Monoclonal CLL B-Cells May be Induced to Grow in an In Vitro B-Cell Colony Assay System

By Robert T. Perri and Neil E. Kay

A simple reproducible in vitro B-cell colony assay system was used to evaluate B-cell growth in controls and patients with chronic lymphocytic leukemia (CLL). All six CLL patients studied formed B-cell colonies. The number of colonies was significantly less in patients than controls (66 ± 18 versus 127 ± 8). CLL colonies were shown to be monoclonal and appeared to reflect the circulating malignant B-cell clone in our patient group, while the six controls studied formed polyclonal B-cell colonies. Wright-Giemsa staining showed typical plasma cells to have developed in the controls but not in the patients. Cells from CLL patients retained a more lymphoid appearance. It is believed that investigations with this B-cell assay will provide the means for further in vitro evaluation of malignant B-cell proliferation in other lymphoproliferative disorders.

THE MALIGNANT CELL in chronic lymphocytic leukemia (CLL) is generally believed to be the B cell. The use of idiootype-specific antisera have confirmed that CLL is a disorder typically involving a monoclonal B-cell clone.1 The clonal expansion of B cells is poorly understood but, as normal mammalian B cells are critically influenced by T-cell subsets,2 it would seem reasonable to evaluate the effect of T cells on CLL B-cell proliferation. However, efforts to manipulate the malignant B cell have been hampered by the inability of CLL B cells to mature following various in vitro inductive signals.4 6 A recent in vitro B-cell colony assay has been developed that permits the evaluation of B-cell growth from both normals and patients with various lymphoproliferative disorders.7

This assay system, which is dependent on conditioned tissue culture media and autologous irradiated T cells, permits not only a quantitative assay of in vitro B-cell growth but also provides for detailed membrane immunoglobulin (Ig) and morphological analysis. Accordingly we adapted this assay in order to delineate whether peripheral blood mononuclear cells from CLL patients could be induced to develop B-cell colonies and then to examine their morphological and membrane Ig patterns.

MATERIALS AND METHODS

Six healthy volunteer donors and six patients with B-cell CLL were studied. Informed consent was obtained from both volunteers and patients. Volunteers and patients were both age and sex matched. All patients were staged as described by Rai.8 Two patients each were stage 4 and one patient each was stage 0, 1, 2, and 3. No patient had any therapy for at least 6 wk prior to being studied. Peripheral blood mononuclear cells were isolated from venous blood by Ficoll-Hypaque. Monocyte depletion was done by adherence in tissue culture flasks for 45 min at 37°C in 5% CO₂ and the nonadherent cells were harvested. Nonadherent cells were then allowed to rosette with AET-sensitized sheep erythrocytes (SRBC) at 4°C for 2 hr. Rosetted cells (≥96% T cells) were separated from the nonrosetted B and null cells (≥95% SIg⁺) by two successive Ficoll gradients. SRBC were removed by hypotonic lysis with distilled water. T lymphocytes were irradiated with 3000 rads. The population of nonrosetted B and null cells used to initiate the B-cell colony assay. In our laboratory, null cells are defined as SIg⁺, SRBC-rosette, and nonspecific esterase negative.

PHA-T-cell conditioned medium (PHA-TCM) was prepared by incubating T cells with 1% PHA in growth media at 37°C with 5% CO₂ for 3 days. Supernatants were then collected, filtered, and stored at 4°C.

The B-cell colony assay was performed as described.1 In brief, the initiating B cells were suspended to 2 × 10⁵/ml in the presence of 3 × 10⁵/ml irradiated T cells in α-MEM with 10% FCS (Gibco, Grand Island, N.Y.), 20% PHA-TCM, and 0.8% methylcellulose. After vortexing, 0.1 ml aliquots were placed into 6-mm flat-bottomed microtiter wells (Linbro, Hamden, Conn.). All experiments were done in triplicate. Plates were secured tightly and incubated at 37°C with 5% CO₂ for 5–7 days.

To quantify colony numbers, we evaluated their numbers after 5 days of in vitro growth by counting the number of colonies present in each microtiter well using an Olympus inverted microscope. A colony was defined as a distinct aggregate of greater than 20 cells. Cells from single colonies were examined morphologically with Wright-Giemsa stain by harvesting cells using a fine Pasteur pipette containing phosphate-buffered saline (PBS). Immunofluorescent studies were performed on cells pooled from multiple colonies by first washing the pooled cells in PBS with 2% BSA and 0.2% sodium azide and then staining for surface immunoglobulin (SIg) using standard techniques.5 Labeled antibodies used were: fluorescein-goat F(ab')₂ anti-human IgG (Fab'), fragment (polyvalent), fluorescein-goat IgG anti-human G and M chains, fluorescein-goat IgG anti-human kappa and lambda chains (Cappel Lab, Cochranville, Pa.).

To test self-renewal capability, colonies were harvested, pooled, washed, and replated as in the primary cultures. After 5–7 days, colony numbers were counted and colonies were characterized.

RESULTS

The suspensions of normal and malignant B cells began forming clusters of several cells (<10 cells per cluster) after 24–48 hr. Distinct colonies appeared at

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72–96 hr in both controls and CLL (Fig. 1 A and B). The B-cell derivation of these colonies in both groups was confirmed by detection of SIg on ≥95% of cell suspensions obtained from the colonies (Table 1). Cells from control or CLL colonies did not rosette with AET-treated SRBC (data not shown). The Ig light chains detected on cell membranes from control colonies were polyclonal (Table 1). However, B-cell colonies of CLL patients were monoclonal and their immunoglobulin class was identical to that of their peripheral blood B cells (Table 1). In two experiments, cells obtained from CLL B-cell colonies were capable of resynthesizing their respective SIg after trypsinization and a further 5-hr incubation at 37°C in 5% CO2.

Wright-Giemsa staining of cells from single colonies showed typical plasma cells to have developed in the controls but not in the patients (Fig. 1 C). Cells from patients with CLL appeared to retain a more lymphoid appearance (Fig. 1 D).

The numbers of B-cell colonies developed by day 5 in our controls (mean ± SEM, 127 ± 8) were significantly greater than in our patients (66 ± 18), p < 0.001. All six CLL patients formed B-cell colonies by day 5. A linear relationship was observed between cell number plated and colony formation in both controls and CLL patients. Colony numbers in controls and patients were lowest (75 ± 6 versus 30 ± 12) when the number of plated initiating cells was lowest (10⁴ cells) and increased in a linear fashion to be greatest (225 ± 12 versus 125 ± 16) when the number of plated initiating cells was greatest (4 × 10⁴ cells). Though only 6 patients were studied, all stages were represented in this study. The number of B-cell colonies formed by one patient (119 ± 8) with Rai stage 0 was comparable to controls (127 ± 8). Two stage 4 patients formed the least number of B-cell colonies (21 ± 5).

Primary colonies obtained were harvested, pooled, washed, and replated as before, to determine the self-renewal capability of these colony cells. Colony morphology, immunoglobulin class, and the kinetics of colony appearance were the same in primary and secondary colonies.

**DISCUSSION**

Investigations of malignant B-cell proliferation in CLL have been limited by the absence of a reliable in vitro assay for B-cell growth. This assay is simple, reproducible, and permits detailed morphological evaluation of cell suspensions from both controls and patient groups.

We have demonstrated that peripheral blood CLL B cells may be induced in vitro to produce B-cell colonies. In addition, these colonies reflect the same isotype in vitro as their circulating counterparts. Thus, the colonies obtained in vitro did appear to reflect the circulating malignant clone of B cells in our patient group. While preliminary, it was also noted that control colony cell morphology was more differentiated than CLL cells.

This latter observation would be consistent with the current belief that CLL represents a clonal expansion of malignant B cells arrested at a stage of differentiation approximating that of the small resting lymphocyte. Totterman et al. have shown that CLL malignant B cells can be induced by phorbol ester to differentiate towards plasma cells. The availability of this assay should permit future in vitro attempts at inducing...
differentiation of the malignant B cells in CLL. This would be of particular importance, as we and others have recently demonstrated major imbalances of both a quantitative and a qualitative nature in CLL T-cell subsets. This assay will also permit further in vitro analysis of CLL and control T-cell modulation of both control and malignant B cells.

Previously described in vitro colony assays have provided clinicians with useful information and assistance (i.e., CFU-S, CFU-E, BFU-E) in helping to subgroup and make therapeutic decisions for patients with various diseases. It would also be the hope that further investigations with this B-cell assay might provide the means of distinguishing various subgroups of patients with CLL whose clinical courses may differ or whose treatment might be influenced by the results of such investigations.

REFERENCES

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