CONCISE REPORT

Interleukin 2 Stimulates Chronic Lymphocytic Leukemia Colony Formation In Vitro

By Ivo Touw and Bob Löwenberg

The requirements of clonogenic cells of B cell-type chronic lymphocytic leukemia (B CLL) for interleukin 2 (IL 2) were analyzed. Using the cells of five patients, we measured IL 2 receptor expression on the cell surface and the colony-forming abilities of the cells in response to IL 2. In four of the cases, significant percentages of the CLL cells expressed IL 2 membrane receptors (as assessed with the monoclonal antibody anti-Tac), indicative of their potential sensitivity to IL 2. Pure recombinant interleukin 2 (r-IL 2) was added to colony cultures that also contained the lectin phytohemagglutinin (PHA) or the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) to activate the CLL cells. Colony formation completely depended on the presence of r-IL 2 and PHA or TPA in culture, with the exception of one case, in which the addition of IL 2 was not required for colony growth in TPA-supplemented cultures. Twenty-five to fifty units of r-IL 2 per milliliter of culture medium provided optimal stimulation. Under these conditions, a linear relationship was observed between plated cell numbers and colony numbers formed. Morphological and immunologic analysis of colony cells indicated that these were monoclonal CLL cells that had matured toward plasma cell-like features.

INTERLEUKIN 2 (IL 2) is the soluble factor required for the in vitro proliferation of activated T lymphocytes. With the availability of pure IL 2 preparations (obtained by recombinant techniques) and the development of monoclonal antibodies reacting with IL 2 receptor molecules on the cell surface membrane, it now becomes clear that IL 2 can also directly act on normal human B lymphocytes as well as on neoplastic B cell progenitors, and induce a proliferative response of those cells.

Earlier, we reported that the proliferation of common and pre-B acute lymphoblastic leukemia (ALL) cells depends on IL 2 plus additional leukocyte factor(s). On this basis, a colony culture technique for ALL has been developed.\(^{8,9}\) A modification of this culture assay, it is shown here that B cell-type chronic lymphocytic leukemia (B CLL) cells, when activated by the lectin phytohemagglutinin (PHA) or the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), form colonies in response to recombinant IL 2 (r-IL 2). In contrast to clonogenic ALL cells, CLL colony formers do not require extra leukocyte stimulation for proliferation.

MATERIALS AND METHODS

Patients and separation of leukemia cells. Nucleated cells of five patients with CLL were isolated from the peripheral blood by Ficoll-Isopaque separation. None of the patients had received cytoreductive therapy for at least 12 weeks prior to examination. Residual T lymphocytes were removed from the Ficoll interface cells by rosetting with 2-aminoethylthiouronium bromide (AET)-treated sheep erythrocytes and sedimentation of the rosetted cells through Ficoll-Isopaque.\(^{9}\) In the non-rosetting (less than 0.5% positive) leukemic cell fractions, 97% to 100% of the cells were characterized morphologically as small lymphocytes.

Colony culture. Colony cultures were performed in 35-mm diameter culture dishes (1.4 mL culture volume), essentially as described for non-T ALL.\(^{10}\) However, one major modification was introduced, i.e., that feeder leukocytes were not included in the culture system. Instead, pure r-IL 2 (Biogen SA, Geneva) added to the culture upper layer served as stimulator in combination with either 0.75% vol/vol PHA (reagent grade, Wellcome, Dartford, England) or 70 ng/mL TPA (Sigma Chemicals, St Louis). Colonies (50 cells or more) were counted on day 7 of culture. Colony cells were then mass-harvested with a Pasteur pipette, washed three times with phosphate-buffered saline, and prepared for immunofluorescence, morphological, and cytogenetic analysis (May-Grünwald-Giemsa, nonspecific esterase and Sudan B staining of cytokeratin cells), and E rosette formation.

Immunofluorescence studies. The presence of immunoglobulin (Ig) chains on the cell surface and in the cytoplasm was assessed by immunofluorescence microscopy as described,\(^{11}\) using goat anti-human (GaHu) immunoglobulin antisera coupled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC). GaHu IgG/TRITC, GaHu IgA/FITC, GaHu IgM/TRITC (Kallestad, Austin, Tex), GaHu IgD/FITC, GaHu IgM/TRITC, and GaHu IgA/FITC (Nordic, Tilburg, The Netherlands) were all used in titers of 1:40. In dual staining procedures, incubations with TRITC-coupled antisera always preceded those with the FITC-coupled antibodies.

Cell surface membrane receptors for IL 2 were assayed in indirect immunofluorescence using the monoclonal antibody anti-Tac\(^2\) and a goat anti-mouse Ig FITC conjugate (GAM/FITC; Nordic).

RESULTS

Colony stimulation. In four of the five cases, a significant proportion of the fresh CLL cells was found to express IL 2 receptors on the cell surface (Table 1). Culture experiments then disclosed that B CLL colony formation was induced by IL 2 when PHA or TPA was added as well. No colonies were formed in cultures containing IL 2 alone, PHA or TPA alone, or without any of these substances (Table 1). As an exception, TPA alone induced colony growth from the cells of patient No. 4. In all instances, colonies consisted of monoclonal B cells (see below); E rosette tests, and Sudan black B and nonspecific esterase stainings of cells harvested from the cultures were negative, indicating that contaminating T lymphocyte and myelomonocytic growth did not occur. To assess the optimal dose of IL 2 required for colony

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formation, IL 2 was added in increasing amounts (0.05 to 1,000 units) to the culture dishes. In PHA-supplemented cultures, colony numbers (patient No. 1) already reached plateau values at 10 units of IL 2 per dish (Fig 1A). In contrast, a maximal size of colonies was obtained at a higher dose, i.e., 50 units per dish. Further increases of IL 2 concentrations in culture (100 to 1,000 units per dish) reduced the colony size (Fig 1A). Similar results were obtained with the cells of patients No. 3 and 5 in TPA-supplemented cultures. A cell titration experiment performed in the presence of 50 units of IL 2 (optimal growth) revealed a linear relationship between cell numbers plated and colony numbers formed (Fig 1B). This feature makes the colony system suitable for quantitative studies.

The polyclonal B cell mitogen Staphylococcus aureus strain Cowan I was ineffective in the induction of IL 2-stimulated B CLL colony growth either alone or in combination with PHA or TPA.

Immunologic and morphological analysis. Immunofluorescence analysis was performed on the cells after colony formation and the immunophenotypes of the colony cells were compared with those of the preculture cells. These studies revealed that high percentages of colony cells expressed the same immunoglobulins with single light chains in the cytoplasm or on the cell surface as the CLL cells prior to culture (Table 2). Bright cytoplasmic immunofluorescent staining suggested plasmacellular maturation within the CLL colonies. Substantial morphological changes were noted during colony growth in the TPA- and IL 2-stimulated cultures, indicative of the differentiation of CLL colony cells into plasma cells (Fig 2, a2 and b2). In the PHA- and IL 2-supplemented cultures, the majority of colony cells resembled plasmacellular lymphocytes (Fig 2, a1 and b1). In all cases, 15% to 20% of the colony cells expressed IL 2 receptors on their cell surface.

**DISCUSSION**

It has been shown previously that B CLL cells produce colonies in vitro following activation by the plant lectin PHA. Successful culturing of the CLL colonies was dependent on the presence of (irradiated) T lymphocytes. The nature of the stimulatory components produced by the T lymphocytes has remained unknown. Moreover, the question of whether the lectins exerted a direct effect on the clonogenic CLL cells or an indirect influence through the T cells (or both) has not been answered. The data presented in this report show that lectin (PHA- or phorbol ester (TPA)-activated B CLL colony formation occurs in the absence of T lymphocytes (or other stimulator cells) when pure IL 2 was added to the cultures as the single source of stimulation (Table 1). Membrane receptors for IL 2 were found on high percentages of the fresh CLL cells, with the exception of patient No. 5 (Table 1). In addition, such receptors were also detectable on the colony cells, including those of patient No. 5. These results provide evidence that IL 2 in the presence of PHA or TPA directly stimulates CLL proliferation.

In common and pre-B ALL, PHA and TPA were found to induce IL 2 receptors on the cell membrane initially not expressed on the untreated cells. The role of these factors in CLL proliferation is not immediately clear, as high percentages of untreated CLL cells may already express IL 2 receptors (Table 1). Recent results from 3H-thymidine incorperated...
poration experiments suggest that PHA (or TPA) activation of the CLL cells is required for the continuous expression of IL 2 receptors on the proliferating cells in culture from day 3 on (Ivo Touw and Bob Löwenberg, unpublished data).

Morphological analysis revealed maturation of the CLL colony cells. In the TPA-supplemented cultures, colony cells expressed the morphology of plasma cells (30% to 75%) or intermediate plasmacellular differentiation stages (Fig 2a2 and b2). In vitro differentiation of CLL cells induced by TPA has been noted by others.13,14 In the colonies formed in the cultures stimulated with PHA, plasma cell development was less prominent (2% to 5%). Nevertheless, the abundant presence of M and κ or λ immunoglobulin chains in the cytoplasm (Table 2) and the plasmacytoid appearance of the colony cells (Fig 2a1 and b1) indicated that maturation occurred to some extent. High concentrations of IL 2 have recently been reported to induce plasmacellular maturation of normal B lymphocytes.15 We wondered whether higher IL 2 concentrations in PHA cultures would drive CLL colony cells toward further differentiation stages, but morphological analysis of the colony cells grown in the presence of 500 to 1,000 units of IL 2 did not provide evidence for this.

The cells of patient No. 4 were exceptional in that they did not require addition of IL 2 to culture for TPA-activated colony formation (Table 1). This raised the question of whether minimal numbers of residual (E-positive) T lymphocytes had been responsible for the effect through the elaboration of IL 2 into the culture medium. This possibility appears unlikely, since a second E rosette depletion of the CLL cells did not eliminate colony growth in cultures with TPA alone (data not shown). More plausible explanations are that the CLL cells of this patient produced stimulatory components themselves or that their colony-forming abilities do not depend on growth factors. These alternatives are presently under investigation.

To this end, it appears that IL 2 has become established as a growth and differentiation inducer of normal and neoplastic B cells,5–9,15 including CLL colony-forming cells. Notably, CLL cells proliferate in vitro without the need of other growth factors, similar to normal B lymphocytes. The existence of other lymphokines with the capacity to stimulate B cell proliferation, referred to as B cell growth factors (BCGFs), has been postulated in a large series of publications, as recently reviewed.16 In order to understand the putative role of BCGFs in normal and neoplastic B cell regulation additional to IL 2, purification to homogeneity of these activities is now of crucial importance.

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