tris-buffered ammonium chloride solution. Tonsillar B-cell fractions were obtained by the depletion of E rosette-forming cells from the mononuclear cell preparations.

Patient Identification. Two patients with CLL and monoclonal protein bands were studied. Patient Ei has been described previously (5). Patient Se is a 62-yr-old woman who had CLL for 3 yr. On her initial presentation, her leukocyte count was >50,000/mm³. An IgMγ band of 4 mg/ml was present in the serum. In addition, lymphocytic infiltrate of liver and lungs was seen in biopsy specimens. Marked splenomegaly was noted. After splenectomy and a short course of chemotherapy, the leukocyte count decreased. At the time of this investigation, her leukocyte count was 12,000/mm³ with 54% lymphocytes. 17% of the peripheral blood mononuclear cells were monocytes identified by the presence of peroxidase granules, 42% were T cells, and 34% stained with an anti-idiotypic antiserum against IgM Se. 0.2% of the mononuclear cells were plasma cells stained brightly with the anti-idiotypic antiserum for intracellular Ig. Other CLL patients had no detectable Ig bands.

Anti-Ig Antisera, Immunofluorescence, and Hemagglutination Antisera. Antisera were prepared in New Zealand Red rabbits against the serum IgM components from these patients after purification by Pevikon block electrophoresis. Rhodamine-conjugated F(ab)₂ fragments specific for μ, γ, α, κ, λ, IgM protein Se (IgM Se) and IgM protein Ei (IgM Ei) were prepared and characterized by methods previously described (5). Unconjugated idiotypic antisera specific for IgM Ei and for IgM Se were also prepared. These antisera were absorbed with pooled normal human serum, pooled IgG, and monoclonal IgM proteins isolated from other patients, and were shown to be highly specific for the protein used in the immunization both by hemagglutination assay and by immunofluorescence. Idiotypic antisera to IgM Se did not react with normal B cells or with cells from patient Ei by fluorescence; the same individual specificity was true for idiotypic antisera to IgM Ei. Immunofluorescence for the detection of membrane Ig, intracellular Ig, hemagglutination, and hemagglutination-inhibition were carried out as described (2, 5).

Cell Cultures and Plaque-Forming Cell (PFC) Assay. Cell cultures in a total volume of 2 ml were set up in 16-mm diameter wells of Linbro culture plates (Linbro Chemical Co., Hamden, Conn.) at 37°C with 5% CO₂ in RPMI 1640 supplemented with 0.2 mM glutamine, 1% trypsinase soy broth, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 10% SRBC-absorbed human AB serum. For the experiments to determine leukemic cell differentiation, the cultures were carried out for 6 days. The cells were then counted, washed, and processed for immunofluorescence microscopy for the determination of the number of plasma cells generated. For the cultures involving tonsillar B cells, 1 X 10⁸ SRBCs were added to the cultures as antigen. At day 5, cells were harvested and assayed for PFCs against SRBC by the Jerne-Nordin PFC assay procedure as modified by Fauci and Pratt (8). Concanavalin A (Con A)-induced suppressor cells were generated according to Haynes and Fauci (9). Pokeweed mitogen (PWM; Grand Island Biological Co., Grand Island, N. Y.) was used at a final dilution of 1:100.

Measurement of DNA Synthesis in Cultured Cells. At the termination of cultures, triplicate 0.1-ml aliquots from each culture were transferred to a flat-bottom Linbro plate (6-mm diameter wells) and pulsed with [³H]thymidine (2 μl of 1.0 mCi/ml of spec act 1.9 Ci/mM; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). After an 8-h incubation at 37°C cells were processed by a semiautomatic harvester for liquid scintillation counting.

Autoradiographic Techniques. Cultured cells were labeled for 8 h with 5 μl of [³H]-thymidine (0.5 mCi/ml of specificity 1.9 Ci/mM; Schwarz/Mann) added to a 2.0-ml culture with 2 X 10⁸ cells in the presence of PWM. For continuous labeling, repeated doses (2 μl) of [³H]thymidine were added at day 0, 3, 5, and 7. Labeled cells were washed extensively and smears were made by cytocentrifugation. The slides were fixe and stained with the rhodamine-conjugated anti-idiotypic antiserum. Autoradiographs were developed according to standard procedures. No counterstaining was done. The labeling procedures did not effect the viability and the rate of differentiation of Se cells. All labeled cells contained >20 grains per cell.

Results

Maturation of Leukemic Lymphocytes and the Allogeneic Helper Cells. Leukemic B cells isolated from patient Ei when cultured alone or with autologous T cells were not able
to mature to plasma cells (Table I). In the first experiment, only $1 \times 10^4$ plasma cells were seen after a 6-day culture of $2 \times 10^6$ leukemic B lymphocytes. In contrast, $5.5 \times 10^5$ plasma cells were detected in a similar culture to which $2 \times 10^6$ allogeneic T cells from a normal individual were added. This represents a 55-fold increase in plasma cell generation. In the second experiment, >100-fold increase was observed. Although most cultures were set up for 6 days, significant numbers of plasma cells ($3.5 \times 10^5$) were generated at day 4 in one experiment. Similar results were obtained with either the rhodamine-conjugated anti-idiotypic antiserum or the anti-\(\mu\) antiserum. In one experiment involving double fluorochromes, the anti-\(\mu\) antiserum stained the same cells as the anti-idiotypic antiserum. The staining of plasma cells by the rhodamine-conjugated idiotypic antiserum is shown in Fig. 1. A rare plasma cell was seen in a control culture in which <0.5% of cells stained brightly with anti-idiotypic antiserum (Fig. 1a). In contrast, many plasma cells were seen in cultures to which allogeneic T cells were added. A representative field of such a culture is shown in Fig. 1b.

IgM Ei secreted into the culture supernate was assayed directly by a passive hemagglutination-inhibition assay involving the idiotypic determinant. Even though the cultures were carried out in medium supplemented with normal AB serum, direct assaying for specific IgM Ei secretion was feasible. Very little IgM Ei secretion (1 \(\mu\)g/ml) was found in the supernate when the leukemic B cells were cultured alone. A 16-fold increase in IgM Ei secretion was detected in the first experiment in the culture of Ei cells to which allogeneic T cells were added. In the second experiment, a 32-fold increase was detected. Similar results were obtained in two additional experiments in which allogeneic T cells were obtained from two different normal donors.

The second patient, Se, was studied in a similar fashion. Leukemic B lymphocytes isolated from her peripheral blood also matured to plasma cells when they were cultured with allogeneic T cells from a normal individual. In a typical experiment, over 100-fold increase in specific IgM Se secretion was detected in the culture to which allogeneic T cells were added.

Isolated T cells from Ei and Se responded to phytohemagglutinin (PHA) in a \[^{3}H\]thymidine uptake assay. Ei T cells gave a reduced response (30-50% of normal T
cells) to Con A and PWM, whereas Se T cells gave a normal response to PWM and a reduced response to Con A. Both T cells gave a reduced response to allogeneic B cells (30-40%) in a mixed lymphocyte culture reaction.

A series of stimulatory substances such as Con A, PHA, streptokinase-streptodornase (SKSD), tetanus toxoid, protein A, and dimethylsulfoxide were also added to Ei B cells. No further differentiation was observed. Limited experiments were done with supernates from mixed lymphocyte cultures. Only a 5- to 10-fold increase in plasma cells were seen.

Fig. 1. Staining of plasma cells with idiotypic antiserum in cultures containing similar numbers of leukemic cells. (a) A rare plasma cell in the control culture containing only Ei leukemic B cells. (b) Plasma cells in a culture of Ei leukemic B cells to which allogeneic normal T cells were added.
PWM-Induced Maturation of Leukemic B Cells. As reported previously (5), repeated attempts to induce further maturation of leukemic B cells from the patient Ei with PWM were unsuccessful. PWM induction of plasma cell generation by normal B cells has been shown to be T-cell dependent (10). To rule out that an insufficient number of T cells in the unseparated mononuclear cell preparation was responsible for these unsuccessful attempts, T cells from the patient Ei were isolated in a sufficient quantity. As many as $1 \times 10^7$ autologous T cells from this patient failed to help $2 \times 10^6$ leukemic B cells to mature further in the presence of PWM.

In the case of Se, unseparated peripheral blood mononuclear cells were able to mature to plasma cells stainable with an anti-idiotype antiserum in the presence of PWM. To study the T-cell dependence of this phenomenon further experiments were carried out on isolated T and B cells from this patient. Se B cells did not respond to PWM with autologous T cells in the absence of PWM. As little as $0.5 \times 10^6$ autologous T cells were sufficient to allow significant numbers of the leukemic B cells to mature to plasma cells in the presence of PWM (experiment 1, Table II). A concomitant increase in specific IgM Se secretion was also detected in these cultures. In the second experiment, similar results were observed.

Experiments were also carried out to determine if cell division was needed for Se leukemic B cells to mature to plasma cells under the influence of PWM and autologous T cells. In an 8-h labeling experiment with $[^3H]$thymidine at day 3, 5, and 7, only 10–15% of the plasma cells identifiable by immunofluorescence had grains by autoradiography. By day 3, >30% of the nonrosetting cells were plasma cells. In a continuous labeling experiment, only 28% of the plasma cells were labeled by day 7.

Lack of Excessive Suppressor Cell Activity in Patients with CLL. Isolated T cells from patient Ei were added to cell cultures in which Ei B cells and allogeneic T cells were cocultured to assess suppressor cell activity of Ei T cells. As many as $1 \times 10^7$ T cells from this patient failed to suppress the generation of plasma cells in this allogeneic culture system.

Experiments were also carried out in the case of Se to rule out excessive suppressor activity in a different assay system. The suppressor activity of a cell preparation was determined by its ability to suppress the responses to PWM by the autologous mononuclear cells. In such a system, $0.75 \times 10^6$ cells from normal individuals cultured

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**TABLE II**

*Effect of PWM on the Differentiation of Se Leukemic B Lymphocytes*

<table>
<thead>
<tr>
<th>Se B cells</th>
<th>Se T cells</th>
<th>Plasma cells/culture</th>
<th>IgM Se secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^6$</td>
<td>None</td>
<td>$10^6$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>$3 \times 10^6$</td>
<td>$10^6$</td>
<td>$3 \times 10^3$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>$3 \times 10^6$</td>
<td>$10^6$</td>
<td>$3 \times 10^3$</td>
</tr>
</tbody>
</table>

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*Culture conditions and assay systems were identical to those described in Table I. PWM was added to a final dilution of 1:100.*
TABLE III

Lack of Suppressor Cell Activity in the Peripheral Blood of Patient Se in a System of PWM-Induced Proliferation and Generation of Plasma Cells*

<table>
<thead>
<tr>
<th>Treatment of cultured cells</th>
<th>Medium alone</th>
<th>[3H]Thymidine uptake</th>
<th>10 μg Con A/ml of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma cells/culture</td>
<td>cpm</td>
<td>Plasma cells/culture</td>
</tr>
<tr>
<td>—</td>
<td>5.7 × 10⁴‡</td>
<td>68,145‡</td>
<td>3.6 × 10⁶</td>
</tr>
<tr>
<td>3.0 × 10⁸</td>
<td>4.2 × 10⁵</td>
<td>43,284</td>
<td>4.2 × 10⁶</td>
</tr>
<tr>
<td>1.5 × 10⁸</td>
<td>4.9 × 10⁵</td>
<td>51,851</td>
<td>4.1 × 10⁶</td>
</tr>
<tr>
<td>0.75 × 10⁸</td>
<td>6.3 × 10⁵</td>
<td>56,670</td>
<td></td>
</tr>
</tbody>
</table>

* Cultures were set up under identical conditions described in Table I.
‡ In a similar culture without the presence of PWM, 2 × 10⁶ plasma cells per culture and 2,188 cpm [3H]thymidine incorporation were observed. Plasma cells were identified with an anti-idiotypic antisera specific for IgM Se.

TABLE IV

Lack of Allogeneic Helper Activity of Isolated T Cells from Patients with CLL, using Tonsillar B Cells as a Test System*

<table>
<thead>
<tr>
<th>T-cell source (2 × 10⁶)</th>
<th>PFC against sheep erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>Normal</td>
<td>1,200</td>
</tr>
<tr>
<td>Ei</td>
<td>6</td>
</tr>
<tr>
<td>Se</td>
<td>3</td>
</tr>
<tr>
<td>Ya</td>
<td>3</td>
</tr>
</tbody>
</table>

* 2 × 10⁶ tonsillar B cells were cultured either alone or with T cells under identical conditions as described in Table I.

in the medium alone for 48 h would suppress significantly the generation of plasma cells when added to a culture of freshly isolated autologous cells stimulated by PWM (20–30% inhibition). A more marked suppression was observed in the Con A-induced suppressor system. As little as 0.375 × 10⁶ cells cultured in the presence of 10 μg/ml of Con A inhibited both cell proliferation by 40% and plasma cell generation by 70% in a similar system. In contrast to the normal control, relatively larger numbers of Se mononuclear cells either cultured alone or in the presence of Con A were needed to induce significant suppression of both proliferative and differentiative responses by freshly isolated Se mononuclear cells to PWM stimulation (Table III). The proliferative response of Se cells to 10 μg/ml of Con A after 48 h was approximately 50% of the normal control response as measured by [3H]thymidine uptake.

Defective T-Cell Helper Function in Patients with CLL. Because of the observation that the provision of appropriate T-helper activities markedly increased the rate of differentiation of leukemic B cells in vitro, the helper function of purified T cells from patients with CLL was examined. The system employed for assaying the helper activity of a given T-cell population was the generation of PFC by normal tonsillar B cells against SRBC cocultured with the T cells (7). In such a system, few plaques were generated in a culture containing 2 × 10⁶ B cells without the addition of allogeneic T cells from normal individuals (Table IV). In contrast, 1,200 plaques were
seen when T cells from a normal person were added. The addition of an equal number of purified T cells from three patients with CLL (Ei, Se, and Ya) generated no additional plaques. Ya is a patient without a monoclonal serum protein band. Further experiments with additional cases are being published separately (11).

Discussion

In the present studies, the leukemic B lymphocytes from two cases of CLL with monoclonal IgM protein in their serum were induced to differentiate further to plasma cells under the influence of the allogeneic helper effect (review in 12) generated in the cultures of these leukemic cells and T cells from normal individuals. In one of the cases, the leukemic B cells were able to mature further in the presence of PWM and autologous T cells. The origin of these plasma cells were proven to be derived from the leukemic cells by the use of idiotypic antisera specific for the membrane Ig of the leukemic cells. In addition, a marked increase in specific IgM secretion was detected in the supernates of the cultures. Evidence was also obtained by the combination of immunofluorescence and autoradiography that the majority of the leukemic B cells were able to mature to plasma cells without cell division. These in vitro observations support further the previous conclusion that some plasma cells were derived from leukemic cells in vivo by a process of differentiation (5). The possibility that these plasma cells might derive from a separate clone has been ruled out.

As many as 50% of the leukemic B cells were induced to mature to plasma cells in some experiments. In comparison this rate of differentiation in vivo (0.1–2.0%) was markedly reduced. This reduced rate could not be due to an intrinsic defect of the leukemic cells. In the present investigation, excessive spontaneous suppressor T-cell activities were not found in the two cases studied. In the case of Se, Con A-induced suppressor activities were also shown to be reduced. These findings are in agreement with the previous report regarding suppressor activity in CLL cases (13). Therefore, it appears that excessive suppressor activities are not responsible for the reduced rate of differentiation in vivo.

The isolated T cells from both patients were found to be defective in the generation of allogeneic effect factors when they were cultured together with normal B cells. This defect in helper T cells has been found in the majority of patients with CLL, even in cases in which lymphocytosis was the sole manifestation of the disease (11). In contrast, this defect in T cells was not found in the patients with Waldenstrom’s macroglobulinemia. In this regard, the cases of CLL with a monoclonal IgM band differ from classic macroglobulinemia. The two cases studied also differ from each other in the ability of their T cells to help autologous leukemic B cells to mature in the presence of PWM. In the case of Se, T cells responded normally to PWM stimulation by cell divisions and by the secretion of helper factors for the maturation of Se leukemic B cells. In the case of Ei, the isolated T cells had a reduced response to the mitogen and did not help Ei B cells. Thus, there is heterogeneity in the T-cell helper defects in these patients. The isolated T cells were also found to be relatively deficient in the subset of T cells bearing Fc receptors for IgM (11). This subset of T cells has been shown to be involved in both helper and suppressor functions in man (14). The lack of T \( \mu \) cells might account for the T-cell defects observed in our cases.

Maino et al. (15) have demonstrated that leukemic lymphocytes synthesize monoclonal light chains and that this synthesis is increased in the presence of phytohemagglutinin (PHA). They did not observe the differentiation described in the present
study; however, cases with monoclonal bands were not specifically studied. PHA had no effect in the differentiation to plasma cells in our two cases. Also it was reported in a case of prolymphocytic leukemia that a B-cell response to PWM occurred, although it was not proven that the leukemic cells were involved or if a monoclonal band was present in the serum (16). Whether leukemic B cells in CLL cases without Ig monoclonal proteins will differentiate further to plasma cells when appropriate helper factors are identified and provided remains to be determined.

The two patients with CLL described above were severely ill with their disease; patient Ei has died recently despite prolonged therapy. There was no apparent way to separate these cases from most other patients with CLL except for the presence of the monoclonal band. The latter was clearly an index of some differentiation in vivo. In these cases, the leukemic B cells resemble normal B cells in the following manner. They have IgM and IgD as their membrane Ig. The majority of the plasma cells derived from these leukemic B cells in vivo have surface Ig but lack Ia-like antigens on their surface (17). They are able to be transformed by Epstein-Barr virus, and permanent lines have been obtained by such transformation (18). Finally, they are responsive to T-cell regulation. Thus far no intrinsic defect in the leukemic B cells has been evident. The demonstrated lack of helper T-cell activities in these cases may contribute to the accumulation of leukemic lymphocytes at their early stages of maturation. How significant this defect is and what role it might play in the pathogenesis of the disease remains to be determined.

Summary

Successful induction of in vitro differentiation and immunoglobulin synthesis of the leukemic lymphocytes was carried out in two cases of chronic lymphocytic leukemia. Few plasma cells and little specific Ig secretion were detected in the cultures of isolated leukemic B cells in either the presence or the absence of autologous T cells. Up to 30% of the leukemic B cells matured to plasma cells, and a 32-fold increase in specific Ig synthesis was observed when T cells from normal individuals were added to the cultures of these leukemic B cells. In one of the two cases, autologous T cells were able to induce >50% of the leukemic B cells to differentiate further to plasma cells in the presence of pokeweed mitogen. This markedly accelerated in vitro differentiation was only achieved with leukemic cells from cases in which there was evidence of slight differentiation in vivo.

No evidence could be obtained for excessive suppressor T cells in these patients. However, a T-cell defect in the generation of allogeneic effect helper factors was identified. This defect may be responsible for the reduced rate of leukemic maturation in vivo.

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