Steinberg, Joseph

THE CHARACTERIZATION OF IMMUNOREGULATORY FACTOR-MEDIATED GROWTH AND DIFFERENTIATION OF LEUKEMIC B CELLS ISOLATED FROM PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

City University of New York Ph.D. 1987

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by

Joseph Steinberg

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09.20.86

Date

Constantin A. Bona, M.D., Ph.D.

Chair of Examining Committee

4-29-87

Peter C. Chabora, Ph.D.

Executive Officer

Robert E. Calhoon, Ph.D.

Vincent J. Merluzzi, Ph.D.

~~Malcolm A.S. Moore, D. Phil.~~
KARL WELTE, M.D.

~~William R. Benjamin, Ph.D.~~

Chris D. Platsoucas, Ph.D.

ABSTRACT

The Characterization of Immunoregulatory Factor-Mediated Growth and Differentiation of Leukemic B Cells Isolated From Patients With Chronic Lymphocytic Leukemia

by

Joseph Steinberg

Adviser: Professor Constantin A. Bona

The well-documented loss of systemic immunocompetence which arises as a frequent manifestation of chronic lymphocytic leukemia (CLL) is viewed here, in part, as a consequence of binding and sequestration of essential soluble immunoregulatory factors by circulating monoclonal B lymphocytes of leukemic origin. Indirect evidence for this contention is furnished through an *in vitro* functional analysis (i.e., growth and maturation) of isolated neoplastic B cells from a group of 20 patients. Individual essentially pure E rosette-negative largely leukemic B cell lines exhibited highly variable proliferative and differentiative responses when cultured with conditioned medium (CM) prepared from PHA-P stimulated normal peripheral blood mononuclear cells. Without exception, all cell lines exhibited some degree of proliferation in four-day old cultures with CM and the B cell polyclonal activator rabbit anti-human antibody (anti-μ). In addition, of the leukemic lines tested, the most ontologically mature populations as judged by surface immunofluorescence could also be induced to secrete significant

quantities of immunoglobulin (Ig) when cultured for six days in the presence of CM alone. Surprisingly, standard concentrations (10 μg/ml) of $F(ab')_2$ fragments of anti-μ were not stimulatory in every case but instead were able to block or induce growth of several spontaneously proliferating B cell lines in a dose-dependent manner. Costimulation with CM plus anti-μ to a large degree helped alleviate the proliferative inhibition. The response of leukemic cells to the commonly utilized polyclonal activators Staphylococcus aureus Cowan I strain (SAC) and to the phorbol ester TPA alone or in combination with CM was also investigated. In acordance with previous findings, the majority of CLL lines tested, unlike normal polyclonal B cells, were not proliferatively responsive to SAC. In the case of several patient lines, SAC unlike anti-μ was unable to proliferatively synergize with CM suggesting that each polyclonal activator mechanistically exerted its influence differently. TPA added at the initiation of cultures was usually found to effectively synergize with CM; however, in several cases where cells were cultured with CM plus anti-μ, the use of TPA actually reduced the level of proliferation. In addition, when utilized to optimize proliferation in preparation for karyotypic analysis, TPA produced mitotic figures of insufficient quality containing chromosomes which were poorly dispersed and highly contracted. By comparison, recombinant IL-2 in parallel experiments when substituted for TPA yielded higher quality mitotic figures indicating that the suboptimal mitotic effects were TPA-specific.

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I wish to convey my deep appreciation for the wisdom and guidance which I received from my sponsor, Dr. Constantin A. Bona, and reserve special thanks to Dr. Chris D. Platsoucas and Dr. M.A.S. Moore for freely lending their expert knowledge and providing me with the encouragement, as well as physical resources, which enabled me to accomplish my task.

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INTRODUCTION

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most prevalent lymphoproliferative disorder among individuals reaching middle age (for reviews, see Rai and Sawitsky, 1981 and Gale and Foon, 1985). Disease incidence is not confined exclusively to this age group, however, since positive diagnoses have been reported in younger individuals (Spier et al., 1985; Rewald et al., 1985; our data). Nonetheless, as an age-related disorder, there exists the expectation that the number of new cases will keep pace with the growing proportion of aged within society. Patients typically present with large numbers of small, monotonous, lymphoid-like cells in the general circulation and as infiltrates of the lymphoid organs including the spleen, various lymph nodes and the bone marrow. Ample evidence exists demonstrating that the leukemic cell populations are composed of B lymphocytes, each arising from an individual clone (Preued'homme and Seligman, 1972; Aisenberg et al., 1973; Rundles and Moore, 1978; Solombi et al., 1982). Hence the disease is commonly designated BCLL. The circulating cells generally express low but variable quantities of surface immunoglobulin (Ig) including mu (μ) or μ and delta (δ) heavy chains in association with either kappa or lambda light chains. The presence of a single light chain isotype on the vast majority of cells comprises a valid means of establishing monoclonality. A far smaller proportion of leukemic clones may express the IgG isotype while some clones do not appear to express any detectable surface Ig (Kimby et al., 1985). Leukemic cells also

typically bear receptors for mouse erythrocytes, receptors for the Fc portion of IgG, complement receptors, particularly C3d, recognized by the monoclonal antibody (MoAb) B2, the Ia antigen, as well as several common B cell antigens including B1 and B4 (Gale and Foon, 1985). Conversely, CLL lymphocytes are unusual in that they display certain T cell characteristics not generally associated with B cells. These include altered surface glycoprotein profiles (Brown et al., 1985), T1 antigen expression on circulating leukemic cells (Wang et al., 1980; Martin et al., 1981; Plater-Zybeck et al., 1985), expression of the T cell marker dipeptidyl peptidase IV (Gotze et al., 1985), and the ability to express endogenous E rosette receptors, although recent observations indicate this characteristic may extend to normal B cells as well (Mills and Cowley, 1985).

Since CLL can manifest a relatively stable course with rates of survival often exceeding 10 years, it is considered a low-grade malignancy by many practioners. However, patient prognosis is essentially independent of therapy such that survival has not been substantially improved. In addition, sudden transformation of clinical features can lead to rapid mortality which often results after an overwhelming bout of infection. Even among patients who manifest a stable course, a high degree of morbidity as a consequence of greatly compromised systemic immune function must often be tolerated. Indeed, generalized immunopathogenesis is a hallmark of CLL. Therefore, the maintenance of a satisfactory degree of immunocompetence among patients is of overriding concern in the clinical management of this disease.

Due to wide variations and uncertainties in prognosis, a number of systems of classification have been devised in an attempt to categorize patients according to disease severity and clinical stability. Various schemes based upon laboratory or clinical criteria have been created. Falling into the former category, classifications based on lymph node morphology, cytochemical characteristics and levels of immune responsiveness have been proposed (Rappaport, 1966; Lukes and Collins, 1977; Lennert, 1987; Vitale et al., 1985). Staging procedures based on tumor-related characteristics such as volume, morphology, and location as well as secondary effects, such as the degree of anemia and thrombocytopenia, have been commonly employed (Rai et al., 1975; Binet et al., 1981). Staging procedures, in particular, have proven of some utility for indicating modalities of therapy but, in general, these schemes have failed to provide satisfactory criteria for gauging disease prognosis or progression, particularly in newly diagnosed cases.

As indicated, general immune impairment is a major contributor to morbidity in CLL and affects both humoral and cell-mediated immune function. The presence of increased numbers of circulating lymphocytes is rather paradoxically accompanied by marked reductions in serum antibody levels (Rubartelli et al., 1983). The resultant hypogammaglobulinemia extends to all classes of Ig, and is indicative of generalized B cell dysfunction. In vitro studies of leukemic lymphocytes have uncovered a plethora of functional and structural defects. It has been demonstrated, for example, that leukemic cells generally do not secrete Ig in the presence of mixed lymphocyte cultures even when incubated with allogeneic Ia-matched T cells

(Halper et al., 1979). Additionally, irregularities in Ig synthesis (Nies et al., 1974) and structural defects in secreted Ig (Nies et al., 1976) have been reported. More recently, cell membrane peculiarities have been uncovered following treatment with the fluorescent dye merocyanin 540 (Valinsky et al., 1982) and the sodium-independent amino acid trans-membrane transport system or L-system has also been shown to be markedly impaired in leukemic cells (Segel et al., 1985). Fluorescent microscopic and flow cytometric observations of weak membrane immunofluorescence patterns following phenotypic staining of surface Ig with appropriate MoAbs (Ligler et al., 1983; Silber et al., 1984), Kimby et al., 1985) constitute yet another example of aberrant membrane patterns. Caligaris et al. (1986), studying the organization of actin-containing microfilaments and vimentin-containing intermediate filaments, concluded that cytoskeletal modifications present in leukemic cells might also be a useful indicator of malignant transformation. In addition, when compared to normal circulating B cells, leukemic cells have been shown to display impaired mitogenic responsiveness, faulty cap formation following attachment of multivalent ligands, decreased levels of tocopherol and the ectoenzyme 5' nucleotidase while demonstrating elevated levels of ascorbic and dehydroascorbic acid (Silber et al., 1984). Reports of low levels of vitamin-D-binding protein, which normally forms a close spatial relationship with surface-bound Ig and which may affect Ig mobility through the cell membrane lipid bilayer (Nel et al., 1985), might account for the poor Ig capping ascribed to leukemic cells.

Irregularities, attributed to T cell dysfunction in CLL, have been the subject of intense interest over a prolonged period (Chiorazzi et al., 1979; Kay, 1981; Keller et al., 1981; Fernandez et al., 1981; Ham et al., 1981; Lauria et al., 1983; Herrmann et al., 1983; Bloem et al., 1985). The most common clinical manifestations suggestive of altered T cell function have included impaired delayed hypersensitivity responses and increased susceptibility to viral infections (Rai and Sawitsky, 1981). Although impaired T cell function is widely acknowledged, its causation and specific manifestations remain controversial. Published submissions supporting decreased helper cell activity, increased suppressor cell activity, both of these and neither of these functional states have been reported (Faguet, 1979; Hensey et al., 1980; Kay, 1981; Fauci et al., 1977; Hans and Daley, 1979; Fernandez et al., 1986). Several recent reports have done little to help resolve the arguments concerning the effectiveness of T cell-mediated suppression in patients with CLL. Bloem et al. (1985) have recently chronicled a lack of antigen-specific suppressor activity in T suppressor cells isolated from patients and incubated in the presence of functional helper T cells. This finding could have a bearing on the prevalence of autoimmune disorders associated with this disease. On the other hand, Mangan and D'Alessandro (1985) noted a gradual accumulation of suppressor lymphocytes in the bone marrow of affected individuals which they correlated with a concomitant decrease in the cell growth of erythroid progenitors. This cellular expansion (T cell) and lowered CFU-E and BFU-E frequencies were noted at the earliest Rai disease stages.

The present state of confusion concerning the role of T cells in the pathogenesis of CLL has been further heightened by an inability to distinguish between dysfunctions due to altered cell numbers (quantitative effects) as opposed to possible intrinsic T cell defects (qualitative effects). Evidence favoring the former possibility has been obtained from lymphocyte surface marker studies. It has been well substantiated that T lymphocyte subset proportions are dramatically altered in this disease. When panels of lymphocyte class and subclass-specific MoAb reagents have been employed, in addition to the dramatic reversals noted in overall patient T cell/B cell ratios, major alterations have also been observed in distributional patterns between T cell subsets (Matutes et al., 1984; Kanter et al., 1982; De-Paoli, 1984). These have typically manifested themselves by dramatic decreases in the proportions of helper T cells (T4+) and concomitant increases in the proportion of suppressor T cells (T8+). While T8+ cells tend to be more numerous in the general circulation, T4+ cells continue to predominate in the bone marrow (De-Paoli, 1984), although the number of T8+ cells, as previously noted, also increases. It is noteworthy that the absolute number of T cells (T4+ and T8+) is higher in patients than in normal subjects (Wybran et al., 1973; De-Paoli, 1984).

Additional indirect evidence lending support to the importance of quantitative T cell changes has been advanced by Johnstone and coworkers (1982). Utilizing unfractionated normal lymphocytes, they demonstrated that rarification of the number of T cells, accomplished through a series of serial dilutions to abnormally low levels, resulted in the cells displaying reduced uptake of ^{3}H-thymidine and

delayed peak incorporation times when exposed to appropriate mitogens. This degree of lessened activity was reminiscent of T cells derived from CLL patients which were subjected to the similar conditions of stimulation.

T cell defects of a qualitative nature have been more elusive to detect. Lending partial support to the notion of an intrinsic T cell defect has been the observation that some T lymphocytes from CLL patients bare novel T4+8+ (doubly marked) phenotypes (Platsoucas et al., 1982). The significance of this finding remains unclear however.

Soluble Immunoregulatory Factors

While it may be argued that active cell-mediated suppression of a wide variety of immune functions is a plausible and perhaps inevitable feedback response to the massive leukemic B cell proliferations which are a consequence of CLL, this explanation by no means exhausts the likely possibilities; particularly in light of recent evidence which indicates that despite numerical increases in both relative and absolute terms, suppressor T cells isolated from patients may be biologically unresponsive (Bloem et al., 1985; Fernandez et al., 1986.

An alternative interpretation can be proposed which attributes causation of immune dysfunction in CLL to deficiencies in the levels of certain crucial immunoregulatory factors. It has been widely acknowledged for some time that the transformation of resting B lymphocytes into plasma cells is in part dependent upon a set of externally derived signals. Following activation, the subsequent

proliferation, maturation and differentiation of precursor B lymphocytes comprises a complex sequale which has recently been the subject of intensive investigation. Events leading to the secretion of Ig require the participation of at least three classes of hemopoietic cells - helper T lymphocytes, monocytes or accessory cells, as well as B lymphocytes (for a review, see Melchers and Anderson, 1984). While the induction of this process often requires a degree of physical contact leading to cognition between cells such as might occur, for example, during the presentation of foreign antigen (Oppenheim et al., 1968), evidence has accumulated over time which underscores an essential role for the involvement of certain antigen-nonspecific factors (i.e., lymphokines) in the triggering of B cell activation events. These soluble substances comprise a broad category of small hormone-like secretory proteins which include such functionally disparate substances as the interleukins, colony stimulating factors, macrophage modulating factors, the interferons and various chemotactic factors (Smith, 1984).

Until fairly recently, substances of non-human origin from a wide variety of biological sources were utilized to activate polyclonal B cell populations. In vitro triggering of B cells with lectins such as pokeweed mitogen (PWM) (Waldmann et al., 1974; Fauci et al., 1976; Fauci and Ballieux, 1979), or Epstein-Barr virus (Kirchner et al., 1979) and Nocardia water-soluble mitogen (Bona et al., 1979) represent several examples. However, in order to understand the nature of the humoral immune response more fully, closer approximations to physiological conditions have been sought.

The utilization of autologous or allogeneic soluble products of hemopoietic origin in the elucidation of B cell responses has recently yielded results at an increasingly rapid pace. An early indication that B cells might require external help in order to secrete antibody came as a result of the work of Mosier (1967) who demonstrated a requirement for T cells in mixed cultures. Later Dutton et al. (1971) and Schimpl and Wecher (1972) made the profound discovery that antibody formation could be induced by culturing B cells in the presence of cell-free supernatants obtained from mitogen or alloantigen stimulated T cell cultures. The activity present within such supernatants was ascribed to a substance or substances referred to as T cell replacing factor or TRF (Schimpl and Wecher, 1975). It was soon recognized that the early signal promoting B cell proliferation (Kishimoto et al., 1975) could be distinguished from triggering of Ig secretion by the later-acting TRF (Schimpl and Wecher, 1972). Thus B cell growth and differentiation were seen to be temporally separable. As a natural consequence, these events were postulated to be under the control of a putative B cell growth factor (BCGF) and a separate B cell differentiation factor (BCDF).

Originally, the pace of research into the role of B cell-active lymphokines was hampered by their unavailability in purified form. Recently, several factors possessing B cell activity have been isolated and partially characterized. However, additional difficulties quite apart from the inherent complexity of the differentiation process itself have arisen or remain to be resolved. Among them have been i) the identification and sequence of action of the physiologically most important lymphokines from among the bewildering

array which have been described to date; ii) the unavailability of long-term cultures of normal target B cells, and iii) the utilization of B lymphocyte isolates sufficiently free of cellular cross-contaminants.

Although much progress has been made, the identity of the factors which play a role in the early, mid and late stages of B cell maturation have not been fully resolved. Generally, supernatants from lectin-stimulated T cell cultures have constituted a primary source of B cell-active factors (Sredni et al., 1981; Benjamin et al., 1984); however, results obtained utilizing crude preparations possessing multiple activities have been difficult to interpret. Cell fusion experiments yielding human T cell hybridomas producing a more restricted range of secretory products have been reported (Okada et al., 1983; Butler et al., 1983); but, unfortunately, such hybridomas, unless carefully subcloned on a regular basis, have invariably been unstable, and have quickly ceased producing active factors due to chromosomal exclusion (J.J. Farrar, personal communication). The partial purification of putative BCGF had been reported by a number of investigators (Yoshizaki et al., 1983; Howard et al., 1982; Okada et al., 1983; Maizel et al., 1983; Paul, personal communication, 1985). Maizel and co-workers (1983) demonstrated that the activity present within their preparation was protease (trypsin) sensitive. Additionally, they found this activity could be abolished by heating to 56°C for 30-60 min. Investigations to determine the isoelectric point of BCGF yielded values ranging from 6.3 to 6.9 (Yoshizaki et al., 1983; Howard et al., 1982; Okada et al., 1983; Maizel et al., 1983), which served to distinguish it from T cell

growth factor (IL-2, IE8.2) (Smith, 1984). Molecular weight estimates derived from gel filtration analysis initially yielded results between 12 Kd and 25 Kd (Yoshizaki et al., 1983); Maizel et al., 1983; Muraguchi and Fauci, 1982; Butler et al., 1983). This range of values was similar to molecular weights reported for human IL-2 (14 Kd) (Smith, 1984) and murine interleukin-1 (IL-1, 14 Kd) (Mizel and Mizel, 1981). Although the biological activities of the various interleukins appear to be physically separable with reference to B cells, the similarities in their molecular weights suggest the possible existence of a degree of sequence homology between them. This, however, has not been borne out.

Most recently, a report detailing the purification to homogeneity of a murine BCGF has been published (Mehta et al., 1985). Biochemical characterization has revealed the protein to have an approximate molecular weight of between 12 and 14 Kd with a major isoelectric point at 6.5. In addition, BCGF appears to be derived from a 60 Kd precursor molecule (Sahasrabuddhe et al., 1984), which was demonstrated by the existence of a 17S mRNA that codes for a biologically-active BCGF. This factor in all probability corresponds to the interleukin commonly known as BCGF1 [or currently B cell-stimulating factor 1 (BSF-1) (Oliver et al., 1985; Rabin et al., 1985). When cultured in the presence of both anti-Ig (or specific antigen) and BSF-1, resting G_o B cells are induced to enter the cell cycle. It has been suggested that this lymphokine acts during the first 12 hr of culture in the presence of anti-Ig (Howard et al., 1982). The initial view held that BSF-1 acted only after resting B cells were preactivated by a mechanism involving cross-coupling of

their surface-expressed Ig (Howard and Paul, 1983; Kehrl et al., 1984). However, several studies utilizing BSF-1 containing supernatants (sup) or partially purified BSF-1 demonstrated that resting B cells subjected to stimulation with BSF-1 alone were observed to swell and express Ia antigens but did not enter the cell cycle (Noelle et al., 1984; Roehm et al., 1984). The current interpretation maintains that murine BSF-1 activates resting B cells which are then rendered receptive to stimulation by specific antigen or anti-Ig antibodies (Oliver et al., 1985; Rabin et al., 1985).

In order for B cell growth to proceed through S phase an additional growth factor referred to as BCGFII is apparently required (Swain and Dutton, 1982). This later-acting T cell-derived factor is distinguishable from BSF-1 by its higher molecular weight (45 Kd) (Okada et al., 1983) and does not co-stimulate with anti-Ig (Okada et al., 1983; Swain et al., 1983) but does require its prior presence. It is worthy of note that this lymphokine, as has been recognized of so many others, possesses multiple activities. In this case, BCGFII of murine origin also apparently stimulates differentiation of mouse eosinophils (Sanderson et al., 1986).

B cell differentiation, as has already been pointed out, is considered to be under the control of a different factor(s) (i.e., historically TRF and presently BCDF). Previous studies have demonstrated that factors inducing Ig secretion in Epstein-Barr virus-transformed B cell lines were present in the culture supernatants of a human T cell hybridoma (Okada et al., 1983) or T cells stimulated with various mitogens (Muraguchi et al., 1981; Teranishi et al., 1982; Yoshizaki et al., 1982; Hirano et al., 1984). This activity

was termed TRF (Muraguchi et al., 1981; Teranishi et al., 1982), BCDF (Okada et al., 1983; Yoshizaki et al., 1982), or BCDF-II (Hirano et al., 1984). Several experimental results indicated that BCDF acted upon B cells during the final stages of maturation leading to antibody secretion and appeared to be the human equivalent of murine TRF as originally reported by Schimpl and Wecher (1972). Several lines of evidence lent support to the contention that this factor(s) was responsible for the induction of plasma cell formation. i) BCDF induced IgG or IgM secretion in Epstein-Barr virus transformed human B cell lines without stimulating cell growth (Muraguchi et al., 1981; Teranishi et al., 1982; Saiki et al., 1983); ii) BCDF promoted an increase in the biosynthesis of secretory Ig heavy cahins as well as in their mRNAs (Kikutani et al., 1985), and iii) this factor induced IgM and IgG secretion in Staphylococcus aureus Cowan 1 (SAC)-activated normal B cells (Okada et al., 1983; Hirano et al., 1984; Teranishi et al., 1984).

Yoshizaki et al. (1983), for example, reported the isolation by chromatofocusing of a 50 Kd protein fraction obtained from a PHA/-phorbol myristate acetate (PMA)-stimulated T cell-conditioned sup which acted synergistically with a 17 Kd BCGF fraction (probably BSF-1) to induce Ig secretion in various B cell targets. Most recently, Hirano et al. (1985) have succeeded in purifying to homogeneity a 19 to 21 Kd protein which induced terminal B cell maturation without fostering cell growth. Past work by Isakson et al. (1982) suggested that there, might in fact, be several distinct BCDF's, each corresponding to a particular Ig class and, furthermore, that switching of secretion to the new Ig class was dependent upon the presence of the

appropriate BCDF. They succeeded in identifying a T cell hybridoma as well as two IL-2-dependent T cell lines, which following stimulation by Con-A, produced factors which induced IgG secretion in surface Ig^- LPS-primed normal murine B cells. All other hybridoma and T cell lines tested produced BCDF, which promoted the secretion of IgM exclusively in the same targets. Thus, there may exist a separate BCDFμ and BCDFγ. Lending support to this contention is the fact that in addition to BCDF, IL-2, interferon and BSF-1 have been regarded as possessing differentiation activity (Leibson et al., 1984; Zubler et al., 1984; Nakagawa et al., 1985).

Until the most recent definitive results were published, the role of IL-2 in the induction of Ig secretion was the subject of some debate. It is well known, for example, that PHA-stimulated T cells produce sups containing joint IL-2, BCGF and BCDF activities. Initial studies had indicated that IL-2 could exert a direct effect on B cells and its presence was considered a necessary precondition for the induction of Ig secretion (Swain et al., 1981). This scheme was challenged (Howard et al., 1982) after it was determined that IL-2 activity could be chromatographically separated from BCGF activity. Additionally, these investigators reported that the IL-2-free fractions so obtained were capable of supporting Ig secretion while IL-2 containing fractions did not. The work of Tsudo and co-workers (1984) helped to reestablish the importance of IL-2 as a direct B cell activator. They demonstrated the expression of Tac antigen (i.e., IL-2 receptor) (Leonard, 1982) on prestimulated normal human B cells. They also found that the addition of exogenous IL-2 to target cell cultures resulted in increased Ig secretion and, furthermore,

that this effect could be blocked by preincubation with anti-Tac MoAb. A large number of studies, following in quick succession, confirmed the ability of IL-2 to promote either proliferation, differentiation or both in prestimulated B cells (Waldmann et al., 1984; Mingari, 1984; Jung et al., 1984; Waldmann et al., 1984; Muraguchi et al., 1985; Mittler et al., 1985; Boyd et al., 1985). With the establishment of the direct modulation of B cells by IL-2 in addition to its well-defined role as a T cell modulator, the natural target cell population of this lymphokine was expanded and these results further underscored the multiple affinities of many lymphokines.

The study of antigen-nonspecific directed maturation of leukemic B cells has proceeded in parallel with observations on normal B cells. The utilization of monoclonal B cell targets has yielded much valuable information which could be extrapolated to normal B cells by obviating the effects of polyclonality in test systems. Several studies have emphasized that CLL cells in contradiction to previous thinking could be driven to differentiate into plasma cells in vitro (with or without accompanying proliferation) following triggering by certain mitogens (Robert et al., 1979; Guglielmi et al., 1980; Tott[illegible]an et al., 1980). Terminal differentiation of CLL lymphocytes with BCDF-rich preparations had been achieved in a number of instances (Fu et al., 1978; Yoshizaki et al., 1982; Okada et al., 1983; Fu, 1982). The patients studied in each case were few and they were chosen on the basis of demonstrated monoclonal Ig in their serum indicating that their leukemic cells were actively engaged in secreting Ig in vivo. Most patients, however, do not exhibit such in

vivo patterns and it was not apparent whether their cells could be driven to differentiate.

Steinberg et al. (1985), utilizing 12 randomly selected patients, demonstrated that highly purified leukemic cells often proliferated vigorously upon stimulation with T cell-conditioned media (CM) or recombinant IL-2 (rIL-2) when cocultured with $F(ab')_2$ fragments of anti-mu antibody (anti-μ). These results were also reported by Lantz et al. (1985) who observed that anti-μ plus rIL-2-induced proliferation could take place independently of other factors, and that this proliferation peaked late (6 days on average). Rubartelli and co-workers (1985) have demonstrated that PMA prestimulated CLL clones could be driven to differentiate in the presence of CM. They concluded that the ability to differentiate in vitro was dependent upon the stage of maturation reached by leukemic cells in vivo.

Kabelitz et al. (1985) found that activation of CLL cells was a necessary precondition for Tac antigen expression - unstimulated leukemic cells did not respond to rIL-2. Maximal stimulation (proliferation and differentiation) was achieved if the activator (PMA) was left in culture. Whether IL-2 promotes differentiation in the absence of proliferation as does purified BCDF remains an open question; however, there is little doubt that it acts as a late-functioning TRF which requires the prior expression of Tac. Mayer et al. (1985), for example, found that the addition of IL-2 to B cell cultures could be delayed for up to 72 hr without affecting the degree of leukemic cell differentiation as indicated by the number of plaque-forming cells produced.

Since leukemic cells apparently express receptors for various T cell-derived factors, these lymphocytes have been studied to determine the range of T cell-secreted products bound. CLL lymphocytes have been shown to effectively adsorb exogenous BCGF from partially purified preparations (Yoshizaki et al., 1982) and to rapidly remove IL-2 activity via their Tac receptors (Foa et al., 1985). This had led to suggestions that neoplastic B cells may act to make unavailable certain essential T cell-derived lymphokines in vivo and comprises an alternative explanation to the argument that lowered immunocompetence observed in this disease is the result of active T cell suppression or lack of helper T cell activity.

Lymphokine Deficiency Model

A scheme is presented which ascribes the overall diminution of immune status in CLL to effector lymphocyte hyporesponsiveness brought about by systemic deficiencies in the levels of certain T cell-derived immunoregulatory factors including BSF-1, BCGFII, BCDF and IL-2. The model has the following features:

i) It is robust in the sense that it accounts for dysfunction in both the humoral or cell-mediated arms of the immune system.

ii) It does not require the invocation of intrinsic cellular defects beyond those confined to the malignant clone.

iii) It partitions primary effects directly attributable to malignant cells (i.e., tumor load) from secondary effects impacting negatively upon normal lymphocytes.

iv) It predicts that activation of leukemic clones, as a result of autocrine effects or specific antigen recognition, will lead to truncated survival rates.

v) It predicts a return to normal immune function following the addition of extraneous soluble factors or following restoration of normal lymphocyte subset proportions.

While it is clear that impaired immune function is associated with increased lymphocytosis in untreated patients (Rai and Sawitsky, 1981), the role of proliferating clones in this process remains unclear. The model's central assumption rests on the premise that under certain conditions malignant lymphocytes express receptors for B cell and T cell immunoregulatory factors. Thus, as a result of massive population densities, CLL lymphocytes may effectively act as sinks which sequester available T cell-derived factors. In the likely event that lower-than-normal levels of these factors are produced by the helper/inducer T cell population (although see Foa et al., 1985), the extent of the lymphokine deficiency and its effects could be expected to be further compounded. It may be recalled that while T cell levels in absolute terms are usually elevated in CLL (Wybran et al., 1983); De-Paoli, 1984), T4+ cell proportions are drastically lowered (Matutes et al., 1981; Kanter et al., 1982; De-Paoli, 1984).

With regard to lymphokine production and utilization, the possible deleterious effects caused by changes in lymphocyte population proportions could arise in two distinct ways - between class and within class differences. 1) Proportional changes between lymphocyte classes are the direct result of neoplastic B cell expansion with

concomitant decreases in the overall percentage of T cells. This might result in shortages of a whole constellation of T cell-derived factors adversely affecting the function of both normal B and T cells. 2) Proportional changes within lymphocyte classes would arise from increases or decreases in the levels of one or a number of lymphocyte subsets. Thus, inversion of T cell ratios, which is a common characteristic of CLL, could have an attenuating effect upon lymphokine production by T4+ cells due to increased numbers of presumably active T8+ cells. Conversely, outgrowth of malignant B cells vis-a-vis polyclonal B cells could be expected to adversely impact lymphokine availability. Either outcome would be expected to have far-reaching consequences with regard to the maintenance of normal immune function in patients.

CLL: Cytogenetics

With the recognition that malignant processes originate in the genes of host cells, studies of leukemic lymphocytes have been undertaken to uncover possible chromosomal aberrations. Early attempts to induce cell division in leukemic cells were generally unsuccessful following use of T cell-specific mitogens such as PHA. Later, after the identification and utilization of somewhat more effective B cell-specific polyclonal activators (Robert, 1979), the capacity to identify abnormal chromosomal patterns associated with this disease was realized (Hurley et al., 1980; Nowell et al., 1981; Morita et al., 1981; Katz et al., 1982; Karl-Henrich et al., 1982; Vahdati et al., 1983). Unlike other leukemias, such as CML, CLL was not amenable to characterization by a single specific chromosomal marker.

Instead, a number of numerical and structural defects were uncovered in more than 50% of the cases reported. The varied nature of these defects, therefore, should give pause to labeling this disease a single entity and more discriminating systems of classification based on genotype may be required.

Numerous studies have verified that the most common numerical chromosomal abberation is a trisomy of chromosome 12 (Gahrton et al., 1979, 1980, 1981; Morita et al., 1981; Karl-Henrich et al., 1982; Han et al., 1984). Studies of karyotypes obtained following mitotic induction with EB virus have revealed the incidence of trisomy 12 to be associated with more aggressive disease and poorer prognosis (Karl-Henrich et al., 1982). Robert et al. (1982) and Juliusson et al. (1985) arrived at similar conclusions and proposed that individuals presenting with this karyotype required earlier treatment.

The occurrence or co-occurrence of abnormalities involving chromosomes 1, 3, 11, 13 and 14 have also been reported (Pittman and Catovsky, 1984). These nonrandom chromosomal aberrations appear unrelated to therapy as they are evidenced in both treated and untreated patients. By a wide margin the most commonly encountered structurally abnormal chromosome is 14, which has been found to be present in at least 50% of all cases in which it was sought. Defects of the 14q+ chromosomal region were originally described by a number of investigators (van den Bergh et al., 1979; Autio et al., 1979; Nowell et al., 1981; Gahrton and Robert, 1982; Valdati et al., 1983; Han et al., 1984) (for a recent review, see LeBeau, 1986).

The most frequently involved locus corresponds to 14q32 which occupies a portion of the site of the Ig heavy chain genes (Croce et al., 1979). Most commonly, this abnormality, lying within the 14q+ region, arises from a translocated portion of chromosome 11. Recently, the chromosomal breakpoints (14q32.3 and 11q13) associated with the 11, 14 translocation have been cloned (Tsuzimoto et al., 1984) and have yielded the surprising result that none of the presently described oncogenes is associated with the breakpoint regions. This previously unknown locus has been designated bcl-1 by Croce and co-workers and appears to originate within a highly localized sequence on chromosome 11 which translocates to the J4-DNA segment on chromosome 14. Its mechanism of action may be to alter in some fashion the V-D-J joining of the immunoglobulin heavy chain gene segments. The ultimate effect on mortality of this genetic alteration is not completely clear, although its presence appears to be linked to shortened survival (Pittman and Catovsky, 1984). In general, there seems to be a consensus that complex karyotypes, especially those including +12, and high percentages of abnormal metaphases predispose toward more rapid mortality (Han et al., 1985; Juliusson and Gahrton, 1985).

AIMS

i) To characterize the proliferative and differentiative responses of highly purified CLL lines from a range of patients which have been induced with the aid of allogeneic T cell-conditioned media (CM) rich in soluble immunoregulatory factors including BSF-1, BCGFII, IL-2 as well as B cell differentiation factors.

ii) To utilize leukemic lymphocytes, where possible, to model certain features of B cell maturation.

iii) To attempt to subclassify leukemic lymphocytes on the basis of correlates between the above functional parameters and the well-documented but variable genetic aberrations reported from analyses of patient karyotypes.

CHAPTER 1

INDUCTION OF PROLIFERATION AND DIFFERENTIATION OF LEUKEMIC B CELLS FROM PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA BY ANTI-μ ANTIBODY AND T CELL CONDITIONED MEDIUM

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is usually a malignancy of B cell origin characterized by the progressive accumulation of small lymphocytes derived by monoclonal expansion (Aisenberg and Block, 1972; Preud'homme and Seligman, 1972; Aisenberg et al., 1973; Fu et al., 1974; Gale and Foon, 1985). Leukemic B cells from patients with CLL express on their surfaces immunoglobulins of monoclonal origin usually of μ and/or δ heavy chain and either κ or λ light chains. This immunoglobulin is usually present in lower density than in normal B lymphocytes (Aisenberg and Block, 1972; Preud'homme and Seligman, 1972; Aisenberg et al., 1973; Fu et al., 1974; Gale and Foon, 1985; Kubo et al., 1974; Foulis et al., 1973; Chen and Heller, 1978; Ligler et al., 1983; Baldini et al., 1985). Leukemic B cells also express Ia antigens (Winchester et al., 1975; Humphreys et al., 1976), B cell differentiation antigens, including B1, B2, B4 and others (Gordon et al., 1983; Knowles et al., 1984; Anderson et al., 1984), Fc receptors (Dickler and Kunkel, 1972; Gupta et al., 1979; Platsoucas et al., 1980) and complement receptors (Ross et al., 1973). Leukemic B cells from patients with CLL can be induced to differentiate into plasma cells synthesizing and secreting monoclonal immunoglobulin in the presence of autologous or allogeneic T cells alone (Fu et al., 1978; Saiki et al., 1980) or in the presence of T cells and polyclonal activators (Robert, 1979; Nowell et al., 1981; Platsoucas, 1985).

The proliferation and differentiation of normal resting B cells to cells synthesizing and secreting immunoglobulin involves a number of stages of activation, proliferation and differentiation that are

also regulated by factors produced primarily by T cells (Muraguchi et al., 1981, 1983, 1984; Nakanishi, 1983; Okada et al., 1983; Parker et al., 1979; Howard and Paul, 1983). Polyclonal activators, such as Staphylococcus aureus Cowan 1 (SAC) or anti-immunoglobulin antibodies, are sufficient to activate and drive normal B cells to proliferate in the absence of accessory cells or their factors. Stimulation of resting B lymphocytes with anti-μ is known to provide an early activation signal (Sell and Gell, 1965; DeFranco et al., 1985; Baeker and Rothstein, 1985; Parker, 1975; Sieckmann et al., 1978; Muraguchi and Fauci, 1982) leading to induction of proliferation and/or differentiation in conjunction with T cell-derived factors (Oliver et al., 1985; Rabin et al., 1985; Steinberg et al., 1985; Lantz et al., 1985; Kabelitz et al., 1985).

We report here that highly purified E-rosette-negative largely leukemic B cells from patients with CLL responded by proliferation to T cell-conditioned medium (CM) derived by stimulating human peripheral blood mononuclear leukocytes with PHA or to $F(ab')_2$ fragments of rabbit anti-human μ chain specific antibody (anti-μ). Mixtures of anti-μ and CM synergized to induce a vigorous proliferative response among these cells. In addition, stimulation of these largely leukemic B cell populations with CM alone resulted in induction of differentiation to plasma-like cells, synthesizing and secreting IgM and in certain cases IgG.

MATERIALS AND METHODS

Patients. Peripheral blood from 15 patients with confirmed diagnosis of CLL of the B cell type was provided by the Long Island Jewish Hospital-Hillside Medical Center, New Hyde Park, NY; Memorial Sloan-Kettering Cancer Center, New York, NY and New Rochelle Hospital, New Rochelle, NY. Only untreated patients or those not having had therapy three months prior to testing were included.

Normal Donors. Leukocyte concentrates from normal donors were obtained from the New York Blood Center and processed as described below. Cells isolated from tonsils were obtained from patients undergoing routine tonsillectomy. The tissue was coarsely minced, passed through a sterilized sieve, washed, and the mononuclear cells were isolated on a Ficoll-Hypaque density cushion. Highly purified tonsilar lymphocytes were provided by Drs. Farrar and Benjamin of Hoffmann-LaRoche, Inc.

Media. RPMI-1640 (RPMI) and Dulbecco's phosphate buffered saline (PBS; pH 7.4) were obtained from the Media Laboratory of the Memorial Sloan-Kettering Cancer Center, New York City, NY. The RPMI was supplemented with 2 mM L-glutamine and 80 µg/ml gentamycin (GIBCO, Grand Island, NY). This medium will subsequently be referred to as RPMI-G. RPMI-G medium supplemented with 10% heat inactivated fetal bovine serum (FBS; Sterile Systems, Inc., Logan, UT), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO) and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO) served as culture medium.

Conditioned Medium (CM). CM was produced by stimulation of mononuclear cells obtained from a pool of 50 buffy-coat preparations (American Red Cross, Lansing, MI) with PHA-P (DIFCO Laboratories,

Detroit, MI). Mononuclear cells were isolated by gravity sedimentation through hetastarch. These cells were suspended to 2 x 10^6 leukocytes/ml in serum-free RPMI-G containing 0.1% PHA-P. The supernatant was collected after 4 hr of culture and concentrated approximately 50-fold by ultrafiltration. The CM was stored at 4°C prior to use. This conditioned medium contained B cell growth and differentiation factors as well as other activities (see Appendix), and it was able to induce proliferation and differentiation of normal B lymphocytes to functional plasma cells, synthesizing and secreting immunoglobulin (see Results). It was kindly provided by Drs. Farrar, Benjamin, and Familletti of Hoffmann-LaRoche, Inc., Nutley, NJ.

Monoclonal Antibodies (MoAbs). MoAb reagents were employed to determine the Ig heavy and light chain isotypes of individual leukemic clones. They were also utilized in the final stages of the purification of leukemic B cells. MoAbs directed against cell surface Ig (heavy and light chains) included anti-γ, α, μ, δ, Kappa and Lambda (Coulter, Immunology, Hialeah, FL and Becton Dickinson, Mountain View, CA). Anti-Leu 5 and anti-Leu 9 (Becton Dickinson) MoAbs were utilized for antibody-dependent complement-mediated cell lysis of contaminating T lymphocytes.

Anti-Ig Antibodies. Affinity-purified $F(ab')_2$ fragments of rabbit or goat heterologous antibodies specific for human μ, γ or δ heavy chains were obtained from Cappell, Malvern, PA.

Isolation of B Cells. Peripheral blood mononuclear cells (PBMC) from patients with CLL or normal donors were isolated by centrifugation on a Ficoll-Hypaque density cushion. The PBMC, recovered from the interface, were washed three times with RPMI-G and adjusted to a

concentration of about 3 x 10^8 cells/ml in (37°C) RPMI-G/5% FBS. Adherent cells were removed by passage through Sephadex G-10 (Pharmacia, Piscataway, NJ) columns according to the method of Jerrels et al. (1980). Briefly, 2 ml of the PBMC suspension was allowed to flow into 10 ml of packed Sephadex G-10 which had been pre-equilibrated with RPMI-G/5% FBS. The Sephadex G-10 columns were then incubated for 30 min at 37°C in a humidified atmosphere containing 5% CO_2. Following incubation, the nonadherent cells were slowly eluted from the columns with 10-15 ml of warm RPMI-G/5% FBS. The eluted lymphocytes were washed once with RPMI-G and suspended to a concentration of 2 x 10^7 cells/ml. E-rosette forming cells were in part depleted by rosetting with (2-aminoethylisothioronium bromide (AET)-treated sheep red blood cells. Four ml aliquots of the lymphocyte suspension from the previous step were mixed with 18 ml of the AET-treated SRBC suspensions and 14 ml RPMI-G supplemented with 1.5% human serum albumin (25% UPS: Armor Pharmaceutical Co., Kanhake, IL). The lymphocyte suspensions were incubated for 15 min at 37°C, centrifuged at 450 xg for 5 min at 4°C and incubated on ice for 1 hr. The pellets were then gently resuspended and the E^+ cells were removed by centrifugation through Ficoll-Hypaque. E rosette-negative cells (E^-) were collected from the interface, washed with RPMI-G and resuspended to a concentration of 2 x 10^7 cells/ml. The procedure was repeated a second time. In order to insure complete depletion of E^+ cells, E^- suspensions were mixed with anti-Leu 5 and anti-Leu 9 MoAbs at a concentration of 10 µg of MoAb 20 x 10^6 cells/ml in RPMI-G. Following incubation at 4°C for 30 min, the cells were washed with RPMI-G and resuspended in a 1:8 dilution of reconstituted normal

rabbit complement (Cedarlane Laboratories, Hamby, Ontario) containing 10 µg/ml DNase I (Sigma, from beef pancreas). Following incubation at 37°C for 45 min, the cells were collected from Ficoll-Hypaque density cushions, washed and resuspended in culture medium. After isolation, cell viability was generally found to be greater than 90% as judged by trypan blue dye exclusion. E-rosette negative cells prepared by this method were completely devoid of T lymphocytes as determined by: (1) rerosetting with AET-treated SRBC; (2) immunofluorescence analysis using an Ortho Model 30L cytofluorograph (Ortho, Westwood, MA) after staining with anti-Leu 5, OKT4 and OKT8 MoAbs (Ortho); (3) lack of a proliferative response to PHA-P. All these criteria proved negative, demonstrating that these purified E^- cells were devoid of T cells. In contrast, unseparated cells from patients with CLL responded vigorously to PHA-P.

Cell Surface Immunofluorescence. Aliquots of 1×10^6 lymphocytes were incubated with 200 µl of the appropriate monoclonal antibodies (1/100 to 1/200 dilution) in PBS for 30 min at 4°C. The cells were washed twice with 1 ml of PBS containing 2% FBS and 0.01% sodium azide (Sigma), centrifuged and resuspended in 200 µl of a 1/50 dilution of FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse Ig antibody (GAM-FITC, Coulter Immunology). Following incubation for 30 min at 4°C, the cells were washed three times with 1 ml aliquots of PBS containing 0.01% sodium azide, resuspended in 1 ml of medium and filtered through fine nylon mesh (40µ) in preparation for enumeration by flow microfluorimetry.

Flow Microfluorometric Analysis. Microfluorometric analysis of lymphocytes was performed on an Ortho Model 30L cytofluorograph

(Ortho, Westwood, MA). Fluorescent cells were scored as the percentage above the highest channel registering background fluorescence when cells were stained with mouse nonspecific Ig (irrelevant antibody) of the class (IgG_1, IgG_{2a}, or IgM) corresponding to the isotype of the MoAb of intent, followed by staining with GAM-FITC. In addition to phenotyping lymphocytes, the purity of isolated B cells was ascertained by staining cells with the T cell-specific MoAbs OKT4, OKT8 (Ortho Diagnostic, Raritan, NJ) and Leu-5. In all cases, following purification, the percentage of B cells was found to be greater than 95% and the percentage of T and adherent cells was judged to be far less than 1% each.

Proliferative Responses of B Cells. Purified E-rosette negative cells from patients with CLL or normal donors were adjusted to a concentration of 2×10^6 cells/ml in culture medium. The cells were cultured in 96-well, flat bottom, microtube plates (Linbro, McClean, VA) at a density of 200,000 cells/well. Various concentrations and combinations of CM and anti-Ig (anti-μ, anti-δ and anti-Y) were used. Each reagent with the exception of CM (100 μl/well) was added in 10 μl/well aliquots. The final volume per well was adjusted to 0.3 ml with culture medium and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 96 hr. Thymidine incorporation was utilized as an index of proliferative activity. Individual wells were pulsed with 1 μCi of [^{3}H]thymidine during the last 16 hr of incubation. The pulsed cells were collected on glass fiber filters (Whatman Ltd., Maidstone, UK) with the aid of an automatic cell harvester (Cambridge Technology, Inc., Cambridge, MA),

and the amount of label incorporated was measured by liquid scintillation counting.

Immunoglobulin Synthesis and Secretion and Lymphokine-dependent Morphological Changes. Leukemic cells were cultured under conditions similar to those described in the preceding section for a period of 6 days in the presence of various reagents. Following culture, the 96-well, flat bottomed plates were centrifuged at 400 xg for 4 min and 200 µl of the supernatant from each well was carefully withdrawn and stored at -20°C until assayed for secreted Ig. Following stimulation, a number of lymphocytes from patients were collected, pooled from triplicate wells and washed several times with PBS. Approximately 1 x 10^5 cells in 0.1 ml PBS were cytocentrifuged (cytospin, Shandon Southern, Runcom, England) for 8 minutes, placed onto glass slides, and stained with Wright's stain. The samples were examined by light microscopy and photomicrographed.

Determination of IgM and IgG by Enzyme-linked Immunoabsorbent Assay (Elisa). These determinations were carried out by a modification of a method originally published by Engvall and Perlmann (1972) and described elsewhere (Kunicka and Platsoucas, 1986). Heavy chain specific (µ or γ) rabbit anti-human Ig antibodies (Accurate Chemicals, Hicksville, NY) were diluted to a concentration of 2 µg/ml in buffer (0.1 M Na_2CO_3, $NaHCO_3$, pH 9.6, with 0.05% NaN_3). One hundred fifty µl of antibody solution was placed into individual wells of 96-well, flat bottomed flexible plates (Falcon 3912) and incubated at 37°C for 3 hr and at 4°C for an additional 18 hr. The plates were then washed three times with PBS containing 0.02% Tween 20 (Sigma). Several dilutions of the unknown immunoglobulin-

containing supernatants were prepared in PBS/0.02% Tween 20 and 150 µl aliquots were placed in each well. The plates were incubated for 5 hr and washed four times with PBS/0.02% Tween 20. Alkaline phosphatase conjugated rabbit anti-human Ig antibody specific for µ or γ chain (Sigma) were added next and the plates incubated for a further 16 hr at room temperature. The plates were washed four times and 150 µl of 1 mg/ml p-nitrophenylphosphate in 1 M diethanolamine-HCl (Sigma) buffer (pH 9.8) containing 0.5×10^{-3} M $MgCl_2$ was added to each well. The plates were incubated for 1 hr at room temperature after which the reaction was stopped by the addition of 50 µl/well of 3 M NaOH. The optical density of the released p-nitrophenolate was measured at 405 nm with the aid of an automatic Elisa reader (Titentek, Flow, McLean, VA). Ig values were estimated from comparisons against standard curves generated with the aid of purified IgM and IgG immunoglobulins (Kallestad, Choska, MN). Suitable ranges for immunoglobulin determinations by this method were 1-80 mg/ml for IgG and 5-100 mg/ml for IgM.

RESULTS

The characteristics of the patients with B cell chronic lymphocytic leukemia investigated in this study are shown in Table I. These patients were either untreated or did not receive treatment for at least three months prior to these studies. All patients exhibited large numbers of small lymphocytes in their peripheral blood which stained weakly positive with anti-immunoglobulin heavy chain specific antibodies and either anti-kappa or anti-lambda

TABLE I

Characteristics of Patients With B-cell Chronic Lymphocytic Leukemia

Patient	WBC*	Cell Surface Immunoglobulin**	T1 Expression	Stage
1	158,000	M,D,κ	positive	III
2	10,000	M,D,λ	positive	I
3	109,000	M,G,κ	positive	II
4	191,000	M,D,λ	positive	I
5	66,000	M,D,κ	positive	II
6	80,000	M,D,κ	positive	IV
7	130,000	M,D,A,κ	positive	III-IV
8	91,000	M,λ	positive	II
9	110,000	M,κ	positive	IV
10	335,000	M,λ	positive	III
11	375,000	M,κ	positive	IV
12	219,000	M,κ	positive	III
13	46,000	ND	positive	IV
14	75,000	M,κ	positive	I
15	230,000	M,κ	positive	IV

*Cells/mm^3.

**Cells were analyzed by immunofluorescence using the cell sorter and all reported phenotypes correspond to percentages of 10% above background staining with irrelevant mouse antibody.

specific reagents, indicating that they were derived from a clonal expansion in a manner typical of leukemic B cells of CLL origin (Table I). Furthermore, peripheral blood lymphocytes from these patients were T1 positive, Ia-positive and negative for the T-cell specific markers (Leu 5, T3, T^4 and T8). The clinical stage of these patients was ascertained as described by Rai et al. (1975).

Highly purified E-rosette negative largely leukemic B cells from these patients were prepared as described in Materials and Methods. These cell isolates were for all practical purposes depleted of T lymphocytes as determined by surface marker analysis and by lack of a proliferative response to PHA, as discussed in Materials and Methods. Purified E-rosette negative largely leukemic B cells from 5 of 15 patients, 12 with CLL (1, 3, 7, 9 and 12) exhibited a modest proliferative response to 10 to 50 μg/ml of $F(ab')_2$ fragments of rabbit anti-human μ-chain specific antibody (anti-μ) (Table II). In these five patients an increase in ^3H-thymidine incorporation in response to anti-μ of at least 100% over control cultures grown in unsupplemented medium was observed. The proliferative response to anti-μ was concentration-dependent (Table II and unpublished results). For example, the leukemic B cells from patient 7 responded significantly to 10 μg/ml of anti-μ in three separate experiments that were carried out over a period of over one year, but not to 50 μg/ml. Highly purified polyclonal B cells isolated from the peripheral blood of two of three normal donor groups responded as anticipated (Muraguchi and Fauci, 1982), by growing in the presence of moderate concentrations of anti-μ (10 μg/ml). However, none of the purified B cells from the three tonsil donors studied responded in

TABLE II

Proliferation of Leukemic B Cells from Patients with CLL in Response to $F(ab')_2$ Fragments of Anti-μ*

Patients	$F(ab')_2$ Fragments of Anti-μ		
	0	10 μg/ml	50 μg/ml
1 exp. 1**	285± 25***	556± 16	ND
exp. 2	179± 8	324± 12	ND
2	ND	116± 2	ND
3	52± 8	111± 2	ND
4	203± 9	230± 44	ND
5	86± 45	86± 80	98± 11
6	353± 46	394±169	ND
7 exp. 1**	977±107	5,487±716	ND
exp. 2	523± 76	1,726±112	527± 42
exp. 3	3,320±295	5,661±468	1,803±211
8	118± 15	142± 16	167± 63
9	333± 29	749± 64	1,189±114
10	324± 2	332± 66	ND
11	106± 11	211± 51	150± 26
12	280± 82	333± 63	509± 52
13	58± 1	79± 29	83± 31
14 exp. 1**	525± 82	313± 32	ND
exp. 2	519±113	258± 11	411± 43
15	186± 41	141± 48	ND

TABLE II (Con't)

Normal controls			
Peripheral blood B cells			
1	376± 52	2,804±165	ND
2	2,591±142	3,243±133	ND
3	3,491±212	3,542±113	ND
Tonsil B cells			
4	1,286± 72	426± 19	806± 56
5	190± 83	151± 8	256± 71
6	318± 79	225± 28	882±212

*Purified E-rosette negative largely leukemic B cells from patients with CLL were cultured with anti-μ as described in Materials and Methods.

**Experiments were performed at different times over a period of one year or more.

***cpm are expressed as the mean ± SD of triplicate culture.

this way to anti-μ alone. In fact, tonsil specimen 4 underwent a slight drop in ^{3}H-thymidine incorporation when exposed to anti-μ (10 μg/ml) and demonstrated slight restoration of proliferation when cultured with 50 μg/ml of anti-μ.

CM alone prepared by stimulating human peripheral blood mononuclear leukocytes with PHA induced significant proliferative responses in purified E-rosette negative largely leukemic B cells from 13 of 14 patients with CLL (Table III). Spontaneous ^{3}H-thymidine incorporation of B cells from these patients in the presence of medium alone is shown in Table II. The extent of these proliferative responses varied substantially from patient to patient and ranged from 2.6 (patient 12) to 91 (patient 4) fold. Significant variability was also observed in the concentration of CM which induced maximum proliferation (range 1:8-1:128). In the case of most leukemic cell lines examined, optimal proliferative responses to CM were obtained at intermediate concentrations. High or low dilutions of CM induced suboptimal proliferation in the majority of patients tested (Table III).

Stimulation of purified cells with both anti-μ (10 μg/ml) and CM (various concentrations) resulted in substantially increased proliferation in all patients (1 to 15) examined (Table III). These responses were significantly higher than those obtained with CM alone in 13 of 15 individuals. Levels of synergy were substantially higher than the sum of those induced by anti-μ and CM alone. Optimal CM concentrations for inducing responses synergistically with anti-μ were 1:32 and 1:128 (in 9 of 11 patients, Table III). Optimal anti-μ concentrations for synergistic interaction with CM were found to lie

TABLE III

Synergism of Anti-μ and Conditioned Medium in Inducing Proliferative Responses of Leukemic B Cells from Patients with CLL*

Patients	Anti-μ (μg/ml)	Proliferative Response (cpm) Conditioned Medium (dilutions) 1:8	1:32	1:128	1:512	1:2048
1 Exp 1**	0	621± 115	1,315± 105	1,140± 50	ND	ND
	10	2,699± 309	13,493±1,716	17,079± 181	ND	ND
Exp 2	0	247± 14	488± 44	557± 71	399± 46	191± 10
	10	709± 48	8,578±1,129	12,591± 396	4,228± 558	1,163± 62
2	0	116± 2	ND	ND	ND	ND
	10	6,568± 52	16,848± 355	25,103± 410	18,930± 451	2,028± 124
3	0	8,157± 338	4,871± 292	2,104± 190	721± 37	325± 43
	10	ND	23,875± 357	8,810± 676	3,115± 79	1,622± 139
4	0	4,511± 48	18,142± 522	18,513± 689	4,824± 341	590± 101
	10	32,087± 799	77,615±1,695	44,096±3,905	15,230± 616	2,857± 235

TABLE III (Continued)

5	0	5,363± 447	11,411±1,699	11,285±1,397	5,601± 382	1,434± 65
	10	23,514±2,996	54,672±4,797	23,687±1,015	12,050± 229	4,562± 809
6	0	4,427±1,078	4,262± 795	3,174± 387	2,275± 84	1,625± 279
	10	14,086±1,034	12,696± 769	7,994± 316	4,428± 214	4,262± 315
7 Exp 1**	0	3,633± 306	6,738± 125	12,311±1,177	5,467± 19	2,387± 99
	10	53,526±5,414	54,121±1,356	44,473±2,658	19,929±1,377	12,266± 699
Exp 2	0	12,367±1,761	15,043±4,936	19,652±2,420	6,187±9,281	3,338± 297
	10	51,043±6,559	54,385±1,074	39,656±1,386	21,764± 751	10,939± 999
Exp 3	0	4,866± 168	15,989±1,216	20,752±2,414	16,016±1,443	5,326± 356
	10	41,038± 174	64,818±2,584	65,477±5,627	36,618±3,945	21,216± 344
8	0	1,231± 191	483± 88	333± 123	235± 53	148± 39
	10	5,346± 925	4,346± 925	2,208± 206	451± 56	174± 15
9	0	428± 32	557± 138	757± 54	544± 30	335± 23
	10	3,856± 288	6,991± 582	4,404± 307	3,233± 301	1,856± 310
10	0	4,852± 484	7,972±1,267	13,752± 244	8,332± 206	1,898± 253
	10	20,441±2,381	30,288±2,100	22,076± 462	18,853±2,273	3,112± 438

TABLE III (Con't)

11	0	100±	2	123±	19	141±	48	140±	30	103±	24
	10	914±	29	2,042±	282	2,515±	359	1,192±	85	503±	112
12	0	344±	20	636±	134	540±	75	359±	80	263±	11
	10	340±	135	739±	169	1,198±	18	634±	198	578±	22
13	0	318±	151	516±	59	451±	67	352±	126	125±	16
	10	ND		1,280±	92	1,303±	125	424±	45	ND	
14 Exp 1**	0	995±	26	1,854±	418	2,053±	256	1,527±	322	1,268±	230
	10	1,197±	122	1,781±	150	1,542±	214	1,195±	116	709±	50
Exp. 2	0	840±	11	1,019±	101	966±	63	818±	44	574±	55
	10	860±	69	1,027±	87	951±	74	918±	22	809±	36
15	0	3,367±	144	5,473±	327	6,344±	246	4,389±	99	1,617±	382
	10	3,589±	156	4,021±	243	4,293±	117	2,306±	120	1,050±	180
Normal Controls											
Peripheral blood B cells											
1	0	3,299±	318	5,550±	429	6,881±	783	5,634±	192	ND	
	10	19,282±	268	54,570±	3,476	28,026±	867	12,170±	1,270	ND	

TABLE III (Con't)

2	0	ND	2,819± 267	3,683± 45	2,632± 338	1,522± 149
	10	ND	24,075± 218	24,159±3,301	15,960± 988	9,313± 944
3	0	2,088± 236	2,587± 315	3,421± 96	2,701± 189	980± 78
	10	19,390± 458	22,065±1,400	24,157±1,870	14,824± 323	9,310± 853
Tonsil B cells						
4	0	963± 157	4,073± 114	6,575± 575	7,223± 591	4,891± 165
	10	17,192±2,709	24,845±2,088	19,617±2,767	11,819±1,193	5,618± 434
5	0	980± 35	1,328± 94	1,670± 200	1,368± 127	1,090± 63
	10	13,324± 144	19,140±1,740	23,196±1,653	15,640±2,271	3,336±3,177
6	0	1,063± 220	1,460± 59	2,343± 479	3,059± 149	1,767± 268
	10	23,231±1,806	23,025±3,917	18,416±1,803	ND	ND

*Purified E-rosette negative largely leukemic B cells from patients with CLL were cultured with various dilutions of conditioned media in the presence of absence of anti-μ as described in Materials and Methods. Spontaneous ^{3}H-thymidine incorporation in the presence of medium alone is shown in Table II.

**Experiments were performed at different times over a period of one year. .

in the range of 10 to 20 μg/ml. A representative experiment is shown in Figure 1 (patient 9).

Highly purified E-rosette negative peripheral blood or tonsil lymphocytes from all normal donors responded by proliferating in the presence of CM alone. In agreement with the results obtained with B cells from patients with CLL, synergism in inducing proliferative responses was observed when CM and anti-μ were added at the start of cultures (Table III). Optimal CM concentrations which yielded synergistic interactions with anti-μ were in the range of 1:8 to 1:128 (Table III). Optimal anti-μ concentrations ranged between 5 and 20 μg/ml (Figure 1).

The proliferative responses of E-rosette negative cells from patients with CLL to $F(ab')_2$ fragments of rabbit anti-human δ-chain specific (anti-δ) antibodies were investigated. Representative results are presented in Table IV. Anti-γ was not able to induce proliferation in any CLL patient so tested (total of 6), whereas anti-δ was effective in inducing proliferation in a single patient (7). This patient's cells expressed IgD as judged by surface marker analysis (see Table I). In contrast to findings obtained with anti-μ, anti-δ and anti-γ did not demonstrate synergy with CM in eliciting proliferative responses in leukemic lines (Table IV). By contrast, both anti-δ and anti-γ effectively synergized with CM to induce proliferation in purified polyclonal B cells from both sets of donor controls (peripheral blood and tonsil).

In addition to eliciting cell growth, CM was highly effective in inducing the majority of leukemic lines so tested (7 of 10) to undergo terminal differentiation resulting in active synthesis and

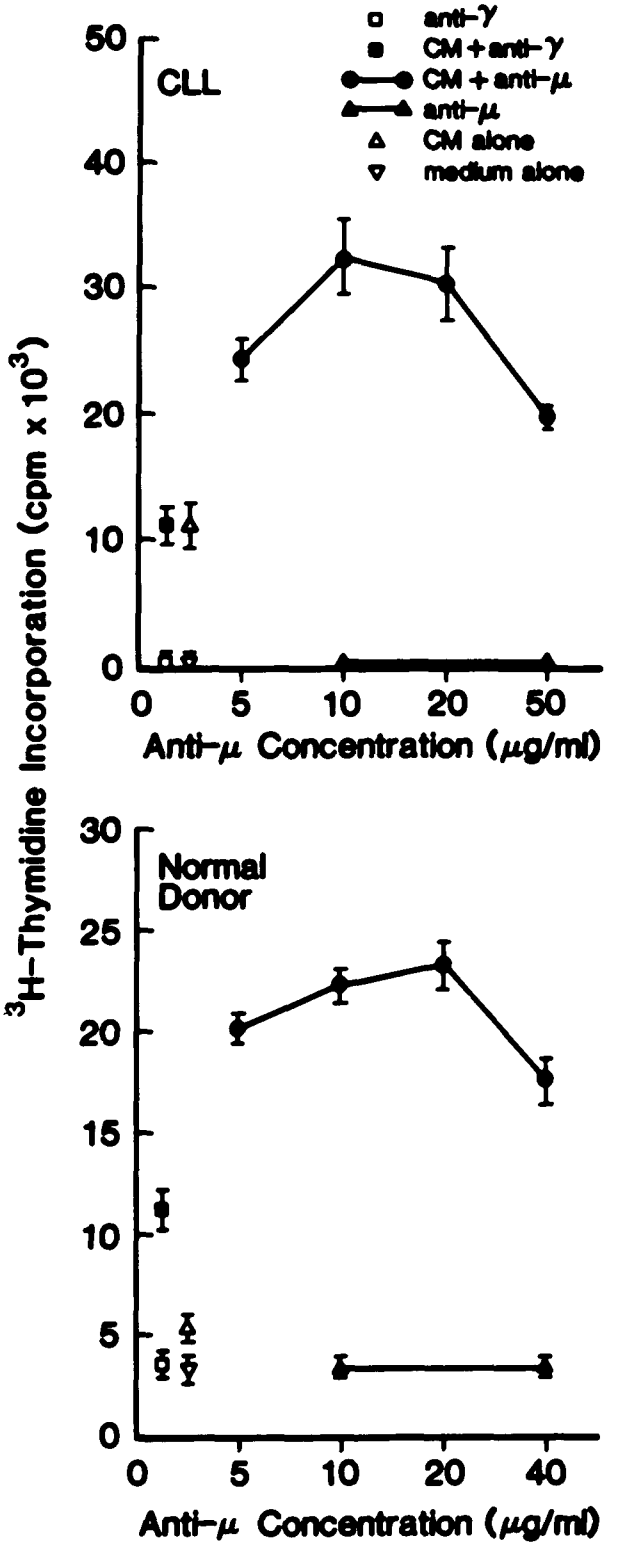

anti-γ
CM + anti-γ
CM + anti-μ
anti-μ
CM alone
medium alone
CLL
50
40
30
20
10
0
0
5
10
20
50
Anti-μ Concentration (μg/ml)
^{3}H-Thymidine Incorporation (cpm x 10^3)
Normal Donor
30
25
20
15
10
5
0
0
5
10
20
40
Anti-μ Concentration (μg/ml)

Figure 1. Proliferative responses of purified E-rosette negative cells from a patient with CLL (#5) (top) and a normal donor (bottom) to CM (△); anti-μ (▲); CM plus anti-μ (●); anti-γ (□) and medium alone (▽). Synergism between CM and anti-μ was observed. CM was used at a dilution of 1:32. Optimal anti-μ concentration for synergism with CM were in the range of 5-20 μg/ml. Anti-γ was used at concentration of 10 μg/ml. Proliferative responses were determined as described in Materials and Methods.

TABLE IV

Effect of Anti-δ^+ and Anti-γ^+ on the Induction of Proliferation of B Lymphocytes* by CM

Patient	Medium	Anti-Ig			Conditioned Medium	Conditioned Medium plus anti-Ig		
		Anti-μ	Anti-δ	Anti-γ		Anti-μ	Anti-δ	Anti-γ
#5	86± 45	86± 80	ND**	80± 8	11,285±1,397	23,676±1,015	ND	10,419± 597
#6	353± 46	384±169	511±73	430± 23	2,275± 84	4,428± 215	2,275± 89	2,174± 324
#7								
Exp. 1***	3,320±297	5,661±968	7,343±822	3,308±136	15,989±1,216	64,818±2,584	15,424± 7	19,303±1,176
Exp. 2	523± 76	1,726±112	ND	444± 21	19,652±2,420	39,656±1,386	ND	16,779± 501
#10	324± 2	332± 66	ND	367±106	13,752± 244	22,076± 462	ND	13,429±1,745
#13	58± 1	79± 29	ND	27± 5	451± 67	1,303± 125	ND	521± 89
#14	196± 63	127± 18	254± 58	289± 76	558± 46	541± 73	545± 29	493± 47
Normal controls								
Peripheral blood B cells								
#1	278± 62	199± 30	1,296± 40	213± 6	5,047± 387	50,560±2,005	41,706±534	ND
#2	3,491±212	3,542±113	ND	3,807±310	5,457± 649	21,829± 487	ND	11,510±1,419

TABLE IV (Con't)

Tonsil B cells								
#3	1,286± 72	425± 19	1,872±166	1,355±259	6,575± 575	19,617±2,767	28,962±3,408	6,229± 688
#4	190± 83	151± 8	284± 36	146± 28	942± 40	11,598± 888	20,893±1,919	959± 114
#5	318± 79	255± 28	412± 71	288± 27	2,343± 479	18,416±1,803	19,128±3,219	2,713± 127

* All anti-Ig were $F(ab')_2$ fragments of rabbit anti-human heavy chain specific μ, γ or δ. They were used at a concentration of 10 μg/ml.

* Purified E-rosette negative largely leukemic B cells from patients with CLL were cultured as described in Materials and Methods.

**Not determined.

***Experiments were performed at different times over a period of one year.

secretion of Ig (Figure 2). Production of IgM by B cells treated with CM for six days was observed in each Ig-secreting line. Three of these patients (6, 7 and 11) apparently produced variable quantities of IgM spontaneously. The level of IgM production was increased substantially following coculture with CM. In three patients tested (6, 7 and 12) a switch to IgG production was observed (Figure 2) although, as mentioned above, secretion of IgM did not cease. Patients (6, 7) who were spontaneous secretors of IgM appeared to be the most readily induced to switch to IgG production. Treatment of purified tonsilar lymphocytes with CM under standard conditions resulted in de novo production of IgM (cells cultured in medium alone: 0 ng/ml; cells cultured with CM:910 ng/ml). This level of Ig production was twice as great as the most active leukemic clone (patient 7) tested.

A morphological examination of highly purified leukemic B cells from several patients prior to and following treatment with CM revealed that they indeed underwent a physical transformation to plasmacytoid-like cells with accompanying cellular proliferation. A representative experiment is shown in Figure 3. By contrast, cell lines which were poor responders to CM (such as patient 14) did not exhibit similar morphological maturation (data not shown).

A summary of results, including all experiments having to do with proliferation of and differentiation of patient CLL lines in response to anti-μ and CM, are presented in Table V.

DIFFERENTIATION OF LEUKEMIC B CELLS FROM PATIENTS WITH CLL TO IMMUNOGLOBULIN SYNTHESIZING AND SECRETING CELLS BY CONDITIONED MEDIA

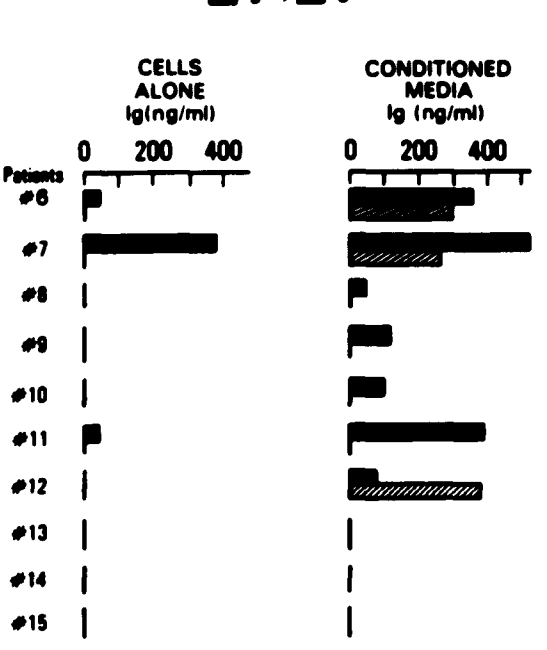


Figure 2: Differentiation of purified E-rosette negative largely leukemic B cells from patients with CLL to immunoglobulin synthesizing and secreting cells by CM. Cells were cultured with CM (1:128 dilution) for 6 days at 37°C, supernatants were collected and IgM and IgG were determined using a heavy chain-specific ELISA, as described in Materials and Methods.

Figure 3: Induction of differentiation by CM (dilution 1:128) of purified E-rosette negative largely leukemic B cells from patients with CLL to plasma-like cells. Top: untreated E-rosette negative largely leukemic B cells from patient #7. Bottom: E-rosette negative cells from the same patient after treatment with CM for 96 hours. Cytocentrifuge preparations were made using a cytocentrifuge and stained with Wright's stain.

TABLE V

Induction of Proliferation and Differentiation of Leukemic B Cells From Patients with CLL with Anti-μ and Conditioned Media*

Patients	Proliferation			Differentiation	
	Anti-μ	Conditioned Media	Conditioned Media and Anti-μ	Conditioned Media IgM	Conditioned Media IgG
# 1	yes+	yes+	yes (synergism)	ND***	ND
# 2	ND	ND	yes	ND	ND
# 3	yes+	yes	yes (synergism)	ND	ND
# 4	no	yes	yes (synergism)	ND	ND
# 5	no	yes	yes (synergism)	ND	ND
# 6**	no	yes	yes (synergism)	yes	yes
# 7**	yes	yes	yes (synergism)	yes	yes
# 8	no	yes+	yes (synergism)	yes	no
# 9	yes	yes+	yes (synergism)	yes	no
#10	no	yes+	yes (synergism)	yes	no
#11**	no	no	yes (synergism)	yes	no
#12	yes+	yes	yes++	yes	yes
#13	no	yes+	yes (synergism)	no	no
#14	no	yes+	yes++	no	no
#15	no	yes	yes++	no	no

TABLE V (CON'T)

*Purified E-rosette negative largely leukemic B cells from patients with CLL were cultured with anti-μ and/or conditioned media as described in Materials and Methods. Proliferative responses were assessed by ^{3}H-thymidine incorporation and differentiation by determining de novo immunoglobulin synthesis and secretion.

**Patients #6, 7 and 11 produced IgM spontaneously. This production was significantly enhanced by conditioned media.

***Not determined.

+Low response.

++Synergism between anti-μ and CM was not observed in these patients.

DISCUSSION

We report here that highly purified E-rosette negative largely leukemic B cells from certain patients with CLL responded by proliferation and differentiation to CM derived from mitogen (PHA)-stimulated human peripheral blood mononuclear cells. CM also effectively synergized with moderate dosages of anti-μ (10 μg/ml) to induce vigorous proliferative responses in both purified leukemic B cell populations and polyclonal donor B cell controls. De novo Ig synthesis and secretion by leukemic B cell lines in response to CM was clearly demonstrated in the majority of patients tested. Furthermore, we noted a switch to IgG production in three IgM-secreting leukemic cell lines (Figure 2). Previously, Saiki et al. (1980) reported induction of IgM secretion and switching to IgG production in human leukemic CLL B cells from a single individual with the help of T cells. We found, in accordance with results of another recent study (Rubartelli et al., 1985), that T cell-derived growth and differentiation factors present in CM were able to replace T cells in the promotion of growth and Ig secretion in leukemic CLL lines, as is known for normal B cells (for a review, see Howard and Paul, 1983). In fact, it appeared to be the rule rather than the exception that purified leukemic cell lines demonstrated qualitatively normal maturational functions (i.e., underwent proliferation and differentiation), albeit at a reduced level as compared to normal B cells.

Morphological examination revealed that leukemic B cells from patients with CLL underwent terminal differentiation in response to CM (Figure 3). Therefore, it is unlikely in our system that small B

lymphocytes were responsible for Ig production without first undergoing further differentiation. Generally, the ability of cells to proliferate was found to be proportional to their ability to undergo differentiation (see Table V). This is somewhat surprising in light of the fact that cell division does not appear to be a necessary prerequisite for the initiation of Ig secretion (Hirano et al., 1985). In addition, leukemic lines which did not proliferate in a synergistic fashion when exposed to CM plus anti-μ (patients 14 and 15) apparently did not possess the ability to secrete Ig in the presence of B cell differentiation factors present in CM alone. Thus, in the majority of cases, the magnitude of a given leukemic line's proliferative response was indicative of its potential to differentiate, both processes appearing to reflect the clone's overall functional integrity.

In general, the best proliferating cell lines (Table III) appeared to be derived from ontologically more mature populations as judged by surface phenotype (Table I). Such leukemic lines, when analyzed by flow microfluorimetry, in most cases, were found to express more than one surface heavy chain isotype (usually μ and δ) as do mature polyclonal B cells. These results are in agreement with the published findings of others (Grossi et al., 1982; Rubartelli et al., 1983; Cossman et al., 1984) who were able to induce Ig production in leukemic clones through the use of polyclonal activators (e.g., phorbol ester) other than anti-μ. Anti-δ and γ, with one exception (Table IV), did not demonstrate proliferative synergism with CM when cocultured with leukemic lines. This stands in direct contrast to the observed ability of these anti-heavy chain isotypes

to effectively synergize with CM for the induction of proliferation in polyclonal B cell cultures. The obvious explanation for this being that these isotypes were either absent from the surface of leukemic cells of responding clones or, particularly in the case of IgD, were present on a small minority of the cells comprising a given leukemic cell population.

Now that a variety of B cell-active factors including IL-1, IL-2, interferon-Y, BSF-I, BCGF II, BCDF, have been identified, the wisdom of utilizing complex mixtures of unknown lymphokines (CM) to induce maturation in populations of normal or leukemic B cells may be called into question. However, taking into account the bewildering variety of factors, their poorly defined modes of action (Cambier, 1986), their apparent multiple modulatory roles (Noma et al., 1986), and the fact that many cloned factors have been derived from murine genes, the most important consideration in our estimation was to closely as possible mimic the physiologic mix of lymphokines to which responding B cells might be exposed to in vivo. For example, we (Steinberg et al., 1985), and Lantz and co-workers (1985), reported that highly purified, largely leukemic B cells from patients with CLL proliferated in response to rIL-2 when used in costimulator experiments with anti-μ antibody. In four-day assays we identified leukemic lines which displayed significant levels of proliferation as compared to cells cultured in unsupplemented medium (unpublished results). However, the CM + anti-μ proliferative response was found to be far greater (Table III) at the end of 96 hr. Lantz et al. (1985) observed that it required 6 days for leukemic B cells to achieve a maximal proliferative response in the presence of

insolubilized (coupled to Sepharose beads) anti-μ plus IL-2 and, even in these cases, the proliferative levels were greatly exceeded when the cells were preactivated with SAC. This serves as an indication that single factor-induced B cells do not represent optimally stimulated systems and may serve as poor models for the simulation of B cell maturation.

CHAPTER 2

INHIBITION OF SPONTANEOUS AND INDUCED PROLIFERATION OF HUMAN LEUKEMIC B CELLS BY ANTI-IMMUNOGLOBULIN ANTIBODIES

INTRODUCTION

Cross-linking of membrane-expressed immunoglobulin (Ig) receptors by anti-μ comprises an early event in the proliferative induction of resting B cells from a variety of mammalian species (Sell and Gell, 1965; Okada et al., 1983; Maizel et al., 1983; Muraguchi et al., 1983, 1984). This activation by one scheme is dependent upon prior exposure to B cell stimulatory factor 1 (BSF-1) (Oliver et al., 1985; Rabin et al., 1985) although the requirement for this lymphokine may not be absolute. Thus, murine lymphocytes proliferate following exposure to high levels (50 μg/ml) (DeFranco et al., 1985) and human leukemic B cells proliferate in the presence of moderate levels (5-10 μg/ml) of anti-μ (Baeker and Rothstein, 1985) in the absence of BSF-1. By contrast, it has been demonstrated that anti-μ may block differentiation of lipopolysaccharide (LPS)-activated polyclonal murine B cells while leaving their ability to proliferate intact (Andersson et al., 1974; Leanderson and Forni, 1984).

In this submission we present evidence demonstrating the inhibitory effect of anti-μ on the proliferation of monoclonal leukemic B cells from a group of patients with chronic lymphocytic leukemia (CLL). Although the characteristics of the inhibition were found to vary among patients, anti-μ partially or completely inhibited proliferation of purified leukemic B cells from three patients whose cells exhibited spontaneous growth in vitro. In each case, the simultaneous addition of T cell-derived factors to cultures partially mitigated the inhibitory effects of anti-μ. Anti-μ-induced inhibition was also demonstrated in a fourth patient whose cells were

highly responsive to stimulation with the polyclonal activator Staphylococcus aureus Cowan I (SAC). In addition, the present submission demonstrates that B cell proliferation may be sensitive to antibodies directed against IgD (anti-δ) as well as anti-μ and that in general anti-Ig-induced proliferative enhancement or attenuation may be evidenced within the same leukemic B cell clone in a dose-dependent manner.

MATERIALS AND METHODS

Patients. Twenty-two randomly selected patients with confirmed diagnosis of CLL of the B cell type were studied (Gale and Foon, 1985). Only untreated patients or those not having had received therapy 3 months prior to testing were included. Of these, 4 patients exhibited the characteristics which are the subject of this submission.

Normal Donors. One-day old leukocyte concentrates prepared from units of donated normal venous blood were obtained from the New York Blood Center. Normally, 5-7 units were depleted of serum, washed, pooled and the mononuclear cells isolated by Ficoll-Hypaque sedimentation and handled as described below.

Media. (Refer to Materials and Methods, Chapter 1.)

T cell-conditioned medium (CM). (Refer to Materials and Methods, Chapter 1.)

Monoclonal antibodies. Monoclonal antibody reagents were employed to determine the Ig heavy and light chain isotypes of individual leukemic clones. They were also utilized in the final stages of the purification of leukemic B cells. Antibodies directed

against cell surface Ig (heavy and light chains) included anti-γ, α, μ, δ, Kappa and lambda (Coulter, Immunology, Hialeah, FL and Becton Dickinson, Mountain View, CA). Antibodies used in the antibody-dependent complement-mediated cell lysis of T cell contaminants included anti-Leu-5 and Leu-9 known to be present on peripheral T cells, large granular lymphocytes and natural killer cells. All monoclonal antibodies were aliquoted in small stock solutions and stored at 0°C prior to use.

Polyclonal activators. Affinity-purified $F(ab')_2$ fragments of rabbit or goat heterologous antibodies specific for human μ, or δ, or γ-heavy chains were obtained from Cappell, Malvern, PA. These antibodies were reconstituted according to directions in PBS, aliquoted and stored at 0°C prior to use. Staphylococcus aureus Cowan I (SAC) was purchased from Bethesda Research Labs, Gaithersburg, MD.

B cell preparation. (Refer to Materials and Methods, Chapter 1.)

Cell surface immunofluorescence. PBM suspensions or B cell isolates were aliquoted into small test tubes at a concentration of 1×10^6 cells/ml. The cells were pelleted by centrifugation at 400 xg for 4 min at 4°C and the supernatants were discarded. The cell pellets were quickly resuspended in 200 μl of the appropriate monoclonal antibody which was prepared by prediluting the required amount of (1/100-1/200) dilution of ascites for each test with PBS to a final volume of 200 μl. The cell suspension was incubated at 4°C for 30 min and washed twice with 1 ml of PBS containing 2% FBS and 0.01% sodium azide (Sigma). The cells were pelleted by centrifugation, the supernatants discarded, and the pellets resuspended in 200 μl of a

1/50 dilution of FITC-conjugated F(ab') fragments of goat anti-mouse Ig antibody (GAM-FITC, Coulter Immunology), which had been previously prepared by prediluting the required amount of second antibody for each test with PBS to a final volume of 200 µl. Following incubation for 30 min at 4°C, the cells were washed three times with 1 ml aliquots of PBS containing 0.01% sodium azide, resuspended in 1 ml of wash medium and filtered through fine nylon mesh (40µ) in preparation for enumeration by flow microfluorometry.

Flow microfluorometric analysis. Microfluorometric analysis of lymphocytes was performed on either an EPICS V cell sorter (Coulter Electronics, Hialeah, FL) or on an Ortho Model 30L cytofluorograph (Ortho, Westwood, MA). Fluorescent cells were scored as the percentage above the highest channel registering background fluorescence when the cells were stained with mouse nonspecific Ig (irrelevant antibody) of the class (IgG_1, IgG_{2a} or IgM) corresponding to the isotype of the monoclonal antibody of intent, followed by staining with GAM-FITC. In addition to phenotyping lymphocytes, the purity of isolated B cells was ascertained by staining cells with the T cell-specific monoclonal antibody Leu-5. In all cases, following purification, the percentage of B cells was judged to be greater than 95% and the percentage of T and adherent cells was judged to be far less than 1% each.

Proliferative responses of B cells. The proliferative response of leukemic cells obtained from the study subjects as well as normal controls was assessed in unsupplemented medium. The highly enriched B cell suspensions were adjusted to a concentration of 2×10^6 cells/ml in culture medium. The cells were cultured in 96-well, flat

bottom, microtiter plates (Linbro, McClean, VA) at a density of 2 x 10^5 cells/well. Anti-Ig (anti-μ or δ or γ) was added to cultures at varying dilutions in the presence or absence of CM. In addition, B cells were incubated with the polyclonal activator SAC (1:10,000 vol:vol) alone or in combination with anti-μ. Each reagent with the exception of CM (100 μl/well) was added in 10 μl aliquots/well. The final volume per well was adjusted to 0.3 ml with culture medium and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 96 hr. Thymidine incorporation was utilized as an index of proliferative activity. Individual wells were pulsed with 1 μCi of [^{3}H]thymidine during the last 16 hr of incubation. The pulsed cells were collected on glass fiber filters (Whatman Ltd., Maidstone, UK) with the aid of an automatic cell harvester (Cambridge Technology, Inc., Cambridge, MA) and the amount of label incorporated was measured by liquid scintillation counting.

RESULTS

Initial observations. As part of an overall study designed to assess the proliferative and maturational potential of leukemic B cells, peripheral blood was collected from 22 patients previously diagnosed with CLL. Following isolation on Ficoll-Hypaque cushions, an examination of gross morphological characteristics by light microscopy revealed highly uniform populations of PBM having the outward appearance of small circulating lymphocytes. An analysis of cell surface markers by indirect immunofluorescence utilizing a battery of T and B cell-specific mouse anti-human monoclonal antibodies indicated that the PBM were comprised almost exclusively of B

cells. While varying degrees of cellular proliferation could be induced within the leukemic B cells from many patients following incubation with anti-μ plus CM, the B cells from four of the patients manifested a unique and unexpected set of characteristics. Instead of being mitogenic, anti-μ under certain conditions of culture was found to partially or completely block thymidine incorporation within this subgroup. An investigation to further define the nature of the B cell proliferative inhibition was undertaken.

Cell surface Ig phenotype of anti-μ sensitive leukemic cells. Highly purified B cells were routinely subjected to flow microfluorimetric analysis in order to determine surface phenotypic expression of heavy and light chain Ig isotypes. In all cases, positively fluorescencing cells were observed to be weakly staining in a manner typical of CLL (Aisenberg et al., 1973; Chen and Heller, 1978; Ligler et al., 1983; Baldini et al., 1985). The exclusive association of one light chain isotype was the primary indication of the monoclonal nature of each leukemic B cell population (Aisenberg et al., 1973; Preud'homme and Seligmann, 1972). The number of peripheral white blood cells (WBC) determined at the time of blood collection and the surface Ig phenotype of each patient's leukemic B cell population are presented in Table VI. As indicated, WBC counts were well within the range commonly encountered in CLL (Rai and Sawitsky, 1981). The surface Ig phenotypes determined for patients 1, 3 and 4 are also typical of CLL and are suggestive of immature B cells as evidenced by their lack of expression of IgD (Baldini et al., 1985). The cells of patient 2 expressed IgD, IgM, and in addition, IgA. The presence of

TABLE VI

Patient White Blood Cell Count and Surface Ig Phenotype Profile

Patient	WBC[a]	Surface Ig[b]
1	136,000	IgM, Kappa
2	130,000	IgM, IgD, IgA, Kappa
3	230,000	IgM, Kappa
4	83,000	IgM, Kappa

[a] Leukocytes/mm^3

[b] Cells were analyzed by flow microfluorimetry, and all reported phenotypes correspond to percentages of at least 10% above background staining with irrelevant mouse antibody.

IgA was unusual since expression of three isotypes on the cell surface of a CLL clone is a rarely encountered event.

Determination of the degree of T cell contamination. Purified E^- largely leukemic B cells prepared by methods described in Chapter 1 contained less than 0.5% E-rosette forming cells as determined by rerosetting with SRBC. In another series of experiments, purified leukemic B cells were stained with OKT4 or OKT8 monoclonal antibodies and then analyzed by flow microfluorometry. The presence of T cells was not detected by this method. In addition, purified leukemic cells were routinely cultured with PHA-P and never evidenced a positive proliferative response (data not shown). These data, taken together, indicate that the cell populations utilized in these experiments were essentially depleted of T cells.

Spontaneous B cell proliferation. As an initial condition, each experiment included the culture of 2×10^5 highly purified B cells under standard conditions with culture medium alone. The purified largely leukemic B cells from 3 of the 4 patients under investigation exhibited moderate (patient 2) to pronounced (patients 1 and 4) proliferation when incubated with culture medium alone (Table VII). Such spontaneous growth has been previously reported in some patients with CLL (Moayeri and Sokal, 1979; Simonsson and Nilsson, 1980) but was not observed in the remaining test subjects (19 patients, data not shown) nor in proliferation experiments utilizing purified normal polyclonal B cells (212 ± 78).

Inhibition of spontaneous leukemic cell proliferation by anti-μ. Purified leukemic B cells from these patients were next cultured under standard conditions with moderate (10 μg/ml) or high

TABLE VII

Spontaneous Proliferation of Purified Leukemic B Cells in Culture Medium[a]

Patient	CPM[b]
1	36,331 ± 1,678
2	3,320 ± 295
3	186 ± 41
4	17,831 ± 2,956

[a] 2×10^5 Cells in 0.3 ml were grown in culture medium alone. Cells were pulsed with 1 μCi [^{3}H]-thymidine during the last 16 hrs of 96-hr cultures.

[b] CPM are expressed as the mean ± SD of triplicate cultures.

concentrations (50 μg/ml) of anti-μ. Surprisingly, when anti-μ (10 μg/ml) was cultured with the cells of two patients (1 and 4) demonstrating the highest levels of spontaneous proliferation (Figure 4), we observed a near total abrogation of thymidine incorporation in patient 1 and a decrease of 50% in patient 4. Although the leukemic cells of patient 2 exhibited a modest increase in thymidine incorporation when cocultured with 10 μg/ml of anti-μ, at higher concentrations (50 μg/ml), cellular proliferation was reduced below that of spontaneous levels. The leukemic cells of patient 3 responded to the presence of anti-μ in a more conventional fashion. This individual's leukemic B cells did not proliferate in culture medium nor in the presence of anti-μ at either concentration.

In order to determine whether the proliferative responses we had observed were specifically attributable to anti-μ, we elected to substitute $F(ab')_2$, fragments of anti-γ (10 μg/ml) in our cultures. We considered anti-γ to be an irrelevant antibody because IgG was not detected on the surface of any of the leukemic clones tested. Table VIII indicates that anti-γ had no effect on the proliferative response of any of the purified leukemic cell populations examined.

Proliferative responses of leukemic B cells to CM. When leukemic cells were cultured with CM alone, the lymphocytes from all 4 patients responded with a proliferative burst which varied in magnitude among individuals (see Figure 5A-D). Patients 1 and 4 whose leukemic cells exhibited the highest capactity to proliferate spontaneously were also the most readily stimulated by CM alone. In both cases, cells from these patients reached proliferative levels (84,757 ± 5,047 and 138,447 ± 361 cpm, respectively) far in excess of

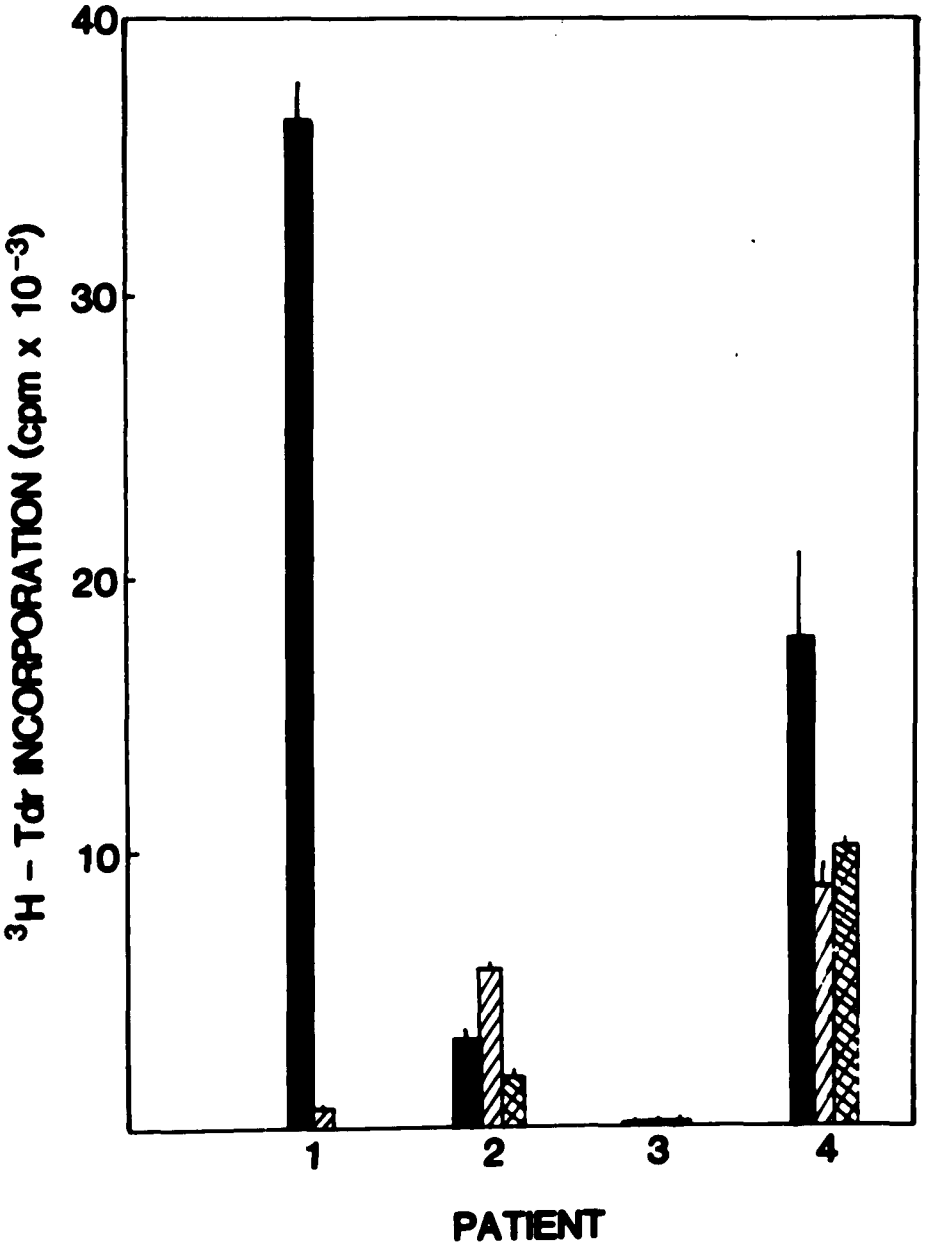

40
30
20
10
^3H - Tdr INCORPORATION (cpm x 10^{-3})
1
2
3
4
PATIENT

Figure 4. Inhibition of spontaneous proliferation displayed by isolated CLL lymphocytes in the presence of anti-μ. PBM were depleted of adherent cells by passage through Sephadex G-10 columns and depleted of T cells by twice rosetting with AET-treated SRBC followed by treatment with pan-T monoclonal mouse antibodies plus rabbit complement, as described in Materials and Methods. Highly purified leukemic B cells (2×10^5 cells/well) in 0.3 ml from each patient were cultured with anti-μ at 10 μg/ml [▨], 50 μg/ml [▩] or in culture medium alone [■], for 96 hr. Cultures were pulsed with 1 μCi [^{3}H]thymidine during the last 16 hr. Results are expressed as the mean ± SD cpm of triplicate cultures.

TABLE VIII

Proliferative Response of Purified Leukemic B Cells to Anti-Y[a]

Patient	cpm[b] Culture Medium	Anti-Y antibody
1	36,331 ± 1,678	--[c]
2	3,320 ± 295	3,308 ± 136
3	186 ± 41	122 ± 20
4	17,831 ± 2,956	18,326 ± 587

[a] 2×10^5 Cells in 0.3 ml were grown in culture medium either alone or containing 10 µg/ml of affinity-purified $F(ab')_2$ fragments of rabbit anti-human Y. Cells were pulsed with 1 µCi [^{3}H]thymidine during the last 16 hrs of 96-hr cultures.

[b] cpm are expressed as the mean ± SD of triplicate cultures.

[c] Not done.

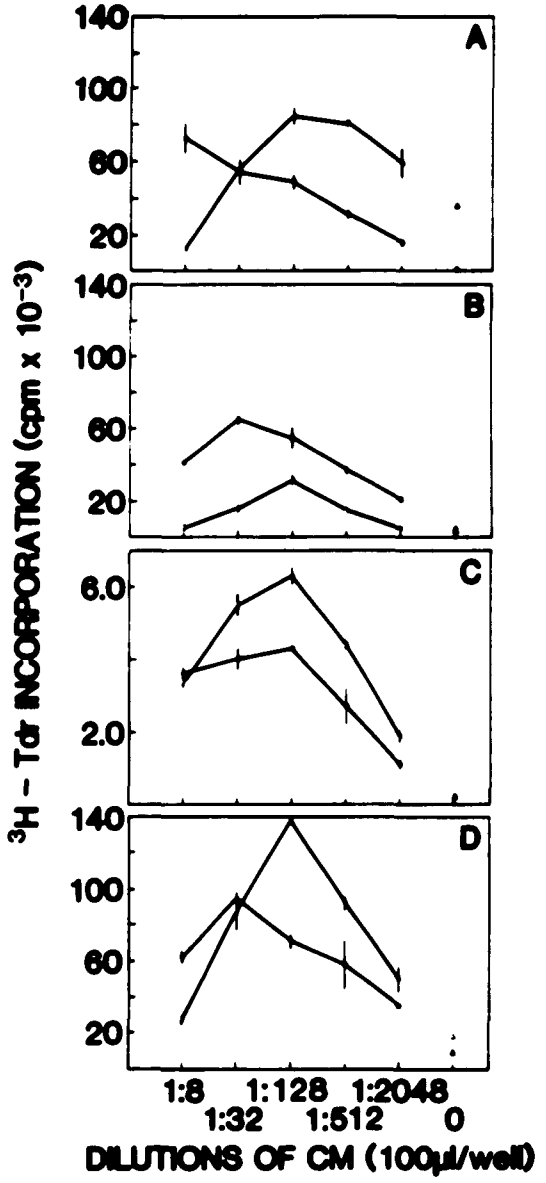

140
100
60
20
A
140
100
60
20
B
6.0
2.0
C
140
100
60
20
D
^3H – Tdr INCORPORATION (cpm x 10^{-3})
1:8
1:32
1:128
1:512
1:2048
0
DILUTIONS OF CM (100µl/well)

Figure 5. Proliferative profiles of leukemic B cells in response to culture medium, anti-μ, CM and CM plus anti-μ. Highly purified leukemic B cells (2×10^5 cells/well) were cultured for 4 days in culture medium [△] alone, with anti-μ (10 μg/ml) [▲]. Cultures were pulsed with 1 μCi [^{3}H]Tdr 16 hr prior to harvesting. Each symbol indicates the mean ± SD cpm of triplicate cultures. Cells used in the experiments illustrated in panels A to D correspond to patients 1 to 4 in Figure 1, respectively.

those achieved by normal polyclonal B cells (3,254 ± 2,426 cpm) in the presence of CM (1:128). The cells of patient 2 also proliferated actively (20,752 ± 2,414 cpm) when cultured with CM (1:128) but did not attain the levels reached by the previous two patients (Figure 5B). Patient 3 posted the weakest response to CM (6,344 ± 246 cpm) and was the sole individual in the group whose cells did not proliferate spontaneously. In general, peak levels of thymidine incorporation were achieved with CM concentrations of 1:128 and fell off rapidly at higher dilutions.

When leukemic cells were cocultured with varying dilutions of CM plus 10 µg/ml of anti-µ, the highest CM concentrations (1:8) utilized resulted in maintenance of levels of proliferation in 3 of 4 patients (1, 3 and 4) which exceeded both spontaneous levels and equaled or exceeded proliferative levels achieved with CM alone (Figure 5). As the CM concentration fell below a dilution of 1:128, the proliferative curves of the three patients assumed negative slopes and, in the case of the first patient, proliferative levels dropped below baseline values (spontaneous proliferation). However, even at the lowest concentrations of CM utilized, some amelioration of anti-µ induced proliferative inhibition was observed in two of the patients (3 and 4) (Figures 5C and 5D).

The isolated leukemic B cells of patient 2 behaved in a more conventional manner under similar conditions of culture. CM (1:128) plus anti-µ (10 µg/ml) synergized to yield a twofold increase in thymidine incorporation. However, when the anti-µ concentration was increased to 50 µg/ml and administered to cultures in combination with CM (1:32), the level of cellular proliferation fell short of

that achieved with standard anti-μ concentrations (Figure 6). Thus, the pattern of anti-μ-induced proliferative inhibition was preserved but required the use of higher antibody concentrations.

Anti-μ dose-dependent modulation of leukemic cell proliferation. The results obtained from patient 2 indicated that varying the dosage of anti-μ (Figure 6) might result in major differences in the leukemic B cell proliferative profile. These findings suggested that the inhibitory effects of anti-μ might be reversible at lower antibody concentrations.

Purified largely leukemic B cells from patient 4 were cultured under standard conditions over a wide range of concentrations (0.31 - 10 μg/ml) of anti-μ alone or of anti-μ plus CM (dilution 1:128) (Figure 7). The use of low anti-μ concentrations (0.31 μg/ml) resulted in minimal increases in the level of thymidine incorporation beyond that representing spontaneous proliferation. Anti-μ was found to activate cells at low concentrations and caused maximal levels of stimulation at concentrations ranging from 0.6 to 1.2 μg/ml. Higher antibody concentrations were not mitogenic. Anti-μ concentrations higher than 3.6 μg/ml were clearly inhibitory. In these experiments the use of CM (1:128) (see Figure 7) was again found to ameliorate the inhibitory effects of anti-μ. The level of protection, as expected, varied inversely with the anti-μ concentration.

Inhibition of proliferation induced by anti-δ. Since microfluorimetric analysis of purified leukemic B cells from patient 2 indicated that they expressed IgD in addition to IgM on their surfaces, we wished to determine if anti-δ was capable of exerting effects similar to those observed with anti-μ. Leukemic cells from

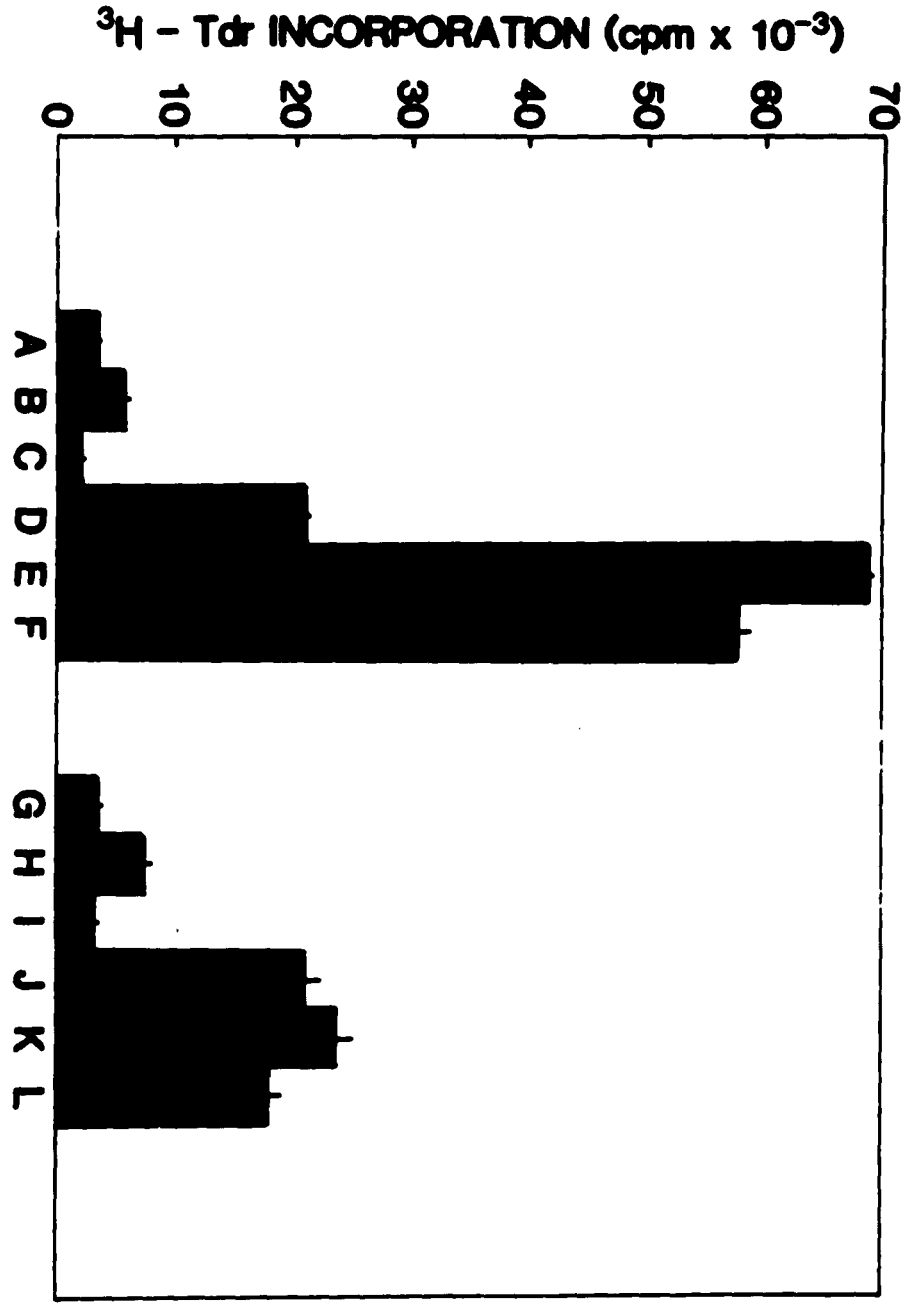

^{3}H – Tdr INCORPORATION (cpm x 10^{-3})
0
10
20
30
40
50
60
70
A
B
C
D
E
F
G
H
I
J
K
L

Figure 6. Enhancement or inhibition of proliferation produced by culturing leukemic cells at two different concentrations of anti-μ or anti-δ. Highly purified leukemic B cells (2 x 10^5 cells/well) from patient 2 were cultured for 4 days in a final volume of 0.3 ml. Culture conditions included (A) culture medium, (B) anti-μ at 10 μg/ml, (C) antiμ at 50 μg/ml, (D) CM (1:32) at 33% vol:vol, (E) CM (1:32) at 33% vol:vol plus anti-μ at 10 μg/ml, and (F) CM (1:32) at 33% vol:vol plus anti-μ at 50 μg/ml. Assay series G-L were cultured under precisely the same conditions except that anti-δ was subetituted for anti-μ. Cultures were pulsed with 1 μCi [^{3}H]Tdr 16 hr prior to harvesting. Results are expressed as the mean ± SD cpm of triplicate cultures.

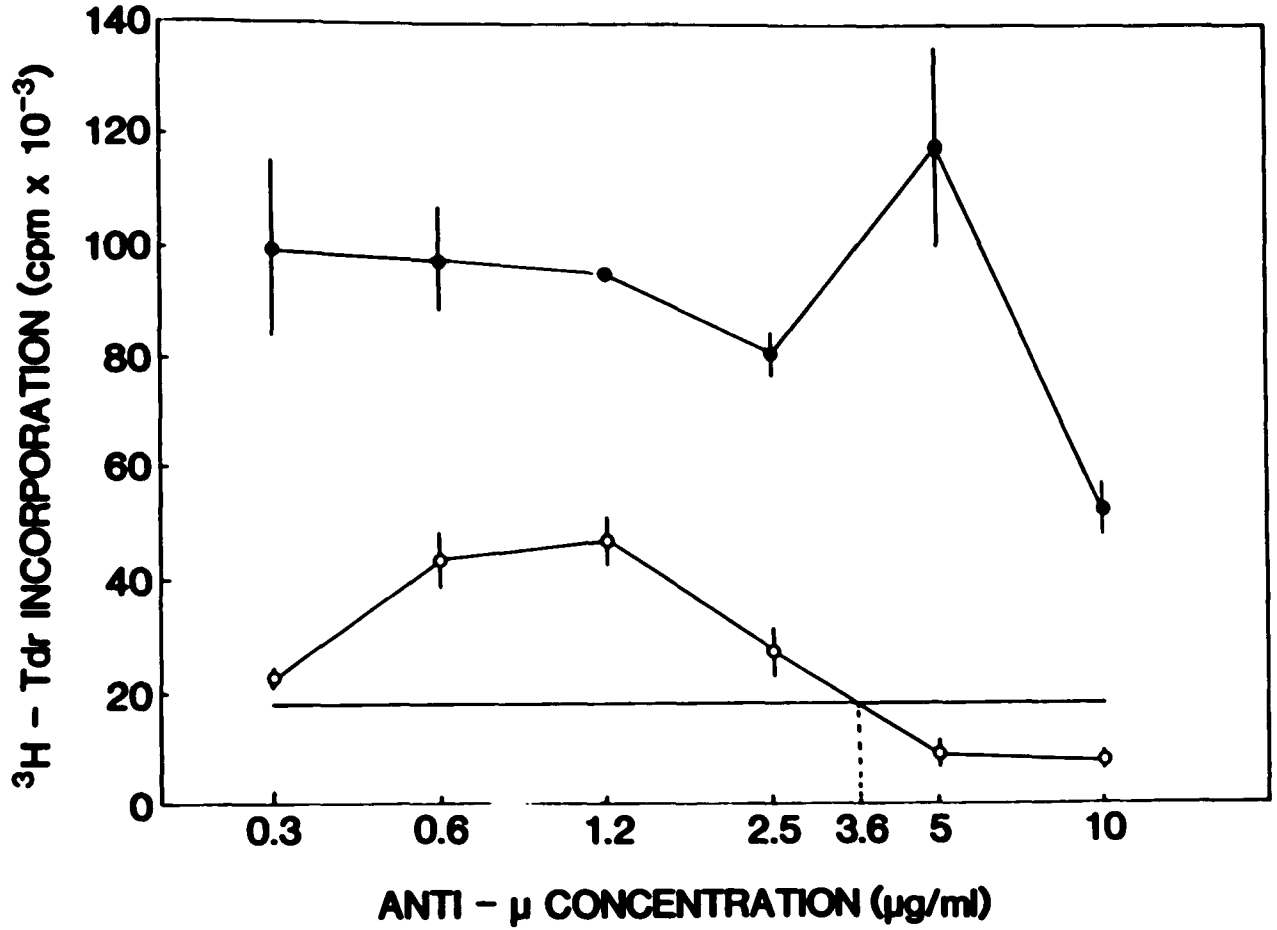
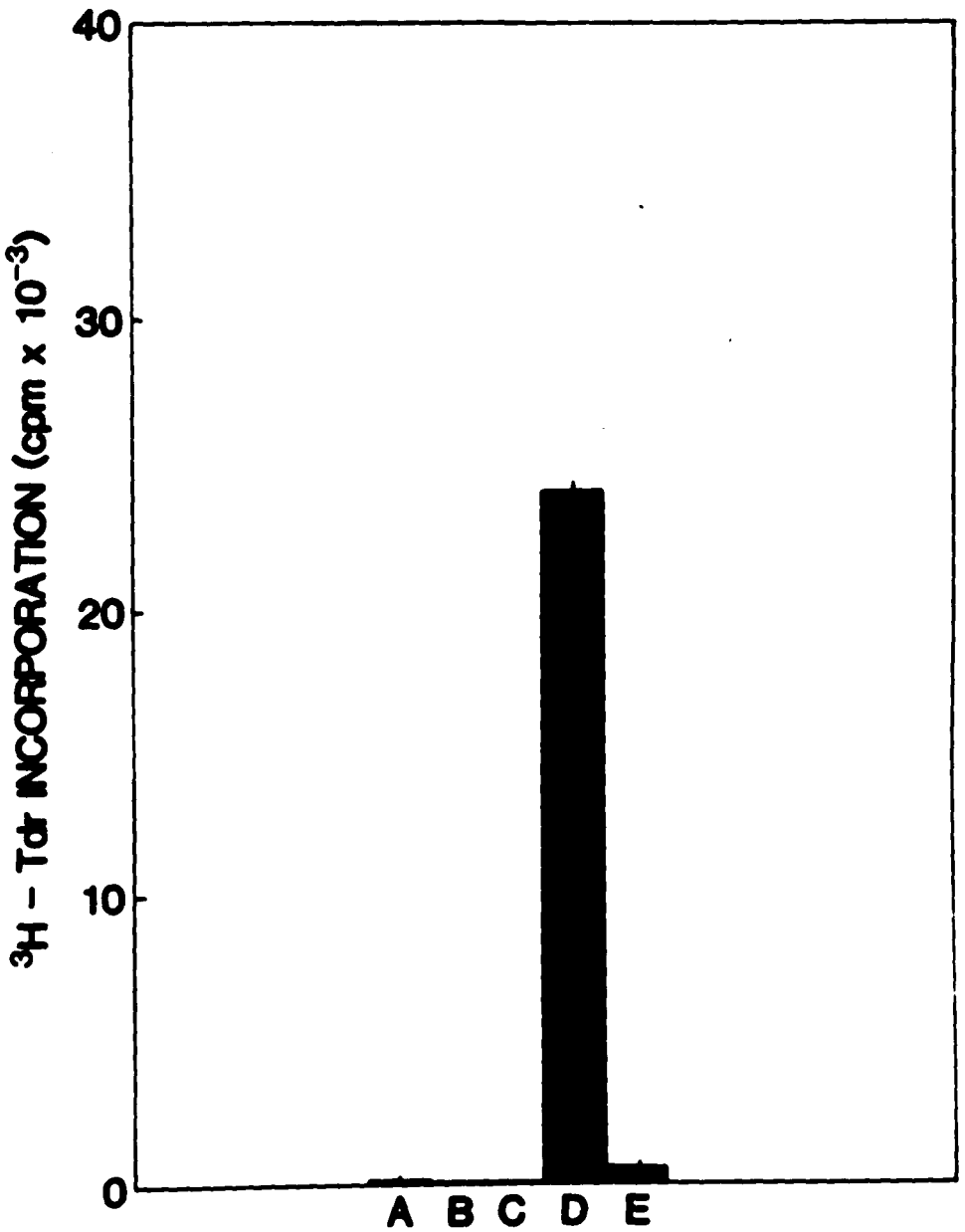

140
120
100
80
60
40
20
0
^3H – Tdr INCORPORATION (cpm x 10^{-3})
0.3
0.6
1.2
2.5
3.6
5
10
ANTI – μ CONCENTRATION (μg/ml)

Figure 7. Dose-dependent effect of varying the anti-μ concentration on the proliferation of leukemic cells from patient 4. Highly purified leukemic B cells (2 x 10^5 cells/well) were cultured for 4 days in culture medium [——] alone, with various doses of anti-μ at 3.3% vol:vol [O] and with various doses of 3.3% vol:vol plus CM (1:128) [●]. Cultures were pulsed with 1 μCi [^{3}H]Tdr 16 hr prior to harvesting. Each symbol indicates the mean ± SD cpm of triplicate cultures.

patient 2 were cultured under standard conditions with 10 or 50 µg/ml of affinity purified $F(ab')_2$ fragments of goat anti-human IgD antibody (δ-chain specific) in the presence or absence of CM (1:32). Although Figure 3 indicates that the magnitude of proliferative enhancement and inhibition varied between anti-µ and anti-δ, the general pattern of cellular responses was somewhat similar in both cases.

When anti-δ was administered at a concentration of 10 µg/ml to the leukemic cells of patient 2, a twofold increase in the magnitude of cellular proliferation was noted. Raising the antibody concentration to 50 µg/ml abrogated this response. Similarly, when cells were cultured with anti-δ (10 µg/ml) in conjunction with CM (1:32), a moderate increase in proliferation was noted while raising the antibody concentration to 50 µg/ml was inhibitory and lowered the proliferative response to levels below those achieved with CM alone. Although the degree of proliferative enhancement and inhibition was low, these results suggest that cross-linking of IgD molecules at the surface of B cells does not invariably result in cellular growth but may be inhibitory under certain conditions.

Abrogation of SAC-induced proliferation. Proliferative responses of CLL lymphocytes to SAC are highly variable (see Chapter 3) and this may, in part, be reflective of their differing ontological status (Gale and Foon, 1985; Grossi et al., 1982; Rubartelli et al., 1983). Although SAC was found to be non-mitogenic when cultured with leukemic cells from the majority of patients tested, the cells of patient 3 (Figure 8) exhibited a strong proliferative response (23,987 ± 527 cpm) which fully matched proliferative levels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A B C D E

Figure 8. Abrogation by anti-μ of SAC-induced leukemic cell proliferation. Highly purified leukemic cells (2 x 20^5 cells/well) from patient 3 were cultured for 4 days in a final volume of 0.3 ml. Culture conditions included (A) culture medium, (B) anti-μ at 10 μg/ml, (C) anti-μ at 50 μg/ml, (D) SAC (1:10,000 vol:vol) at 3.3% vol:vol, and (E) SAC (1:10,000 vol:vol) at 3.3% vol:vol plus anti-μ at 10 μg/ml. Cultures were pulsed with 1 μCi [^{3}H]Tdr prior to harvesting. Results are expressed as the mean ± SD cpm of triplicate cultures.

achieved by SAC-stimulated normal polyclonal B cells (21,732 ± 14,000). The proliferative response of this individual's B cells to CM (1:128) (6,344 ± 246 cpm) was significantly lower than that induced by SAC. The SAC-dependent response was completely abolished, however, by anti-μ (10 μg/ml) when both were added at the initiation of culture (see Figure 8). This patient was the only individual included in the study whose cells did not proliferate spontaneously in culture medium but grew well under the influence of SAC. These data suggest that polyclonal activation by SAC as compared to anti-Ig may proceed via alternate mechanisms.

DISCUSSION

Soluble anti-Ig antibodies in concert with T cell-derived factors can trigger early B cell maturational events (Okada et al., 1983; Maizel et al., 1983; Muraguchi et al., 1983, 1984). Anti-IgM as well as anti-IgD antibodies can function in this capacity (Parker, 1975; Sieckmann et al., 1978; Pure and Vetetta, 1980; Finkelman and Vitetta, 1984). Although in vitro activation of resting B cells may consist of a two-step process (Oliver et al., 1985; Rabin and Paul, 1985) involving 1) BSF-1-initiated blastogenic transformation followed by 2) cross-linking of surface Ig by anti-Ig antibodies, recent studies have indicated that this sequence of events may be circumvented. Murine B cells, in particular, may be triggered to proliferate in the presence of high concentrations (50 μg/ml) of anti-μ alone (DeFranco et al., 1985). Conversely, a wide range of dosages of anti-Ig antibody are known to tolerize some B cells (Cambier et al., 1977; Scott et al., 1977; Vitetta et al., 1977).

LPS-induced polyclonal murine B cell differentiation can be blocked by anti-μ (Andersson et al., 1974; Leanderson and Forni, 1984). The fact that cellular proliferation in the LPS-driven system proceeds unabated accords well with recent evidence that purified B cell differentiation factor can induce Ig secretion in nonproliferating cells (Hirano et al., 1985) and strongly suggests that proliferation and differentiation are independent events. Inhibition of allo-antigen-specific human B cell differentiation by anti-idiotypic antibody (anti-Id) has also been described. Bona and Fauci (1980), utilizing a plaque-forming assay, demonstrated that treatment with anti-Id prevented the secretion of antisheep-RBC IgM antibody by leukemic B cells of CLL origin which had previously displayed this characteristic. In the same vein, utilizing a sensitive radioimmuno-assay, Geha (1983) demonstrated that treatment with serum auto-anti-Id inhibited the PWM-induced synthesis of IgG anti-tetanus toxoid antigen.

Since an effective humoral immune response undoubtedly requires accumulation of adequate numbers of responding cells, it is not unreasonable to presume that in vivo B cell differentiation is preceded by marked clonal expansion. Our studies indicate that B cell proliferation is sensitive to levels of activating antibody and, by extension, suggests that B cell tolerance may be subject to control at the level of cellular proliferation as well as differentiation.

In this submission, we have demonstrated the ability of $F(ab')_2$ fragments of anti-Ig to inhibit the proliferative response in essentially monoclonal populations of leukemic B cells. That this effect has not been observed previously with human polyclonal B cells may be

due to masking of such effects by the overwhelming majority of clones represented in such populations and points to the utility of analyzing monoclonal B cells of leukemic origin for such purposes. We have identified 4 out of a total of 22 patients afflicted with CLL whose purified largely leukemic B cells were inhibited to varying degrees from proliferating in the presence of anti-Ig. In 3 cases in which we observed significant levels of spontaneous proliferation, we could partially or completely block further proliferation with appropriate concentrations of anti-μ. Since the presence in vivo of spontaneously proliferating clones is a poor prognostic indicator (Juliusson et al., 1985), these findings may be of some therapeutic relevance. In general, we noted that the degree of anti-μ-induced proliferative inhibition paralleled a given clone's ability to proliferate spontaneously (see Table IX). Thus, although the cells of patient 2 exhibited the same magnitude of inhibition as patient 4, the anti-μ concentration required to achieve this level of inhibition was five times greater. Standard concentrations of anti-μ (10 μg/ml) being stimulatory in this case (Figure 6).

CM prevented or partially reversed the inhibition of spontaneous proliferation by anti-μ. Restoration of activity was dose-dependent such that the highest concentration of CM yielded the highest levels of protection from the effects of anti-μ (see Figures 5A-D). These results are in general agreement with findings which indicate that T cell-derived factors restore LPS-prestimulated murine B cells to their full maturational potential (Leanderson and Forni, 1984).

TABLE IX

Anti-μ-induced Inhibition Expressed as a Percentage of Spontaneous Growth[a]

Patient	Anti-μ concentration required to achieve inhibition	Spontaneous proliferation cpm[b]	Inhibition cpm	% Change[c]
1	10 μg/ml	36,331±1,678	699±124	98
2	50 μg/ml	3,320± 295	1,803±210	46
4	10 μg/ml	17,831±2,956	8,714±822	51

[a] 2×10^5 Cells in 0.3 ml were grown in the presence of culture medium alone or with α - μ. Cells were pulsed with 1 μCi [^{3}H]thymidine during the last 16 hrs of 96-hr cultures.

[b] cpm are expressed as the mean ± SD of triplicate cultures.

[c] The percent change was calculated as follows:

$$1 - \frac{\text{cpm[inhibition]}}{\text{cpm [spontaneous proliferation]}} \times 100$$

However, even at the highest concentration of CM (1:8) employed, B cell proliferation was not fully restored. Our finding that CM (presumably BSF-1) bestows a measure of protection from anti-Ig-induced proliferative inhibition requires further testing utilizing normal B cells representing various differentiative states.

We have demonstrated that activation or inhibition signals furnished by anti-μ are not necessarily absolute with respect to individual CLL clones. The nature and magnitude of the response is instead strongly dose-dependent. This conclusion was first suggested by results obtained from patient 2 (Figure 6) where lower concentrations of anti-μ resulted in proliferative enhancement while higher concentrations (50 μg/ml) caused reduced levels of proliferation. In addition, when the leukemic cells from patient 4 were cultured with anti-μ over a broad range of concentrations, a spectrum of responses was revealed. Low concentrations of anti-μ (0.3 μg/ml) resulted in a slight increase of proliferation above spontaneous baseline levels. As the anti-μ concentration was raised, the degree of proliferative stimulation rose and peaked at an anti-μ concentration (1.2 μg/ml), an order of magnitude below the standard concentration. Higher levels of anti-μ resulted in lessened proliferative enhancement until a state of net inhibition was reached at a concentration above 3.6 μg/ml. In this patient, however, spontaneous proliferation was never fully abolished at high anti-μ concentrations (50 μg/ml). The inclusion of CM (1:128) in cultures greatly ameliorated the inhibitory response as expected and its presence helped promote proliferation at all anti-μ concentrations, although the magnitude of the positive response dropped off at increasing anti-μ concentrations.

Our results indicate that proliferative inhibition by anti-Ig is concentration-dependent and may be independent of isotype. Thus, patient 2 whose leukemic cells expressed IgD in addition to IgM was susceptible to inhibition by either anti-Ig. Moreover, the pattern of inhibition which required high levels (50 μg/ml) of antibody was the same for both isotypes. These results could also signify the existence of rare CLL clones bearing surface IgG which would be sensitive to inhibition by anti-γ. Since the molar concentration of surface-expressed IgD has been shown to far exceed the levels of surface-expressed IgM on normal murine B lymphocytes (Havran et al., 1984), the sensitivity of this patient's cells to anti-δ could be attributed to low levels of total surface Ig expressed as judged by the weak intensity of surface immunofluorescence (data not shown) which is a well-documented characteristic of CLL (Aisenberg et al., 1973; Chen and Heller, 1978; Ligler et al., 1983; Baldini et al., 1985). These results, although not directly comparable, may have implications for general models of B cell tolerance induction. Data has been published which demonstrates that the expression of IgD on the surface of maturing B cells renders them less susceptible to tolerance induction by specific antigen (McFadden and Vitetta, 1984; Waldschmidt and Vitetta, 1985). This effect may be due in large measure to increased overall levels of surface-expressed Ig and not necessarily to the emergence of a given isotype (i.e., IgD).

As previously reported in this submission, leukemic B cells of patient 3 (Figure 8) did not proliferate spontaneously but exhibited a high level of responsiveness to the polyclonal activator SAC (23,987 ± 587 cpm). In this respect, the behavior of this

individual's leukemic cells resembles those of normal polyclonal B cells (Muraguchi and Fauci, 1982). Overall, we have found that the response to SAC of purified leukemic B cells from patients with CLL is highly variable (see Chapter 3), although the cells from most patients are unresponsive. It is noteworthy that this individual's active demonstrated responsiveness to SAC was essentially abolished by 10 μg/ml of anti-μ. Inhibition of the proliferative response to SAC appears to parallel abrogation of LPS-induced cellular differentiation in murine B cells. The cells in each case have received a strong activation signal which was unambiguously terminated by anti-μ. This finding may be extended to spontaneously proliferating leukemic cells, where the activation signal is presumably internal. It is of interest that the ability of anti-μ and SAC to elicit opposite responses within the same clonal population suggests that the mechanism of action of both polyclonal activators appears to differ, although other effects cannot be entirely ruled out (see Chapter 3).

Since these results could not be obtained in a polyclonal B cell system, the need for an understanding of the long-term propagational requirements of B cells deserves high priority. In its absence, monoclonal leukemic B cells offer an attractive alternative.

CHAPTER 3

POLYCLONAL ACTIVATION OF LEUKEMIC LYMPHOCYTES BY SAC AND ANTI-μ: EVIDENCE FOR DIFFERENT MODES OF ACTION

INTRODUCTION

In addition to anti-μ antibody (anti-μ), one of the most commonly utilized human B cell polyclonal activators over the past 5 years has been killed Cowan I strain Staphylococcus aureus (SAC). Several experimental protocols have been developed in which SAC has been effectively utilized to stimulate B cell proliferation (Muraguchi and Fauci, 1982). Resting B cells may be incubated with SAC and T cell-derived products at the initiation of cultures or if longer periods of proliferation are desired, SAC can be used to prestimulate resting B cells which are induced to enter the cell cycle and undergo several rounds of proliferation. At this point (usually 3 days) SAC is removed and sources of B cell growth or differentiation factors are added to promote further proliferation or terminal differentiation.

Forsgren et al. (1976) demonstrated that staphyloccal protein A (SPA), which is expressed on the cell wall of the staphyloccal bacterium, was the component responsible for SAC-induced mitogenic activity. SPA is known to bind to two widely separated regions on the immunoglobulin (Ig) molecule. These correspond to the Fc (Forsgren and Sjoquist, 1966; Kronvall and Williams, 1969) and $F(ab')_2$ regions (Inganes et al., 1980) of Ig. Although Fc binding by SPA has found wide practical application for the purification of Ig molecules, it is its interaction with the exposed $F(ab')_2$ regions of surface Ig which is responsible for B cell activation and is thought to closely approximate conditions of specific antigen binding on B cell receptors. Romangnani et al. (1981a) convincingly attempted to support this conclusion by furnishing evidence indicating that anti-μ,

presumably through a mechanism of steric hindrance, partially or completely blocked the mitogenic effect of SAC on both normal and leukemic CLL lymphocytes.

The extent of SAC-induced activation has been elucidated by Falkoff et al. (1982), who found that while there was elicitation of a strong proliferative response, terminal differentiation resulting in Ig secretion was strictly dependent upon T cells or their secretory products. This conclusion was at variance with that of others (Pryjma et al., 1980; Schnurman et al., 1980; Dosch et al., 1980), who reported the induction of B cell differentiation as well.

In this submission, the maturational responses of highly purified CLL lymphocytes to SAC or to SAC in combination with anti-μ and T cell-derived factors present in conditioned medium (CM) were evaluated. We determined, in accordance with the results of Romagnani et al. (1981b) and Tamaki et al. (1986), that neoplastic B cells from the majority of CLL patients are unresponsive to stimulation by SAC alone. The inhibition of SAC-induced proliferation by anti-μ was also noted; however, several cases of SAC/anti-μ-induced synergistic interactions were also observed. In addition, SAC in several instances was able to promote Ig production in polyclonal B cells and CLL lymphocytes.

MATERIALS AND METHODS

Patients. Eighteen randomly selected patients with confirmed diagnosis of CLL of the B cell type (Gale and Foon, 1985) were studied. Only untreated patients or those not having received

therapy over the previous three months prior to testing were included.

Normal Donors. (refer to Materials and Methods, Chapters 1 and 2).

Media. (refer to Materials and Methods, Chapter 1).

T cell Conditioned Medium (CM). (refer to Materials and Methods, Chapter 1).

Polyclonal Activators. Affinity-purified $F(ab')_2$ fragments of rabbit or goat heterologous antibodies specific for human μ heavy chain was obtained from Cappell, Malvern, PA. This antibody was reconstituted according to directions in PBS, aliquoted and stored at 0°C prior to use. Staphylococcus aureus Cowan 1 (SAC) was purchased from BRL, Gaithersburg, MD, and washed well to remove preservative and reconstituted in PBS.

B Cell Isolation. (refer to Materials and Methods, Chapter 1).

Monoclonal Antibodies (MoAbs). MoAbs were utilized in the final stages of the purification of leukemic B cells from many of the patients. (refer to Materials and Methods, Chapter 1).

Proliferative Responses of B Cells. (refer to Materials and Methods, Chapter 1).

Ig Secretion of B Cells. (refer to Materials and Methods, Chapter 1).

RESULTS

Response of Polyclonal B Cells to SAC. In order to determine the optimal SAC concentration to be used for stimulation of leukemic cells, under our conditions a dose-response profile utilizing normal polyclonal B cells was generated. B cells were obtained from peripheral blood and each purified isolate grew minimally in unsupplemented medium. SAC was found to be an effective inducer of cellular proliferation over a broad range of concentrations yielding a relatively flat dose-response curve (Figure 9). A SAC concentration of 1:10,000 (vol:vol) resulted in maximal stimulation and was used in all subsequent experiments.

Induction of Proliferation in Leukemic Cells by SAC. It has been well established that SAC is an effective inducer of cell growth in resting B cells (Falkoff et al., 1982; Muraguchi and Fauci, 1982). It was of some interest to determine the effectiveness of SAC in promoting growth in leukemic cells. In accordance with the findings of others (Romagnani et al., 1981b; Tamaki et al., 1986), we noted that the majority of CLL lines tested were not responsive to stimulation by SAC (Figure 10). Out of a total patient population of 18 individuals, only 3 patient cell lines (3, 10, 13) responded well to proliferative induction by SAC while a fourth patient (18) proliferated to a marginal degree. The remaining individuals were unresponsive. By contrast, leukemic cells from nearly all test subjects, as has been previously shown (see results, Chapter 1), exhibited varying degrees of growth (Table I) when exposed to CM plus anti-μ

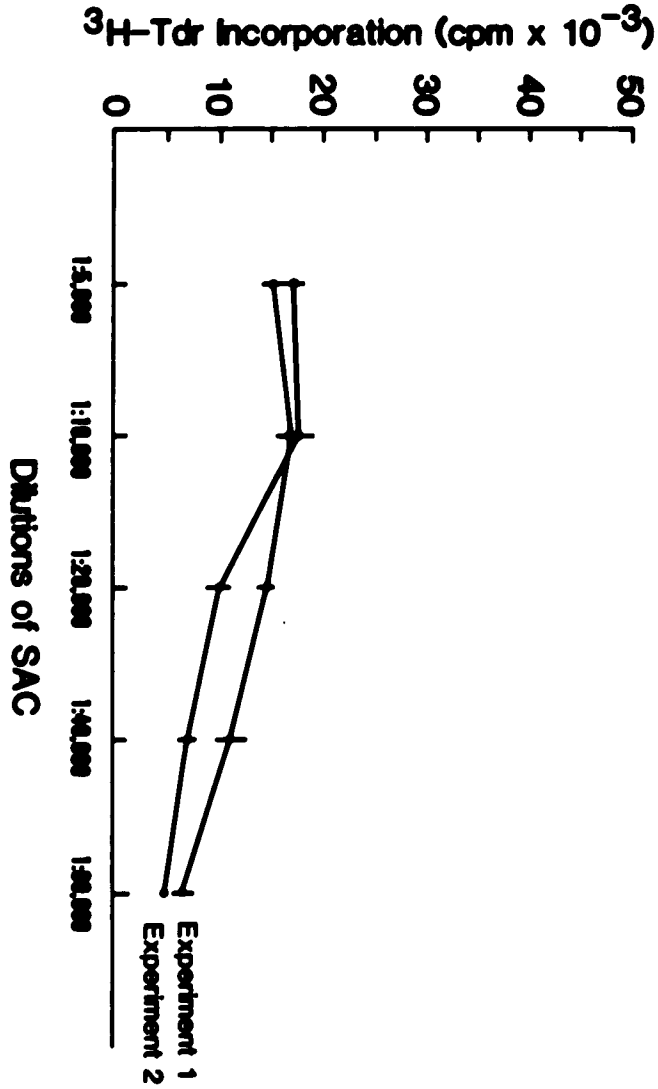
^{3}H-Tdr Incorporation (cpm x 10^{-3})
0
10
20
30
40
50
Dilutions of SAC
Experiment 1
Experiment 2

Figure 9. Proliferative response of normal B cells to varying concentrations of SAC. MC were depleted of adherent cells by passage through Sephadex G-10 columns and depleted of T cells by twice rosetting with AET-treated SRBC followed by treatment with mouse pan-T MoAbs plus rabbit complement, as described in Materials and Methods, Chapter 1. In the two separate experiments depicted, highly purified leukemic B cells (2 x 10^5 cells/well) in 0.3 ml medium were cultured with halving dilutions of SAC for 96 hr. Results are expressed as the mean ± SD cpm of triplicate cultures.

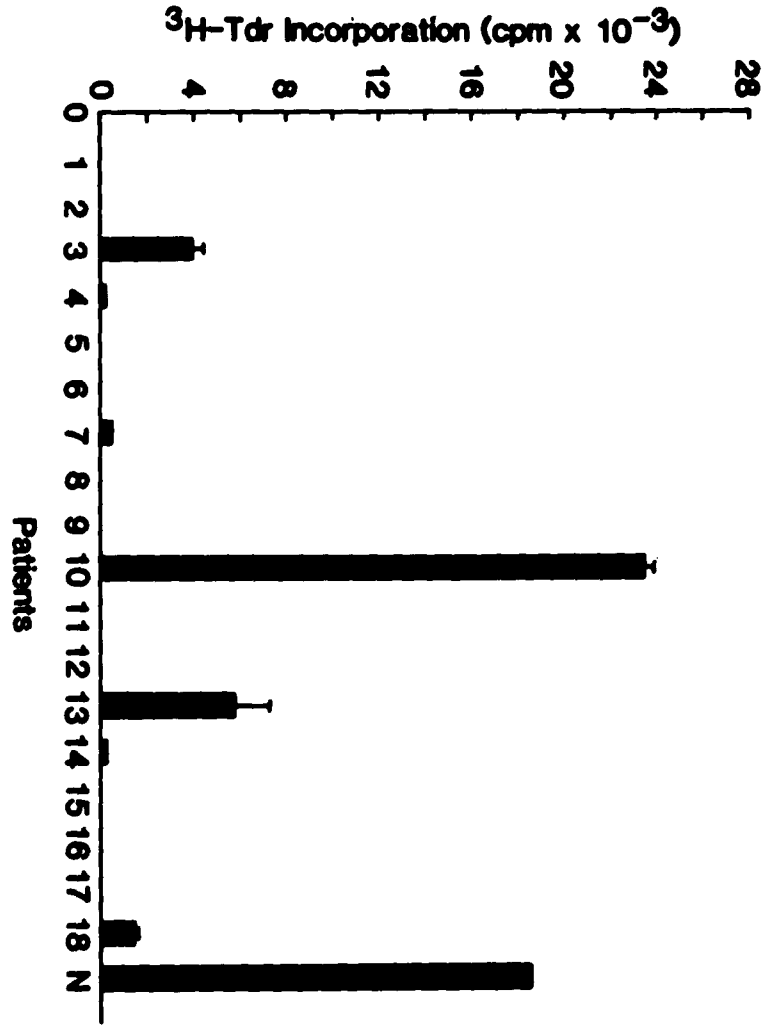
^{3}H-Tdr Incorporation (cpm x 10^{-3})
0
4
8
12
16
20
24
28
Patients
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 N

Figure 10. Proliferative response of leukemic lymphocytes and control B cells to SAC. MC of patients were depleted of adherent cells by passage through Sephadex G-10 columns and depleted of T cells by twice rosetting with AET-treated SRBC followed by treatment, in most cases, with mouse pan-T MoAbs plus rabbit complement, as described in Materials and Methods, Chapter 1. The isolation of control B cells from tonsil tissue is described in Materials and Methods, Chapter 2. Highly purified leukemic B cells (2×10^5 cells/well) in 0.3 ml medium were cultured for 4 days in the presence of SAC (1:10,000 vol/vol) at 3.3% vol:vol. Control B cells (N) were handled in the same manner. Cultures were pulsed with 1 µCi [^{3}H]Tdr 16 hr prior to harvesting. The control histogram is a mean composite of three separate experiments (range: 26,452 ± 693 - 11,856 ± 1,218). Patient histograms depict individual experiments and have been corrected for cells cultured in medium alone and represent the mean ± SD cpm of triplicate cultures.

(10 μg/ml). In addition, many leukemic lines grew in the presence of CM alone.

Response of Leukemic Cells to Stimulation With and Without SAC. Table X indicates that most leukemic cell lines did not grow in unsupplemented medium with one exception (patient 5). Two patients were moderately stimulated by anti-μ (10 μg/ml) while the others did not proliferate. As previously stated (Figure 10), only four out of 18 patients exhibited growth under the influence of SAC while the cells of patients 5 and 14, which proliferated in the presence of anti-μ, did not do so when cocultured with SAC. As is the case with normal B cells (data not shown), the SAC-responsive leukemic lines (3, 10, 13, 18) did not grow in the presence of anti-μ (10 μg/ml).

When anti-μ (10 μg/ml) and SAC (1:10,000 vol:vol) were used together in costimulator experiments, of the patients demonstrating no appreciable differences when cultured with unsupplemented medium, anti-μ alone or SAC alone (Table I), 3 patient lines (9, 13, 14) displayed cooperative growth with anti-μ plus SAC while conversely, one patient's (10) very substantial SAC response was entirely abrogated under these conditions (see Chapter 2).

Patients demonstrating strongly elevated (4, 5, 8, 9) to moderate (1, 3, 6, 7, 10, 12) growth in CM demonstrated marked synergy when cultured with CM plus anti-μ under standard conditions. The cells of two patients (7 and 10) displayed no such cooperativity, and in the case of patient 10, was perhaps slightly inhibited by anti-μ. Where tested (5 patients), unlike normal B cells (Muraguchi and Fauci, 1982), SAC did not effectively synergize with CM in 4 cases (4, 5, 11, 12) and displayed mild synergy in only

TABLE X

Proliferative Characteristics of Leukemic B Cells in the Presence of SAC Under Various Conditions of Culture[a]

	Proliferation in response to								
Patient	medium cpm[b]	Anti-μ cpm	SAC cpm	Anti-μ + SAC cpm	CM cpm	CM + anti-μ cpm	CM + SAC cpm	CM + anti-μ + SAC cpm
1	285± 25	556± 16	221± 39	ND[c]	1,140± 50	17,079± 181	ND	ND
2	ND	116± 2	261± 40	ND	ND	25,103± 410	ND	ND
3	52± 8	111± 2	4,099± 458	ND	2,104± 190	8,810± 676	4,008± 246	ND
4	203± 9	230± 44	412± 21	ND	18,513± 689	44,096±3,905	21,437±1,895	ND
5	3,320±295	5,661±468	2,412± 189	3,952±451	20,752±2,414	55,477±5,627	14,333±2,338	33,891±1,787
6	294± 22	237± 38	207± 47	171± 13	2,984± 264	5,757± 589	ND	3,288± 215
7	525± 82	313± 32	716± 128	423± 32	2,053± 256	1,542± 214	ND	2,977± 143
8	324± 2	332± 66	119± 18	104± 32	13,752± 244	22,076± 402	ND	ND
9	86± 45	86± 80	185± 22	1,184±258	11,285±1,397	23,687±1,015	ND	ND
10	186± 41	141± 48	23,987± 527	517± 37	6,344± 246	4,293± 117	ND	ND

TABLE X (Continued)

11	58±	1	79±	29	53±	18	63±	2	451±	67	1,303±	125	228±	8	4,411±	816
12	353±	46	394±	169	339±	41	46±	19	3,174±	387	7,994±	316	905±	72	9,234±	874
13	131±	18	106±	16	5,991±	1,502	8,655±	160	238±	24	800±	88	ND		ND	
14	151±	44	2,394±	9	404±	53	5,976±	510	222±	36	17,668±	2,180	ND		ND	
15	106±	11	211±	51	162±	23	226±	54	141±	48	2,515±	359	ND		ND	
16	118±	15	142±	16	103±	25	131±	40	333±	123	2,208±	206	ND		ND	
17	333±	29	749±	64	182±	13	395±	46	757±	54	4,404±	307	ND		ND	
18	280±	82	333±	63	1,682±	204	1.180±	170	540±	75	1,198±	98	ND		ND	

[a] 2×10^5 cells in 0.3 ml were grown in the presence of the following substances: Medium (unsupplemented); 3.3% anti-μ (10 μg/ml); 3.3% SAC (1:10,000 vol/vol) and 33% CM (1:128 dilution). Cells were pulsed with 1 μCi [^{3}H]thymidine during the last 16 hr of 96-hr culture.

[b] cpm are expressed as the mean ± SD of triplicate cultures.

[c] Not determined.

one case (patient 3), which was exceeded by growth induced with anti-μ plus CM under the same conditions. When SAC was added in costimulator assays along with CM plus anti-μ a decremental change in proliferative levels generated with CM plus anti-μ was observed in 2 of 5 patients (5, 6) while increased proliferation was achieved in two lines (7, 11), and the level of the last patient's (12) cell growth was essentially unaffected.

Ig Production in Response to SAC. The supernatants from 6-day old triplicate cultures which had been incubated in the presence of various agents including SAC was carefully collected and subjected to Elisa analysis (refer to Materials and Methods, Chapter 2). As Table XI indicates, Ig secretion was found to be highly variable within both patient and control samples.

Spontaneous secretion was noted in one patient (15) and one experimental control (1). Significant quantities of IgM (42 and 110 ng/ml) were produced in both cases. Cells cultured with anti-μ, by and large, did not differentiate and this apparent unresponsiveness was attributable to two causes, one theoretical and the other technical. It is well known that stimulation by anti-μ does not lead to Ig secretion without the help of T cells or their soluble products (Boyd et al., 1985; Baeker and Rothstein, 1985; Boyd et al., 1986). Secondly, the fact that heterologous anti-μ was present in μg quantities in cultures where ng quantities of IgM were being produced makes it highly likely that secreted IgM would be bound at many determinants and either precipitated or neutralized, thereby rendering this isotype impossible to detect under our conditions.

TABLE XI

Ig Secretion of Leukemic and Control B Cells in Response to SAC[a]

	Medium		SAC		CM	
Patient	IgM[b]	IgG	IgM	IgG	IgM	IgG
6	0	3	220	100	830	2
7	0	0	0	0	0	0
8	0	0	0	0	98	0
11	0	0	0	0	0	0
15	42	0	85	0	360	0
16	0	0	0	0	39	0
17	0	0	0	0	115	0
18	0	0	0	0	65	370
Control[c]						
1	110	0	185	0	910	5
2	0	0	225	0	50	0
3	0	0	0	0	0	0

Footnotes to Table XI:

[a] 2×10^5 cells in 0.3 ml were cultured under standard conditions (see Materials and Methods, Chapter 2) in the presence of the following substances: medium (unsupplemented); 3.3% SAC (1:10,000 vol:vol) and 33% CM (1:128 dilution). Following 6 days of culture the supernatants of triplicate wells were carefully removed and stored at 0°C until assayed. Secreted IgM and IgG were determined by Elisa (refer to Materials and Methods, Chapter 1).

[b] IgM and IgG concentrations expressed as ng/ml.

[c] Control B cells were obtained from tonsil tissue and were generously provided by Dr. W.R. Benjamin, Hoffmann-LaRoche, Nutley, NJ.

One of the most unexpected results obtained was the enhancement and de novo elicitation of Ig secretion in leukemic and control B cell populations by SAC alone. Incubation of cells for 6 days, solely in the presence of SAC, resulted in increases in pre-existing (spontaneous) Ig synthesis in two cases (patient 15 and control 1). In addition, another cell line (patient 6) was induced to secrete both isotypes de novo following stimulation with SAC. Induction with SAC was also observed in a control sample (2) as evidenced by the high level of IgM produced. It should be further noted that the leukemic cells of patient 6 did not proliferate in response to SAC (see Table X) but did differentiate in its presence. In the cases where SAC either enhanced spontaneous differentiation or induced Ig secretion, with a sole exception (Control 2), it was not as effective in this regard as CM..

DISCUSSION

Anti-Ig, particularly anti-μ, as well as SAC, have been the two most frequently utilized polyclonal activators for the induction of growth in B cell cultures. Both agents are widely believed to exert their influence through cross-linkage of surface Ig, and this interaction of mitogen with surface Ig receptor has served as a general model for B cell activation resulting from exposure to specific antigen (Sell and Gell, 1965; Falkoff et al., 1982). Both polyclonal activators, under appropriate conditions, are capable of inducing blastogenic changes in B cells but only SAC promotes proliferation of human or murine B cells at moderate concentrations. Unlike SAC, anti-μ is usually ineffective in this regard unless it is used at

high concentrations (Chiorazzi et al., 1980; Muraguchi et al., 1983; DeFranco et al., 1985) or is coupled to an insoluble matrix (Boyd et al., 1985, 1986). Romagnani et al. (1981a) showed that both SPA and anti-μ preparations can compete for the same binding site at the Fab portion of the surface Ig receptor. This is functionally manifested by anti-μ inhibition of SAC induced proliferation. Since insolubilized anti-μ is also capable of promoting a proliferative response, there is a tendency to consider these two activators as interchangeable. Several lines of evidence, however, suggest that this may not be so. First, SPA is also mitogenic for T cells while anti-μ is not (Boyd et al., 1986). In addition, this study revealed instances (Table X, patients 9, 13, 14) where SAC plus anti-μ synergized to enhance proliferation triggered by one or the other agent or acted together to foster proliferation when both agents alone were ineffective (patient 9). SAC, where tested, in many instances failed to synergize with CM (patients 4, 5, 11, 12) as had anti-μ which represents a striking departure from its synergy with T cell-derived factors when cultured with normal B cells (Muraguchi and Fauci, 1982).

Some earlier studies indicated that SAC stimulation could initiate B cell differentiation as well as proliferation as gauged by Ig secretion. Falkoff et al. (1982) reported a strict dependence for a T cell-derived signal in order to achieve terminal differentiation and reported SAC as being incapable of inducing Ig secretion in human polyclonal B cells. Presumably, difficulties with the earlier studies could be traced to contaminating T cells which were activated and induced to secrete B cell differentiation factors in response to

SAC. We have evidence utilizing highly purified monoclonal and polyclonal B cell systems of SAC acting to cooperatively enhance ongoing Ig synthesis (Table XI, patient 15 and control 1). Even more striking, was the ability of SAC alone to induce de novo Ig synthesis in one patient (6) and one control sample (2). Presuming these preparations to be free of T cells and not prestimulated in some manner, these results reinforce the notion that the nature of polyclonal activation of B cells is highly dependent upon the mitogen used, the activation state of the cells, and more to the point, that SAC and anti-μ may trigger B cells by different mechanisms; their sharing of a common binding site not withstanding.

CHAPTER IV

CYTOGENETIC ANALYSIS OF LEUKEMIC CELLS WHICH HAVE BEEN PROLIFERATIVELY INDUCED WITH T CELL-DERIVED FACTORS

INTRODUCTION

Cytogenetic analysis of lymphoproliferative malignancies have been conducted with increasing frequency of late. In the case of CLL, there have been several motives underlying this level of increased activity. Initially, there existed a need to catalogue the type and frequency of structural chromosomal abnormalities occurring in this disease. This, in turn, stimulated efforts aimed toward elucidating the mechanisms which gave rise to such aberrations (for a review, see LeBeau, 1986). One of the least investigated aspects, however, involves measuring the impact of genetic aberrations on the functional properties (i.e., proliferation or differentiation) of affected cells.

Unlike other hematologic malignancies such as chronic myelocyte leukemia (Nowell and Hungerford, 1960), no single diagnostic chromosomal marker associated with CLL has been uncovered, although a number of nonrandom aberrations have been reported (Han et al., 1984; Pittman and Catovsky, 1984; Gahrton et al., 1985; Tsujinoto et al., 1984). Because CLL lymphocytes are classified as mature cells, spontaneous cell divisions are infrequent in the great majority of cases and mitogenic stimulation must be utilized in order to induce metaphases for chromosomal analysis. Early studies during the previous decade were conducted with either PHA, Con A or PWM. All of these substances are primarily T cell mitogens which stimulate admixed B cells indirectly through the action of T cell-derived factors. Results from such studies, as often as not, have yielded information derived from contaminating T cells and not leukemic cells.

More recently, specific B cell polyclonal activators have been utilized for purposes of stimulation. Epstein-Barr virus and LPS have both been used successfully (Robert, 1979; Gahrton et al., 1979). With these agents chromosomal aberrations have been uncovered in about 50% of the cases studied (Gahrton et al., 1980, 1981; Morita et al., 1981; Han et al., 1982; Valdati et al., 1983; Nowell et al., 1981; Hurley et al., 1980).

A number of difficulties still persist in the cytogenetic analysis of lymphocytic malignancies: 1) Non-B cell-specific activators such as PWM are still commonly employed; 2) T cell contamination of B cell cultures is the rule rather than the exception, and 3) certain specific B cell polyclonal activators (Epstein-Barr virus) as well as nonspecific activators such as phorbol esters may actually induce structural chromosomal changes of their own. In an effort to eliminate these difficulties, so that functional phenotypic expression of chromosomal aberrations can be studied, especially that of trisomy 12, we employed stringent measures to deplete leukemic cell isolates of T cells, maximize leukemic cell proliferation through the use of T cell-conditioned medium (CM), interleukin-2 (IL-2), and use of specific B cell polyclonal activators not suspected of fostering mutagenesis (anti-μ).

MATERIALS AND METHODS

Patients. Thirteen randomly selected patients with confirmed diagnosis of CLL of the B cell type were studied (Gale and Foon, 1985). Only untreated patients or those not having received therapy over the previous three months prior to testing were included.

Media. (refer to Materials and Methods, Chapter 1).

T cell Conditioned Medium (CM). (refer to Materials and Methods, Chapter 1).

Polyclonal Activators. Affinity-purified $F(ab')_2$ fragments of rabbit or goat heterologous antibodies specific for human μ heavy chain were purchased from Cappell, Malvern, PA. This antibody was reconstituted according to directions in PBS, aliquoted and stored at 0°C prior to use. The phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA, Sigma) was dissolved in 70% ethanol at a concentration of 1 mg/ml, was aliquoted in small stock solutions, and was stored in the dark at -20°C. The final concentration of ethanol under all experimental conditions was less than 0.007%, a concentration which does not demonstrably affect B cell viability and function (Suzuki et al., 1985). Recombinant interleukin-2 (rIL-2) was a generous gift of Dr. J. J. Farrar, Hoffmann-LaRoche, Inc., Nutley, NJ. The freeze-dried material was reconstituted in PBS containing 1% FBS to a concentration of 10^6 U in 5 ml. All subsequent dilutions were carried out in RPMI-G/10% FBS.

B cell Isolation. (refer to Materials and Methods, Chapter 1).

Mitotic Stimulation. In order to achieve a maximal 4 day proliferative response, leukemic cells were cultured with CM, anti-μ,

rIL-2 with or without TPA. Briefly, enriched E-nonrosetting cells isolated as previously described (B cell Isolation, Materials and Methods, Chapter 1) and adjusted to a concentration of 2 x 10^6 cells per ml of culture medium. The 1 ml cell suspensions were placed into small round bottom plastic test tubes (Falcon) and incubated with CM (1:128, 33% vol:vol), anti-μ (10 μg/ml, 3% vol:vol) and rIL-2 (30 U/ml, 3% vol:vol) with or without TPA (1 ng/ml, 3% vol:vol). All cell cultures were harvested at 96 hr following a 45-60 min incubation with colcemid (GIBCO). After 10 min at 37°C in hypotonic KCl, the cells were fixed in methanol:acetic acid (3:1), washed three times in fixative and spread onto glass slides. The slides were carefully examined for metaphase cells and their positions noted. Q banding of chromosomes was performed in the Department of Cytogenetics, MSKCC, by qualified personnel, according to standard techniques (Chaganti et al., 1983).

RESULTS

Response to TPA. Suzuki et al. (1985) demonstrated that TPA concentrations of 1 to 10 ng/ml yielded maximal stimulation of polyclonal B cells in 72-hr cultures. In light of the recalcitrance of CLL cells to undergo activation with classic mitogens and the weak proliferation which some lines demonstrate in response to T cell-derived factors, we undertook an investigation to ascertain the level of responsiveness of highly purified leukemic B cells from a range of patients to TPA alone or as a costimulator in standard proliferative assays utilizing CM plus anti-μ. In agreement with the results of Tötterman et al. (1980), Table XII indicates that the response of CLL

Table XII

Proliferative Response of Leukemic B Cells in the Presence of TPA Under Various Conditions of Culture[a]

	Proliferation in Response to			
Patient	Medium cpm[b]	CM + anti-μ cpm	TPA cpm	CM + anti-μ + TPA cpm
6	17,831±2,956	80,307±8,736	5,702± 1,227	5,215± 600
7 (Exp 2)	525± 82	2,663± 59	5,098± 370	1,542± 214
8 (Exp 2)	69± 3	2,200± 370	85± 31	6,922± 438
14	151± 44	17,668±1,372	464± 22	4,121± 502
15	106± 11	2,515± 359	ND[c]	11,477± 661
16	118± 15	2,208± 206	ND	48,726±1,862
17	333± 29	4,404± 307	191,794±16,499	10,490± 879
18	280± 82	756± 56	736± 103	1,875± 223

[a] 2×10^5 cells in 0.3 ml were grown in the presence of: medium (unsupplemented); 3.3% anti-μ (10 μg/ml); 3.3% TPA (1 ng/ml) and 33% CM (1:128 dilution). Cells were pulsed with 1 μCi [^{3}H]-thymidine during the last 16 hr of 96-hr cultures.

[b] cpm are expressed as the mean ± SD of triplicate cultures.

[c] not determined.

lines to TPA is positive but highly variable. In the case of two patients (7, 17), TPA alone in culture was found to be stimulatory as compared to unsupplemented medium. Patient 17, moreover, demonstrated a remarkable level of growth with TPA (191,794 ± 16,499) which greatly exceeded its ability to synergize (10,490 ± 879) with CM [1:128 plus anti-μ (10 μ/ml)]. TPA was found to strongly block growth (5,702 ± 1,227; 68% reduction) of spontaneously proliferating cells (17,831 ± 2,956) in the case of patient 6 who was discussed at length in Chapter 1. In the case of two remaining patients (8, 18) TPA alone was found to have little or no effect.

In general, TPA was an effective costimulator of leukemic cells when used in conjunction with CM and anti-μ. Patients 8, 15, 16, 17, and 18 all displayed increased levels of proliferative activity. Conversely, several patient lines were inhibited under the same conditions. The addition of TPA at the initiation of cell cultures containing CM plus anti-μ resulted in very substantial reductions in levels of proliferative activity of patient 14's (77%) as well as patient 6's (94%) cells. Our findings, that TPA can inhibit the proliferative response are in accordance with other recently published results (Mizuguchi et al., 1986).

Enhancement of Anti-μ plus CM-induced Proliferation by rIL-2.

Recent findings that IL-2 is a potent maturation promoting lymphokine of normal B cells (Waldmann et al., 1984; Mingari et al., 1984; Tsudo et al., 1984; Jung et al., 1984; Murazuchi et al., 1985; Ralph et al., 1984) as well as CLL lymphocytes (Steinberg et al., 1985; Lantz et al., 1985; Kabelitz et al., 1985; Mayer et al., 1985) suggested that its inclusion in cell cultures might augment the

activity of other lymphokines present in our CM. We were aware, of course, that IL-2 was a natural component of CM and that its addition to culture constituted further supplementation which was compatible with our goal of maximizing leukemic cell proliferation.

In order to assess the concentration of rIL-2 resulting in near maximal stimulation while not depleting our rather limited stock, we cultured leukemic cells under standard conditions over a range of IL-2 concentrations. The results from Table XIII indicate that rIL-2 alone was mildly stimulatory when cultured with purified cells from four patients tested; furthermore, a final (per well) concentration of 4 U/ml yielded levels of proliferation which did not differ markedly from values obtained with rIL-2 at concentrations nearly an order of magnitude greater (32 U/ml). Thus, this lymphokine was used at a concentration of 4 U/ml in all subsequent experiments.

We next wished to determine whether IL-2 added at the initiation of cultures could effectively increase proliferation in costimulator experiments with CM plus anti-μ. Our results indicate (see Table XIV) that rIL-2 at a final concentration of 4 U/ml was able to yield slightly elevated levels of proliferative activity in all cases tested with the exception of one (patient 6). This was especially important when stimulating poorly responding lines (patients 7, 11) since the presence of IL-2 increased proliferative activity and in turn increased the probability of isolating cells in mitosis.

Karyotypic Analysis of Patients. In order to achieve maximal proliferation, CLL lines were cultured with anti-μ plus exogenous IL-2 (4 U/ml final concentration). Cell isolates from each patient were also cultured in parallel under the same conditions but with the

TABLE XIII

Dose-Response Profile of Several Randomly Selected Patients to rIL-2[a]

Patient	Dosage rIL-2 (U/ml)[b]					
	Medium	32	16	8	4	2	1
	cpm[c]	cpm	cpm	cpm	cpm	cpm	cpm
7 (Exp 1)	977±107	3,941±215	3,860±486	3,329±111	3,364± 425	ND[d]	ND
8 (Exp 2)	324± 2	ND	ND	1,437± 28	1,153±1,081	1,011±54	856±49
9	86± 45	ND	1,690±187	1,718±154	1,799± 123	ND	ND
10	186± 41	ND	706± 88	577± 49	596± 47	ND	ND

[a] 2 x 10^5 cells in 0.3 ml were grown in the presence of: Medium (unsupplemented) or 3.3% rIL-2 over a range of concentrations. Cells were cultured under standard condition.

[b] U/ml represent final rIL-2 concentration.

[c] cpm are expressed as the mean ± SD of triplicate cultures.

[d] Not determined.

TABLE XIV

Response of Leukemic Cells to IL-2[a] as a Costimulator with CM plus Anti-μ[b]

	Proliferation in response to					
Patient	Medium cpm[c]	Anti-μ cpm	CM cpm	CM + anti-μ cpm	IL-2 cpm	IL-2 + CM + anti-μ cpm
5 (Exp 1)	977±107	5,487±716	6,738± 125	54,121±1,356	3,364±114	58,804± 735
5 (Exp 3)	3,320±295	5,661±468	14,564±1,348	36,618±3,945	7,464±447	51,098±1,851
6	294± 22	237± 38	2,984± 264	5,757± 589	1,282± 98	3,702± 287
7 (Exp 3)	196± 3	127± 18	592± 57	576± 35	540± 23	1,078± 46
11	58± 1	79± 29	352± 126	424± 45	106± 18	1,087± 189
12	353± 46	394±169	2,275± 84	4,428± 215	595± 47	5,569± 530

Footnotes to TABLE XIV

[a] rIL-2 was a generous gift of Drs. W.R. Benjamin and J.J. Farrer, Hoffmann LaRoche, Inc., Nutley, NJ. This lymphokine was added at a concentration of 4 U/ml.

[b] 2×10^5 cells in 0.3 ml were grown in the presence of: Medium (unsupplemented) or 3.3% anti-μ (10 μg/ml) or 33% CM (1:32 or 1:128 or 1:512) or CM plus anti-μ or 3.3% rIL-2 (4 U/ml) or CM + anti-μ + rIL-2. Cells were cultured for 96 hr and were pulsed with 1 μCi [^{3}H]thymidine during the last 16 hr prior to harvesting.

[c] cpm are expressed as the mean ± SD of triplicate cultures.

additional presence of 1 ng/ml (final concentration) of TPA. Following 96-hr cultures, the cells were harvested, fixed and spread onto glass slides as described in Materials and Methods (this chapter) for identification of mitotic figures. The following patients were tested: 5, 6, 7, 11, 12, 13, 14, 15, 16, 17 and 18. One patient not previously mentioned in this study was excluded following rediagnosis of lymphoma.

An examination of spreads from all 12 patients under study including the case of lymphoma revealed mitotic activity in all but a single case (patient 6). Oddly, patient 6 was a spontaneous proliferator (see Chapter 2) and the lack of mitotic activity can probably be attributed to the inclusion of anti-μ in culture which, as we have demonstrated, has the effect of reducing the level of proliferative activity induced by T cell-derived factors in our CM.

The appearance of dividing cells cultured with or without TPA was substantially dissimilar. Cells cultured with TPA often demonstrated less mitotic activity as judged by the total numbers of metaphases encountered than cells cultured with anti-μ + CM + rIL-2 only. In addition, and more importantly, the quality of the metaphase chromosomes differed. TPA-induced mitotic figures consisted of much more highly condensed chromosomes which, as suspected by this investigator and later confirmed by trained analysts, were exceedingly difficult to interpret. The condition of mitotic figures induced without TPA was superior. The chromosomal spreads were more loosely arranged and individual chromosomes appeared to be less condensed. Thereafter, only cells cultured in the absence of TPA were utilized for analysis. The location of each mitotic figure was

carefully noted and a minimum of 5 metaphases per slide were identified and forwarded for evaluation.

Table XV is a compendium of karyotypic results of all patients thus tested. One patient (6) out of a total population of 11 individuals failed to yield detectable metaphases. The mitotic figures of an additional three patients (11, 14, 18) because of poor quality were impossible to characterize, thus leaving a total of 7 CLL lines amenable to analysis. Of these, two lines (15, 16) demonstrated a lack of gross chromosomal abnormalities which were detectable at the level of chromosomes, although this does not rule out localized aberrations which might be elucidated through higher resolution DNA sequencing techniques. This is particularly true of the 11, 14 translocation known to be a common chromosomal abnormality in CLL (Tsujimoto et al., 1984). The remaining leukemic lines bore a wide variety of identifiable gross chromosomal aberrations, all of which have been previously described in the literature (Pittman and Catovsky, 1984). However, correlations between these aberrations and the important functional parameter-cellular proliferation were not apparent. This was no doubt due to the small size of the sample population.

However, in the case of chromosome 12, which has been the subject of considerable debate with regard to its importance as a prognostic indicator. Han et al. (1985) and Juliusson and Gahrton (1985) reported its incidence to be associated with a reduced proliferative response. Patient 7 displayed the most aberrant karyotype including trisomy 12 and was a poor proliferator (1,078±206 cpm). Patient 12 possessed a slightly less aberrant karyotype (pseudodiploid)

TABLE XV

Cytogenetic Analysis, Q Banding Technique[a]

Patient	Proliferative[b] Response	Karyotype
5	high	46 xy abnormal (12) - very small
6	high	no mitoses found
7	low	48 xx tri (12) tri (9) trans 11, 14
11	low	failure of analysis[c]
12	moderate	46 xy tri (12) mono (9)
13	moderate	45 xy del (1)
14	high	failure of analysis
15	low	46 xy
16	low	46 xy
17	moderate	46 xy abnormal (3)
18	low	failure of analysis

Footnotes for Table XV:

[a] Karyotypic analysis performed by Dr. Jahnwar, Dept. of Cytogenetics, MSKCC, according to the method of Chaganti et al. (1983).

[b] Proliferative response of individual BCLL lines cultured under standard conditions. Maximal response measured at optimal conditions with 33% CM (1:32 or 1:123) + 3.3% anti-μ (10 μg/ml) + 3.3% rIL-2 (4 U/ml). Responder index: High responders (cpm >15,000, moderate responders (cpm > 4,000), low responders (cpm >1,000). For purposes of comparison polyclonal B cells average >25,000 cpm.

[c] Stemming from poor quality of mitotic figures (poor separation within metaphase spreads or highly condensed individual chromosomes.

and these cells were found to be proliferatively more responsive (5,569±530 cpm) while patient 5, with a near-normal karyotype but with an abbreviated chromosome 12, demonstrated the highest proliferative levels (51,098±1,851). An alternative explanation for these findings could be based on the existence of a quantitative gradient of chromosomal defects; however, other patients (15, 16) with ostensibly normal karyotypes displayed low levels of cellular proliferation.

DISCUSSION

This study had as its major objectives two goals: 1) The utilization of T cell-derived factors in place of certain commonly utilized mitogens to maximize cell division in recalcitrant populations of luekemic cells thereby rendering them more amenable to chromosomal analysis, and 2) determining whether variability in karyotypes could be mirrored in functional clonal subtypes as they relate to cellular proliferation. In our attempts to maximize cellular proliferation we observed that although TPA could deliver a potent activation signal (see Table XII), particularly in conjunction with CM and anti-μ, its use for purposes of chromosomal analysis held several caveats. TPA was found to inhibit proliferation of several anti-μ stimulated lines. This result parallels a recent report in a murine B cell model (Mizugichi et al., 1986) but has not been described to date in human cells. This finding has further implications which will be discussed further (see Conclusion). We also determined that TPA-induced proliferative induction yielded poor quality mitotic figures with individual chromosomes so highly

condensed that they were impossible to analyze. In addition, because of its toxicity and substantial multipotential effects, TPA-induced chromosomal aberrations cannot be ruled out.

The supplemental use of exogenous IL-2 within our culture system (CM + anti-μ), on the other hand, avoids TPA-associated drawbacks and results in enhanced levels of proliferation (see Table XIV). Undoubtedly, poorly responsive leukemic cell lines could benefit from the use of higher dosages of rIL-2 (4 U/ml) than we employed and could lead to dramatic enhancement in rates of cell growth (Ralph et al., 1984). This combination of factors was found to be highly stimulatory to leukemic B cells, specificity being achieved by rigorous depletion of T cells. The lack of contaminating T cells could be expected to dramatically reduce the percentage of false normal karyotypes as has recently been demonstrated by Knuntila et al. (1986). If further gains in yields of dividing cells are required, synchronization of cell cultures could be employed. Synchronization using methotrexate (Yunis et al., 1981) applied over the last 17 hr of culture was found to raise the mitotic index and yielded longer metaphase chromosomes with increased numbers of bands per haploid set (Yunis et al., 1982).

Due to a paucity of data (Table XV), it was not possible to draw conclusions concerning possible correlations between proliferative function and karyotype. Clearly, a far more extensive collection of data is required to render such an analysis meaningful. However, as previously stated, our data weakly hints that trisomy 12 may adversely affect the degree of cellular proliferation. It can be speculated that in the absence of an association between karyotype

and the functional parameters proliferation and differentiation, that the high degree of functional heterogeneity evidenced by CLL cells could be due to external influences and not to intrinsic abnormalities. Lymphokine availability altering ontologic development would be a prime candidate for such exogenous influences.

CONCLUSION

In addition to fostering improved patient care, the study of monoclonal B cells of leukemic origin and, in particular CLL, has led over the past several years to their utilization as functional models of B cell development (Kishimoto, 1983). The general lack of availability of cloned antigen-specific human B cells, the large number of neoplastic cells obtainable from small quantities of patient peripheral blood, the relative commonness of this hematologic malignancy, the extended survival time demonstrated by patients and the development of techniques yielding cell isolates consisting of nearly pure populations of leukemic lymphocytes has rendered the use of CLL lymphocytes as investigational tools not only attractive but, moreover, compelling. These considerations, coupled with the enormous gains which have recently been made and continue to be made in unraveling the physiological conditions necessary for inducing B cell maturation, have been primary motivations for the investigational use of these leukemic cells. Central to the issue of B cell activation and subsequent maturation continues to be the ongoing identification, characterization and availability of allogeneically-derived soluble factors - lymphokines - having the capability of triggering monoclonal leukemic B cells to directly proliferate and differentiate in the absence of a complicating need for T cell help and presumably in a manner paralleling *in vivo* conditions.

These studies reinforce the recently favored view that CLL lymphocytes can be induced to proliferate and, in some cases, differentiate *in vitro* in response to stimulation by B cell immunoregulatory factors (Fu *et al.*, 1979; Yoshizaki *et al.*, 1982).

As such, they are not the quiescent, developmentally frozen entities which they had previously been perceived as being (Fu et al., 1974) by virtue of their general unresponsiveness to various previously utilized T cell-dependent mitogens including PHA, Con A and PWM. This responsiveness to B cell-active factors, although highly variable and frequently of modest proportions in comparison to polyclonal B cells, nonetheless has important implications for understanding the etiology of CLL. For example, it has recently been demonstrated by means of absorption experiments that prestimulated highly purified leukemic B lymphocytes from patients with CLL can directly deplete B cell growth factor (most likely BSF-I; Kishimoto et al., 1984) and IL-2 (Foa et al., 1985) in vitro from culture supernatants. We have also determined that in addition to IL-2 (Steinberg et al., 1985), leukemic cell growth is enhanced by BCGF II (obtained as the exclusive secretory product of a mouse-mouse hybridoma kindly provided by Dr. David Warren) when used as a co-stimulator in experiments with CM plus anti-μ. Even of greater importance, perhaps, is our observation that leukemic lines isolated from several patients (unpublished results) were highly proliferatively responsive to such diverse substances as human recombinant pluripoietin (kindly provided by Dr. Malcolm Moore) and to one or more undefined agents present in the concentrated urine of a normal two-year old child (kindly provided by Dr. Kenneth Landreth). It has also been reported that leukemic cells grow and differentiate in response to alpha, beta and gamma interferon (Ostlund et al., 1986). In light of the recognition and binding of these lymphokines by CLL lymphocytes, it can reasonably be postulated that binding and

sequestration of B cell-active factors by extensive populations of leukemic cells in accordance with our model (see Introduction) either directly or by means of their shed receptors could result in major immunoregulatory factor-deficits, which in turn could have far-reaching negative consequences for systemic immune function as clinically manifested by most patients to varying degrees. Shortages of BSF-I which plays a role in B cell activation (Rabin et al., 1985; Oliver et al., 1985), differentiation (Vitteta et al., 1985) and most recently has been shown to promote growth of helper T cell lines (Fernandez-Botran et al., 1986), as well as IL-2 whose widespread effects in a range of cell types other than lymphocytes has been established, could have major negative consequences for systemic patient immunocompetence as it relates to T cell and polyclonal B cell function. This information could be furnished in explanation of many observations previously cited in the Introduction, eluding to defective T cell function in patients. BCGF II is now also known to possess multiple activities apparently regulating differentiation of eosinophils (O'Garra et al., 1986) in addition to serving as an enhancer of B cell proliferation. The interferons, of course, possess a broad, if ill-defined, range of cellular activities as well, and their loss or physiological reduction could affect antiviral activity. Perhaps the most intriguing immunoregulatory factor in potential deficit in CLL patients is pluripoietin. If our findings are substantiated, reduced levels of this factor could go a long way toward explaining common late-stage bone marrow abnormalities leading to greatly diminished blood platelet counts and

hemoglobin deficiencies which have formed the basis for the most widely accepted clinical staging procedure (Rai et al., 1975).

In addition to binding a wide variety of immunoregulatory ligands, leukemic B cells appear to harbor several autocrine activities mediated by cytokinetic factors. It is now known, for example, that CLL lymphocytes can secrete IL-1 (Pistoia et al., 1986) as well as a second previously undescribed factor which is defined by its ability to inhibit migration of neutrophils (Siegbahn et al., 1986). It may be anticipated that both of these activities further perturbe immune-homeostasis. It is tempting to speculate, for example, that the fevers and night sweats, which are common early manifestations of this disease prompting individuals to visit their doctors, are the result of cyclical secretion of IL-1 by vast numbers of activated malignant lymphocytes.

Finally, CLL is characterized at the clinical, functional (i.e., cellular proliferation and differentiation) and cytogenetic level by its high degree of heterogeneity which is unmatched by other known lymphoproliferative disorders. This study was intended, in part, to catalogue this diversity. The key unanswered question, in this regard, lies in the origination of the functional (and by extension, clinical) variation demonstrated by various CLL lines. Is what is observed a function of the genotypic composition of individual neoplastic clones, or does the external (in vivo) environment modified by unbalanced lymphokine levels and altered T cell proportions contribute equally to the large degree of variation so predominant in this family of diseases? The problem clearly reduces to a classic case of nature vs nurture at the cytological level.

APPENDIX

Compendium of Lymphokine and Non-Lymphokine Associated Activities Present Within the Conditioned Medium (CM)

Activator	Activity*
Lymphokine	
Interleukin-2	9,000 U/ml
Interferon-gamma	6,000 U/ml
Interferon-alpha	Not detected
Interferon-beta	Not detected
B cell growth factor	500 U/ml
B cell differentiation factor	1,000 U/ml
Interleukin-1	trace
Polyclonal Activator	
PHA-P	Not detected
PMA	Not present
SAC	Not present

* All assays were conducted by the Immunopharmacology Department of Hoffmann La-Roche, Inc., Nutley, NJ.

REFERENCES

Aisenberg, A.C. and K.J. Block. 1972. Immunoglobulins on the surface of neoplastic lymphocytes. N. Engl. J. Med. 287:272.

Aisenberg, A.C., K.J. Block and I.C. Long. 1973. Cell-surface immunoglobulins in chronic lymphocytic leukemia and allied disorders. Am. J. Med. 55:184.

Anderson, K.C., M.P. Bates, B.L. Slaugenhoupt, G.S. Pinkus, S.F. Schlossman and L.M. Nadler. 1984. Expression of human B cell-associated antigens on leukemias and lymphomas: a model of human B cell differentiation. Blood 63:1424.

Andersson, J., W.W. Bullock and F. Melchers. 1974. Inhibition of mitogenic stimulation of mouse lymphocytes by anti-mouse immunoglobulin antibodies. Eur. J. Immunol. 4,715.

Autio, K., O. Turunen, O. Penttila, E. Eramaa, A. De La Chapelle and J. Schroder. 1979. Human chronic lymphocytic leukemia: karyotypes in different lymphocyte populations. Cancer Genet. Cytogenet. 1:147.

Baeker, T.R. and T.L. Rothstein. 1985. Proliferation of human malignant lymphocytes induced by anti-IgM independent of B cell growth factor. J. Immunol. 134:3532.

Baldini, L., R. Mozzana, A. Cortelezzi, A. Neri, F. Radaelli, B. Cesana, A.T. Maiolo and E.E. Polli. 1985. Prognostic significance of immunoglobulin phenotype in B cell chronic lymphocytic leukemia. Blood 65:340.

Benjamin, W.R., J. Fuller-Farrar and J.J. Farrar, 1984. Growth factors for B cells. In: Practical Methods in Clinical Immunology (In press).

Binet, J.L., A. Auquier, G. Dighiero, C. Chastang, H. Piguet, J. Goasguen, G. Vaugier, P. Colona, F. Oberling, M. Thomas, G. Tcherenia, C. Jaquillat, P. Boivin, C. Lesty, M.T. Duault, M., Monconduit, S. Belabbes and F. Gremy. 1981. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 48:198.

Bloem, A.C., C.J. Heijnen, B.J. Bast and R.E. Ballieux. 1985. T cells in B cell chronic lymphocytic leukemia. II. Lack of antigen-specific T suppressor cells and their progenitors. J. Immunol. 135:4261.

Bona, C., S. Broden, A. Dimetriu and T.A. Waldmann. 1979. Polyclonal activation of human B lymphocytes by Nocardia water soluble mitogen (NWSM). Immunol. Rev. 45:69.

Bona, C.A. and A.S. Fauci. 1980. In vitro idiotypic suppression of chronic lymphocytic leukemia lymphocytes secreting monoclonal immunoglobulin M anti-sheep erythrocyte antibody. J. Clin. Invest. 65:761.

Boyd, A.W., K.C. Anderson, A.S. Freedman, D.C. Fishen, P. Slaighenhoupt, S.F. Schlossman and L.M. Nadler. 1985. Studies of in vitro activation and differentiation of human B lymphocytes. J. Immunol. 135:516.

Boyd, A.W., A.S. Freedman, J.C. Horowitz, K.C. Anderson, D.C. Fisher, K.J. Rosen, S.F. Schlossman and L.M. Nadler. 1986. Studies of the in vitro activation and differentiation of human B lymphocytes. Cell. Immunol. 99:228.

Brown, V.A., S.K. Smith, A.E. Dewar, G. Stockdill and A.H. Maddy. 1985. Surface glycoproteins as markers of the cellular status of B chronic lymphocytic leukaemia lymphocytes. Clin. Exp. Immunol. 62:95.

Butler, J.L., A. Maraguchi, H.C. Lane and A.S. Fauci. 1983. Development of a human T-T cell hybridoma secreting B cell growth factor. J. Exp. Med. 157:60.

Caligaris Cappio, F., L. Bergui, L. Tesio, G. Corbascio, F. Tousco and P.C. Marchisio. 1986. Cytoskeleton organization is aberrantly rearranged in the cells of B chronic lymphocytic leukemia and having cell leukemia. Blood 67:233.

Cambier, J.C. 1986. Seeing the way to B-cell growth. Nature 319:620.

Cambier, J.C., E.S. Vitetta, J.R. Kettman, G.M. Wetzel and J.W. Uhr. 1977. B-cell tolerance. III. Effect of papain-mediated cleavage of cell surface IgD on tolerance susceptibility of murine B cells. J. Exp. Med. 246:107.

Chaganti, R.S.K., S.C. Jhanwar, B. Koziner, Z. Arlin, R. Mertelsmann and B.D. Clarkson. 1983. Specific translocations characterize Burkitt's-like lymphoma of homosexual men with acquired immunodeficiency syndrome. Blood 61:1269.

Chen, Y.H. and P. Heller. 1978. Lymphocyte surface immunoglobulin secretion in vitro in chronic lymphocytic leukemia (CLL). Blood 52:601.

Chiorazzi, N., S.M. Fu, G. Moutagen, H.G. Kunkel, K.R. Rai and T. Gee. 1979. T cell helper defect in patients with chronic lymphocytic leukemia. J. Immunol. 122:1087.

Chiorazzi, N., S.M. Fu and H.G. Kunkel. 1980. Stimulation f human B lymphocytes by antibodies to IgM and IgG. Functional evidence for the expression of IgG on B lymphocyte surface membranes. Clin. Immunol. Immunopathol. 15:301.

Cossmann, J., L.M. Nechers, R.M. Braziel, J.B. Trepel, S.J. Korsmeyer and A. Baklshi. 1984. In vitro enhancement of immunoglobulin gene expression in chronic lymphocytic leukemia. J. Clin. Immunol. 73:587.

Croce, C.M., M. Shander, J. Martinis, L. Cicure, G.G. D'Ancona, T.W. Dolby and H. Koprowski. 1979. Chromosomal location of the genes for human immunoglobulin heavy chains. Proc. Natl. Acad. Sci. USA 76:3416.

DeFranco, A.L., E.S. Raveche and W.E. Paul. 1985. Separate control of B lymphocyte early activation and proliferation in response to anti-IgM antibodies. J. Immunol. 135:87.

DePaoli, P., G.L. Molaro, S. Battistin, M. Reitano, C. Castiglia and G.C. Santini. 1984. Peripheral blood and bone marrow lymphocytes of B chronic lymphocytic leukemia patients. Boll. 1st. Sieroter. Milan 63:534.

Dickler, H.B. and H.G. Kunkel. 1972. Interaction of aggregated immunoglobulin with B lymphocytes. J. Exp. Med. 136:191.

Dosch, H.M., R.K.B. Shuurman and E.W. Gelfand. 1980. Polyclonal specific mitogens. J. Immunol. 125:827.

Dutton, R.W., R. Falkoff, J.A. Hirst, M. Hoffman, J.W. Kappler, J.R. Kettman, J.F. Leshey and D. Vann. 1971. Is there evidence for a non-antigen specific diffusable chemical mediator from the thymus-derived cell in the initiation of the immune response? Prog. Immunol. 1:355.

Faguet, G.B. 1979. Mechanisms of lymphocyte activation. The role of suppressor cells in the proliferative response of chronic lymphatic leukemia lymphocytes. J. Clin. Invest. 63:67.

Falkoff, R.J.M., L.P. Zhu and A.S. Fauci. 1982. Separate signals for human B cell proliferation and differentiation in response to Staphylococcus aureus: Evidence for a two-signal model of B cell activation. J. Immunol. 129:97.

Fauci, A.S. and R.E. Ballieux. 1979. Antibody Production in Man: In Vitro Synthesis and Clinical Implications. Academic Press, New York.

Fauci, A.S. and K.R. Pratt. 1976. Activation of human B lymphocytes. I. Direct plaque-forming cell assay for the measurement of polyclonal activation and antigenic stimulation of human B lymphocytes. J. Exp. Med. 144:674.

Fauci, A.S., K.R. Pratt and G. Whelan. 1977. Intrinsic B cell defect in the immunoglobulin deficiency of chronic lymphocytic leukemia. Clin. Res. 25:482A.

Fernandez, L.A., J.M. MacSween and G.R. Langley. 1981. Impaired T-cell responses in chronic lymphocytic leukemia. Lack of suppressor cell effect. Immunol. Immunopathol. 18:168.

Fernandez, L.A., J.M. MacSween and G.R. Langley, 1986. Immunoregulation of B lymphocyte colony formation by T cell subsets in patients with chronic lymphocytic leukemia. Blood 67:294.

Finkelman, F.D. and E.S. Vitetta. 1984. Role of surface immunoglobulin in B lymphocyte activation. Fed Proc. 43:2624.

Foa, R., M. Giovarelli, C. Jemma, M.T. Fierro, P. Lusso, M.L. Ferrando, F. Lauria and G. Forni. 1985. Interleukin 2 (IL2) and interferon-gamma production by T lymphocytes from patients with B-chronic lymphocytic leukemia: evidence that normally released IL2 is absorbed by the neoplastic B cell population. Blood 66:614.

Forsgren, A. and J. Sjoquist. 1966. Protein A from S. aureus. I. Pseudoimmune reaction with human gamma-globulin. J. Immunol. 97:822.

Forsgren, A., A. Svedjelund and H. Wizzell. 1976. Lymphocytic stimulation by protein A of Staphylococcus aureus. Eur. J. Immunol. 6:207.

Foulis, A.K., A.J. Cochran and J.R. Anderson. 1973. Surface immunoglobulin of leukemic cells. Clin. Exp. Immunol. 14:481.

Frenandez-Botran, R., P.H. Krammer, T. Diamantstein, J.W. Uhr and E.S. Vitetta. 1986. B cell-stimulatory factor (BSF-1) promotes growth of helper T cell lines. J. Exp. Med. 164:580.

Fu, S.M. 1982. Chronic lymphocytic leukemia: Immunoglobulin synthesis by leukemia B cells and some important pathogenetic factors. In: B and T Cell Tumors: Biological and Clinical Aspects. UCLA Symposium on Molecular and Cellular Biology, 24: galley proof, (Vitetta, E., ed.). Academic Press, NY.

Fu, S.M., R.J. Winchester, T. Feizi, P.D. Walzer and H.G. Kunkel. 1974. Idiotypic specificity of surface immunoglobulin and the maturation of leukemic bone marrow derived lymphocytes. Proc. Natl. Acad. Sci. USA 71:4487.

Fu, S.M., N. Chiorazzi, H.G. Kunkel, J.P. Halper and H.S. Harris. 1978. Induction of in vitro differentiation and immunoglobulin synthesis of human leukemic B lymphocytes. J. Exp. Med. 140:1570.

Fu, S.M., N. Chiorazzi and H.G. Kunkel. 1979. Differentiation capacity and other properties of leukemic cells of chronic lymphocytic leukemia. Immunol. Rev. 48:23.

Gahrton, G. and K.H. Robert. 1982. Chromosomal aberrations in chronic B-cell lymphocytic leukemia. Cancer Genet. Cytogenet. 6:171.

Gahrton, G., L. Zech, K.H. Robert and A.G. Bird. 1979. Mitogenic stimulation of leukemic cells by Epstein-Barr virus. N. Engl. J. Med. 301:438.

Gahrton, G., K.H. Robert, K. Friberg, L. Zech and A.G. Bird. 1980. Extra chromosome 12 in chronic lymphocytic leukemia. Lancet 1:146.

Gahrton, G., K.H. Robert and L. Zech. 1981. Incidence of specific chromosome aberrations in chronic lymphocytic leukemia. Blood 58:859.

Gahrton, G., G. Juliusson, K.H. Robert and L. Zech. 1985. Review: Specific chromosomal markers in B- and T-cell chronic lymphocytic leukemia. Tumour Biol. 6:1.

Gale, R.P. and K.A. Foon. 1985. Chronic lymphocytic leukemia: Recent advances in biology and treatment. Ann. Int. Med. 103:101.

Geha, R.S. 1983. Presence of circulating anti-idiotype-bearing cells after booster immunization with tetanus toxoid (TT) and inhibition of anti-TT antibody synthesis by auto-anti-idiotypic antibody. J. Immunol. 130:1634.

Gordon, J., H. Mellstedt, P. Aman, P. Biberfeld, M. Bjorkholm and G. Klein. 1983. Phenotypes in chronic B-lymphocytic leukemia probed by monoclonal antibodies and immunoglobulin secretion studies: identification of stages of maturation arrest and the relation to clinical findings. Blood 62:910.

Gotze, D., H. Fiebig, H.W. Mansfeld, U. Mey, E. Schon, R. Uhle and S. Ansorge. 1985. Comparison of different markers on blood lymphocytes of chronic lymphocytic leukemia. Folia. Haematol. 112:233.

Grossi, C.E., A. Zicca, A. Leprini, A. Cadoni, V. Pistoia and M. Ferrarini. 1982. Acid hydrolases as markers of maturation in B-cell chronic lymphocytic leukemia. Blood 60:220.

Guglielmi, P., J.L. Preud'homme, R. Ciordaru-Barot and M. Seligmann. 1982. Mitogen-induced maturation of chronic lymphocytic leukemic B lymphocytes. J. Clin. Immunol. 2:186.

Gupta, S. C.D. Platsoucas and R.A. Good. 1979. Receptors for IgA on a subpopulation of human B lymphocytes. Proc. Natl. Acad. Sci. USA 76:4025.

Halper, J., S.M. Fu, A. Gottlieb, R.J. Winchester and H.G. Kunkel. 1979. Poor stimulatory capacity of leukemia cells of CLL patients despite the presence of Ia antigens. J. Clin. Invest. 64:1141.

Han, T., H. Ozer, E.S. Henderson, B. Dadey, A. Nussbaum-Blumerson and M. Barcos. 1981. Defective immunoregulatory T cell function in chronic lymphocytic leukemia. Blood 58:1182.

Han, T., N. Sadamori, J. Takeushi, H. Ozer, S.E. Henderson, A. Bhargava, J. Fitzpatrick and A.A. Sandberg. 1982. Clonal chromosomal abnormalities in patients with Waldenstroms and CLL-associated macroglobulinemia: significance of trisomy 12. Blood 62:525.

Han, T., H. Ozer, N. Sadamori, L. Emrich, G.A. Gomez, E.S. Henderson, M.L. Bloom and A.A. Sandberg. 1984. Prognostic importance of cytogenic abnormalites in patients with chronic lymphocytic leukemia. N. Engl. J. Med. 310:288.

Han, T., L.J. Emrich, H. Ozer and A.A. Sandberg. 1985. Prognostic implication of trisomy 12 and non-trisomy 12 karyotypes in B cell chronic lymphocytic leukemia. Letter to Editor. Blood 66:470.

Hans, R. and B. Dadey. 1979. In vitro functional studies of mononuclear cells in patients with CLL. Cancer 43:109.

Harran, W.L., D.L. DiGiusto and J.C. Cambier. 1984. mIgM:mIgD ratios on B cells: mean mIgD expression exceeds mIgM by 10-fold on most splenic B cells. J. Immunol. 132:1712.

Hensey, P., J. Wetherspoon, G. Reid and F.W. Gunz. 1980. Hypogammaglobulinemia associated with abnormalities of both B and T lymphocytes in patients with chronic lymphatic leukemia. Clin. Exp. Immunol. 39:698.

Herrmann, F., G. Sieber, Z. Chen, B. Enders, B. Komischke and H. Ruhl. 1983. Further evidence for T-cell abnormalities in chronic lymphocytic leukemia of the B cell type. Clin. Exp. Immunol. 53:109.

Hirano, T., T. Teranishi, B.H. Lin and K. Onove. 1984. Human helper T cell factor(s). IV. Demonstration of a human late-acting B cell differentiation factor acting on Staphylococcus aureus Cowan I-stimulated B cells. J. Immunol. 133:798.

Hirano, T., T. Taga, N. Nakano, K. Yasukawa, P. Kashiwamura, K. Shimigu, K. Nakajima, K.H. Pyrun and T. Kishimoto. 1985. Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proc. Natl. Acad. Sci. USA 82:5490.

Howard, M. and W.E. Paul. 1983. Regulation of B cell growth and differentiation by soluble factors. Annu. Rev. Immunol. 1:307.

Howard, M., J. Farrar, M. Hilfiken, B. Johnson, K. Takatsu, T. Hamaola and W.E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. J. Exp. Med. 155:914.

Humphreys, R.E., J.M. McCune, L. Chess, H.C. Herrman, D.J. Malenka, D.L. Mann, P. Parham, S.F. Schlossman and J.L. Strominger. 1976. Isolation and immunologic characterization of a human B lymphocyte specific cell-surface antigen. J. Exp. Med. 144:98.

Hurley, J.N., S. Man Fu, H.G. Kunkel, R.S.K. Chaganti and J. German. 1980. Chromosome abnormalities of leukemic B lymphocytes in CLL. Nature 283:76.

Jerrells, T.R., J.H. Dean, G.L. Richardson and R.B. Heberman. 1980. Depletion of monocytes from human peripheral blood mononuclear leukocytes: comparison of the Sephadex G-10 column method with other commonly used techniques. J. Immunol. Methods 32:11.

Johnstone, A.P., J.C. Jersenius, R.E. Milland and L. Hudson. 1982. Mitogen-stimulated immunoglobulin production by chronic lymphocytic leukemia lymphocytes. Clin. Exp. Immunol. 47:697.

Juliusson, G. and G. Gahrton. 1985. Prognostic implication of trisomy 12 and non-trisomy 12 karyotypes in B cell chronic lymphocytic leukemia. Letter to Editor. Blood 66:471.

Juliusson, G., K.H. Robert, B. Nilsson and G. Gahston. 1985. Prognostic value of B-cell mitogen-induced and spontaneous thymidine uptake in vitro in chronic B-lymphocytic leukaemia cells. Brit. J. Haematol. 60:429.

Jung, L.K.L., T. Hara and S.M. Fu. 1984. Detection and functional studies of p60-65 CTac antigen on activated human B cells. J. Exp. Med. 160:1597.

Junichiro, M., M.A. Beaven, J.H. Li and W.E. Paul. 1986. Phorbol myristate acetate inhibits anti-IgM-mediated signaling in resting B cells. Proc. Natl. Acad. Sci. USA 83:4474.

Kabelitz, D., K. Pfefer, D. von Steldem, P. Bartmann, O. Brudler, C. Nerl and H. Wagner. 1985. In vitro maturation of B cells in chronic lymphocytic leukemia. I. Synergistic action of phorbol ester and interleukin 2 in the induction of Tac antigen expression and interleukin 2 responsiveness in leukemic B cells. J. Immunol. 135:2876.

Kanter, R.J., J. Steinberg, K.R. Rai, R.E. Calhoon and A. Sawitsky. 1982. T-cell determinants in B-cell CLL. Abstract, Amer. Fed. Clin. Res. 30:351A.

Karl-Henrich, R., G. Gahston, K. Friberg, L. Zeck and B. Nilsson. 1982. Extra chromosome 12 and prognosis in chronic lymphocytic leukemia. Scand. J. Hematol. 28:163.

Katz, R.L., A. Cork, I. Cox, S.G. Murphy and J.M. Trujillo. 1982. A comparative study of the effectiveness of different mitogens for karyotypic analysis in chronic B cell leukemia. Leukemia Res. Ch. 6:183.

Kay, N.E. 1981. Abnormal T-cell function in CLL. Blood 57:418.

Kehrl, J.H., A. Muraguchi and A.S. Fauci. 1984. Differential expression of cell activation markers after stimulation of resting human B lymphocytes. J. Immunol. 132:2857.

Keller, R.H., D.G. Blake, S. Lyman and R. Siebenlist. 1981. Immunoregulatory abnormalities in chronic lymphocytic leukemia. I. Correlation of immunoregulatory subpopulations with stage of disease. J. Clin. Lab. Immunol. 6:201.

Kimby, E., H. Mellstedt, M. Bjorlcholm and G. Holm. 1985. Surface immunoglobulin pattern of the leukemic cell population in chronic lymphocytic leukaemia (CLL) in relation to disease activity. Hematol. Oncol. 3:261.

Kirchner, H., G. Tosato, R.M. Blaese, S. Broden and I.M. Magrath. 1979. Polyclonal immunoglobulin secretion by human B lymphocytes exposed to Epstein-Barr virus in vitro. J. Immunol. 122:1310.

Kishimoto, T., T. Miyake, Y. Nishijawa, T. Watanabe and Y. Yamamura. 1975. Triggering mechanism of B lymphocytes. I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B cells. J. Immunol. 115:1179.

Kishimoto, T., K. Yoshizaki, M. Okada, Y. Miki, T. Nakagawa, N. Yoshimura, H. Kishi and Y. Yamamura. 1982. Activation of human monoclonal B cells with anti-Ig and T cell-derived helper factor(s) and biochemical analysis of the transmembrane signaling in B cells. Proc. U.C.L.A. Symp. Mol. Cell. Biol. 24:375.

Kishimoto, T., K. Yoshizaki, M. Kimoto, M. Okada, T. Kuritani, H. Kikutani, K. Shimizu, T. Nakagawa, N. Nakegawa, Y. Miki, H. Kishi, K. Fukumaga, T. Yoshikubo and T. Tada. 1984. B cell growth and differentiation factors and mechanism of B cell activation. Immunol. Rev. 78:97.

Knowles II, D.M., B. Tolidjian, C.C. Marboe, R.S. Mittler, M.A. Talle and G. Goldstein. 1984. Distribution of antigens defined by OKB monoclonal antibodies on benign and malignant lymphoid cells and on nonlymphoid tissues. Blood 62:886.

Knuntila, S., E. Elonen, L. Teerenhovi, L. Rossi, R. Leskinen and C.D. Bloomfield. 1986. Trisomy 12 in B cells of patients with B-cell chronic lymphocytic leukemia. N. Engl. J. Med. 314:865.

Kronvall, G. and R.G. Williams. 1969. Differences in anti-protein A activity among IgG subgroups. J. Immunol. 103:828.

Kubo, R.T., H.M. Grey and B. Pirofsky. 1974. IgD: A major immunoglobulin on the surface of lymphocytes from patients with chronic lymphatic leukemia. J. Immunol. 112:1952.

Kukutani, H., T. Taga, S. Akira, H. Kishi, Y. Miki, O. Saiki, Y. Yamamura and T. Kishimoto. 1985. Effect of B cell differentiation factor (BCDF) on biosynthesis and secretion of immunoglobulin molecules in human B cell lines. J. Immunol. 134:990.

Lantz, O., Grillot-Corvalin, C. Schmitt, J.P. Fernand and J.C. Broucet. 1985. Interleukin 2 induced proliferation of leukemic human B cells. J. Exp. Med. 161:1225.

Lauria, F., R. Foa, V. Mantovani, M.T. Fierro, D. Catovski and S. Tura. 1983. T-cell function and abnormality in B-chronic lymphocytic leukaemia: evidence of a defect of the T-helper subset. Br. J. Haematol. 54:277.

Leanderson, T. and L. Forni. 1984. Effects of v-specific antibodies on B cell growth and maturation. Eur. J. Immunol. 14:1016.

LeBeau, M.M. 1986. Chromosomal fragile sites and cancer-specific rearrangements. Blood 67:849.

Lennert, K. 1979. Malignant lymphomas other than Hodgkin's disease. In: Handbuch der Speziellen Patologischen Anatomil und Hestologie. I. Band, Teil 3B. Springer, Berlin.

Leonard, W.J., J.M. Depper, T. Uchiyama, K.A. Smith, T.A. Waldmann and C. Green. 1982. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor: partial characterization of the receptor. Nature 300:267.

Liebson, H., M. Gefter, A. Zlotnik, P. Marrack and J. Kappler. 1984. Role of gamma interferon in antibody-producing responses. Nature 309:799.

Ligler, F.S., J.R. Ketlman, R.G. Smith and E.P. Frenkel. 1983. Immunoglobulin phenotype and B cells correlate with clinical stage of chronic lymphocytic leukemia. Blood 62:256.

Lukes, R.J. and R.D. Collins. 1977. Lukes-Collins classification and its significance. Cancer Treat. Rep. 61:971.

Maizel, A.L., J.W. Morgan, S.R. Mehta, N.M. Koutab, J.M. Bator and C.G. Sahasrabuddhe. 1983a. Long-term growth of human B cells and their use in a microassay for B-cell growth factor. Proc. Natl. Acad. Sci. USA 80:5047.

Maizel, A., S. Mehta, N. Kouttab, J. Morgan, R. Ford and C. Sahasrabuddhe. 1983b. Proliferation of human B lymphocytes mediated by a soluble factor. Fed. Proc. 42:2753.

Mangan, K.F. and L. D'Alessandro. 1985. Hypoplastic anemia in B cell chronic lymphocytic leukemia evolution of T cell-mediated suppression of erythropoiesis in early-stage and late-stage disease. Blood 66:533.

Matutes, E., A. Wechsler, R. Gomez, H. Chenchi and D. Catovsky. 1981. Unusual T-cell phenotype in advanced B-chronic lymphocytic leukemia. Brit. J. Haematol. 49:635.

Mayer, L., M.K. Crow and C. Thompson. 1985. Synergy between B cell differentiation factors and interleukin 2, using a monoclonal system. J. Immunol. 135:3272.

McFadden, S.F. and E.S. Vitetta. 1984. sIgD-negative B cells from neonatal mice do not respond to the thymus-independent antigen TNP-BA in limiting dilution cultures. J. Immunol. 132:1717.

Mehta, S.R., D. Conrad, R. Sandler, J. Morgan, R. Montagna and A. Maizel. 1985. Purification of human B cell growth factor. J. Immunol. 135:3298.

Melchers, F. and J. Andersson. 1984. B cell activation: Three steps and their variations. Cell 37:715.

Mills, K.H. and J.C. Cawley. 1985. Stimulated lymphoid cells of B lineage but not plasma or myeloid cells can express E receptor. Leukemia Res. 9:375.

Mingari, M.C., F. Gerosa, G. Carra, R.S. Acolla, A. Moretta, R.H. Zubler, T.W. Waldmann and L. Moretta. 1984. Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. Nature 312:640.

Mittler, R., P. Rao, G. Olini, E. Westberg, W. Newman, M. Hoffmann and G. Goldstein. 1985. Activated human B cells display a functional IL-2 receptor. J. Immunol. 134:2393.

Mizel, S.B. and D. Mizel. 1981. Purification to apparent homogeneity of murine interleukin 1. J. Immunol. 126:834.

Mizuguchi, J., M.A. Beaven, J.H. Li and W.E. Paul. 1986. Phorbol myristate acetate inhibits anti-IgM-mediated signaling in resting B cells. Proc. Natl. Acad. Sci. USA 83:4474.

Moayeri, H. and J.E. Sokal. 1979. In vitro leukocyte thymidine uptake and prognosis in chronic lymphocytic leukemia. Am. J. Med. 66:773.

Morita, M., J. Minowada and A.A. Sandberg. 1981. Chromosomes and causation of human cancer and leukemia. XIV. Chromosome patterns in stimulated lymphocytes of chronic lymphocytic leukemia. Cancer Genet. Cytogenet. 3:293.

Mosier, D.E. 1967. A requirement for two cell types for antibody formation in vitro. Science 158:1573.

Muraguchi, A. and A.S. Fauci. 1982. Proliferative responses of normal human B lymphocytes. Development of an assay system for human B cell growth factor (BCGF). J. Immunol. 129:1104.

Muraguchi, A., T. Kishimoto and Y. Miki. 1981. T cell replacing factor (TRF)-induced IgG secretion in a human lymphoblastoid cell line and demonstration of acceptors for TRF. J. Immunol. 127:412.

Muraguchi, A., T. Kishimoto, Y. Miki, T. Kuritani, T. Kaieda, K. Yoshizaki and Y. Yamamura. 1981. T cell-replacing factor (TRF)-induced IgG-secretion in a human B blastoid cell line and demonstration of acceptors for TRF. J. Immunol. 127:412.

Muraguchi, A., J.L. Butler, J.H. Kehrl and A.S. Fauci. 1983. Differential sensitivity of human B cell subsets to activation signals delivered by anti-μ antibody and proliferation signals delivered by a monoclonal B cell growth factor. J. Exp. Med. 157:530.

Muraguchi, A., J.L. Butler, J.H. Kehrl, R.J.M. Falkoff and A.S. Fauci. 1983. Selective suppression of an early step in human B cell activation by cyclosporin A. J. Exp. Med. 158:690.

Muraguchi, A., J.H. Kehrl, J.L. Butler and A.S. Fauci. 1984. Sequential requirements for cell cycle progression of resting human B cells after activation by anti-Ig. J. Immunol. 132:176.

Muraguchi, A.J., H. Kehrl, D.L. Longo, D.J. Volkman, K.A. Smith and A.S. Fuci. 1985. Interleukin 2 receptors on human B cells. Implications for the role of interleukin 2 in human B cell function. J. Exp. Med. 161:181.

Nakagawa, T., T. Hirano, N. Nakagawa, K. Yoshizaki and T. Kishimoto. 1985. Effect of recombinant IL-2 and γ-IFN on proliferation and differentiation of human B cells. J. Immunol. 134:959.

Nakanishi, K., M. Howard and A. Muraguchi. 1983. Soluble factors involved in B cell differentiation: identification of two distinct T cell replacing factors (TRFs). J. Immunol. 130:2219.

Nel, A.E., M. Navailles, D.L. Emerson, P. Goldschmidt-Clermont, S.K. Pathak, K.Y. Tsang and R.M. Galbraith. 1985. Altered configuration of Gc on the plasma membrane of transformed and malignant human B lymphocytes. Clin. Immunol. Immunopathol. 37:191.

Nies, K.M., J. Marshall, M.A. Oberlin, M.S. Halper and J.C. Brown. 1976. Chronic lymphocytic leukemia with gamma chain cytoplasmic inclusions. Amer. J. Clin. Invest. 65:948.

Noelle, R., P.H. Krammer, J. Ohara, J.W. Uhr and E.S. Vitetta. 1984. Increased expression of Ia antigens on resting B cells: an additional role of B-cell growth factor. Proc. Natl. Acad. Sci. USA 81:6149.

Nowell, P.C. and D.A. Hungerford. 1960. A minute chromosome in human chronic granulocytic leukemia. Science 132:1497.

Nowell, P., T.V. Shankey, J. Finan, D. Guerry and E. Besa. 1981. Proliferation, differentiation and cytogenesis of chronic leukemic B lymphocytes cultured with mitomycin-treated normal cells. Blood 57:444.

O'Garra, A., D.J. Warren, M. Holman, A.M. Popham, C.J. Sanderson and G.G.B. Klaus. 1986. Interleukin 4 (B-cell growth factor III eosinophil differentiation factor) is a mitogen and differentiation factor for preactivated murine B lymphocytes. Proc. Natl. Acad. Sci. 83:5228.

Okada, M., N. Sakaguchi, N. Yoshimura, H. Hara, K. Shimizu, H. Yoshida, K. Yoshizaki, S. Kishimoto, Y. Yamamura and T. Kishimoto. 1983. B cell growth factors and B cell differentiation factor from human T hybridoma: two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J. Exp. Med. 157:583.

Oliver, K., R.J. Nodle, J.W. Uhr, P.H. Krammer and E.S. Vitetta. 1985. B-cell growth factor (B-cell growth factor 1 or B-cell-stimulatory factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. Proc. Natl. Acad. Sci. 82:2465.

Oppenhein, J.J., B.G. Levanthal and E.M. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. J. Immunol. 101:262.

Östlund, L., S. Einhorn, K.-H. Robert, G. Juliusson and P. Biberfeld. 1986. Chronic B-lymphocytic leukemia cells proliferate and differentiate following exposure to interferon in vitro. Blood 67:152.

Parker, D.C. 1975. Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin. Nature 258:361.

Parker, D.C., J.F. Fothergill and D.C. Wadsworth. 1979. B lymphocyte activation by insoluble anti-immunoglobulin: induction of immunoglobulin secretion by a T cell-dependent soluble factor. J. Immunol. 123:931.

Pistoia, V., F. Cozzolino, A. Rubartelli, M. Torcia, S. Roncella and M. Ferrarini. 1986. In vitro production of interleukin 1 by normal and malignant human B lymphocytes. J. Immunol. 136:1688.

Pittman, S. and D. Catovsky. 1984. Prognostic significance of chromosome abnormalities in chronic lymphocytic leukaemia. Brit. J. Haematol. 58:649.

Plater-Zyberk, C., R.N. Maini, K. Lam, T.D. Kennedy and G. Janossy. 1985. A rheumatoid arthritis B cell subset expresses a phenotype similar to that in chronic lymphocytic leukemia. Arthritis Rheum. 28:971.

Platsoucas, C.D. 1985. Functional and phenotypic heterogeneity of electrophoretically separated leukemic B cells from patients with chronic lymphocytic leukemia. Clin. Exp. Immunol. 80:474.

Platsoucas, C.D., G. Fernandes, S.L. Gupta, S. Kempin, B. Clarkson, R.A. Good and S. Gupta. 1980. Receptors for immunoglobulin isotype on T and B lymphocytes from untreated patients with chronic lymphocytic leukemia. Clin. Exp. Immunol. 40:256.

Platsoucas, C.D., M. Galinski, S. Kempin, L. Reich, B. Clarkson and R.A. Good. 1982. Abnormal T lymphocyte subpopulations in patients with B cell chronic lymphocytic leukemia: an analysis by monoclonal antibodies. J. Immunol. 129:2305.

Preud'homme, J. and M. Seligmann. 1972. Surface bound immunoglobulins as a cell marker in human lymphoproliferative diseases. Blood 40:777.

Pryzma, D., J. Munoz, R.M. Galbraith, H.H. Fundenberg and G. Virella. 1980. Induction and suppression of immunoglobulin synthesis in culture of human lymphocytes: effects of pokeweed mitogen and Staphylococcus aureus Cowan I. J. Immunol. 124:656.

Pure, E. and E.S. Vitetta. 1980. Induction of murine B cell proliferation by insolubilized anti-immunoglobulins. J. Immunol. 125:1240.

Rabin, E.M., J. O'Hara and W.E. Paul. 1985. B-cell stimulatory factor 1 activates resting B cells. Proc. Natl. Acad. Sci. USA 82:2935.

Rai, K.R. and A. Sawitsky. 1981. Studies in clinical staging, lymphocyte function, and markers as an approach to the treatment of chronic lymphocytic leukemia. In: Studies in Contemporary Hematology. A.S. Gordon, R.D. Silber and J. LoBue (Eds.), Plenum Publishing, NY 2:227.

Ralph, P. G. Jeong, K. Welte, R. Mertelsmann, H. Rabin, L.E. Henderson, L.M. Souja, T.C. Boone and R.J. Robb. 1984. Stimulation of immunoglobulin secretion in human B lymphocytes as a direct effect of high concentrations of 12-2. J. Immunol. 133:2442.

Rappaport, H. 1966. Tumor of the hematopoietic system. In: Atlas of Tumor Pathology, Section 3, Fascicle 8. Armed Forces Institute of Pathology, Washington, DC.

Rewald, E., M.E. Estevez and L. Sen. 1985. Monoclonal B-cell lymphocytosis in early childhood. A case report. Am. J. Pediatr. Hematol. Oncol. 7:331.

Robert, K.G. 1979. Induction of monoclonal antibody synthesis in malignant human B cells by polyclonal B cell activators. Immunol. Rev. 48:123.

Robert. K.H., G. Gahrton, K. Friberg, L. Zech and B. Nilsson. 1982. Extra chromosome 12 and prognosis in chronic lymphocytic leukaemia. Scand. J. Haematol. 28:163.

Roehm, N.W., H.J. Leibson, A. Zlotnick, J. Kappler, P. Marrach and J.C. Cambier. 1984. Interleukin-induced increase in Ia expression by normal mouse B cells. J. Exp. Med. 160:679.

Romagnani, S., M.G. Giudizi, R. Biaziotti, F. Almerigogna, E. Maggi, G. Del Prete and M. Ricci. 1981a. Interaction of SPA with surface IgG. J. Immunol. 127:1307.

Romagnani, S., M.G. Giudizi, F. Almerigogna, R. Biagiotti, G. Bellesi, F. Bernardi and M. Ricci. 1981b. Protein A reactivity of IgM- and IgD-bearing lymphocytes from some patients with chronic lymphocytic leukemia. Clin. Immunol. Immunopathol. 19:139.

Ross, G.D., E.M. Rabellino, M.J. Polley and H.M. Grey. 1973. Combined studies of complement receptor and surface immunoglobulin-bearing cells and sheep erythrocytes rosette-forming cells in normal and leukemic human lymphocytes. J. Clin. Invest. 52:377.

Rubartelli, A., R. Sitia, A. Zicca, C.E. Grasi and M. Ferrarini. 1983. Differentiation of chronic lymphocytic leukemia cells: correlation between the synthesis and secretion of immunoglobulins and the ultrastructure of the malignant cells. Blood 62:495.

Rubartelli, A., S. Roberto, C.E. Grossi and M. Ferrarini. 1985. Maturation of chronic lymphocytic leukemia B cells: correlation between the capacity of responding T-cell factors in vitro and the stage of maturation reached in vivo. Clin. Immunol. Immunopathol. 34:296.

Sahasrabuddhe, C., J. Morgan, S. Sharma, S. Mehta, B. Martin, D. Wright and A. Maizel. 1984. Evidence for an intracellular precursor for human B cell growth factor. Proc. Natl. Acad. Sci. USA 81:7902.

Saiki, O. and P. Ralph. 1983. Clonal differences in response to T cell replacing factor (TRF) for IgM secretion and TRF receptors in a human B lymphoblast cell line. Eur. J. Immunol. 13:31.

Saiki, O., T. Kishimoto, T. Kuritani, A. Muraguchi and Y. Yamamura. 1980. In vitro induction of IgM secretion and switching to IgG production in human B leukemic cells with the help of T cells. J. Immunol. 124:2609.

Sanderson, C.J., A. O'Garra, D.J. Warren and G.G.B. Klaus. 1986. Eosinophil differentiation factor also has B-cell growth factor activity: proposed name interleukin 4. Proc. Natl. Acad. Sci. USA 83:437.

Saxon, A., J. Feldhaus and R.A. Robbins. 1976. Single step separation of human T and B cells using AET-treated SRBC rosettes. J. Immunol. Methods 12:285.

Schimpl, A. and E. Wecher. 1972. Replacement of T-cell function by a T-cell product. Nature 237:15.

Schimpl, A. and E. Wecher. 1975. A third signal in B-cell activation given by TRF. Transplant. Rev. 23:176.

Schuurman, R.K.B., E.W. Gelfand and H.M. Dosch. 1980. Polyclonal activation of human lymphocytes in vitro. I. Characterization of the lymphocyte response to a T cell-independent B cell mitogen. J. Immunol. 125:820.

Scott, D.W., J.E. Layton and G.J.V. Nossal. 1977. Role of IgD in the immune response and tolerance. I. Anti-δ pretreatment facilitates tolerance induction in adult B cells in vitro. J. Exp. Med. 146:1473.

Segel, G.B., A.M. Tometsko and M.A. Lichtman. 1985. Y+- and L-system amino acid transport in normal and chronic lymphocytic leukemia lymphocytes photoinhibition by fluoronitrophenylazide. Arch. Biochem. Biophys. 242:347.

Sell, S. and P.G.H. Gell. 1965. Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with anti-allotype serum. J. Exp. Med. 122:423.

Sieckmann, D.G., R. Asofsky, D. Mosier, I. Zitron and W.E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. 1. Parameter of the proliferative response. J. Exp. Med. 147:814.

Siegbahn, A., A. Rosen, P. Venge, P. Aman and K. Nilsson. 1986. Production of chemokinetic inhibitory factor (CIF) by normal blood and spleen B lymphocytes. Leukemia Res. 10:179.

Silber, R., R.L. Stahl, C.M. Farber, D. Kanganis, A. Astrow and L.F. Liebes. 1984. Chronic lymphocytic leukemia lymphocytes: membrane anomalies and H202 vulnerability. Blood Cells 10:233.

Simonsson, B. and K. Nilsson. 1980. ^{3}H-thymidine uptake in chronic lymphocytic leukemia cells. Scand. J. Haematol. 24:169.

Smith, K.A. 1984. Lymphokine regulation of T cell and B cell function. In: Fundamental Immunology, W.E. Paul (Ed.), pp. 559-576. Raven Press, New York.

Solambi, D.S., P.R. McCurdy and R.P. MacDermott. 1982. Chronic lymphocytic leukemia: a monoclonal disease. Am. J. Hematol. 13:159.

Spier, C.M., C.R. Kjeldsberg, D.R. Head, K.C. DiFiore and B. Tudor. 1985. Chronic lymphocytic leukemia in young adults.

Sredni, B., D. Sieckmann, S. Kumagi, S. House, I. Green and W.E. Paul. 1981. Long-term culture and cloning of nontransformed human B lymphocytes. J. Exp. Med. 154:1500.

Steinberg, J., F.P. Siegel, C.A. Bona and C.D. Platsoucas. 1985. Proliferative responses of leukemic B cells in chronic lymphocytic leukemia to B- and T-cell growth factors. Clin. Res. 33:354A.

Suzuki, T., J.L. Butler and M.D. Cooper. 1985. Human B cell responsiveness to B cell growth factor after activation by phorbol ester and monoclonal anti-μ antibody. J. Immunol. 134:2470.

Swain, S.L. and R.W. Dutton. 1982. Production of a B cell growth-promoting activity (DL) BCGF from a cloned T cell line and its assay on the BCL_1 B cell tumor. J. Exp. Med. 156:1821.

Swain, S.L., G. Desmert, J.F. Warner and R.W. Dutton. 1981. Culture supernatants of a stimulated T cell line have helper activity that synergises with IL-2 in the response of B cells to antigen. PNAS 78:2517.

Swain, S.L., M. Howard, J. Kappler, P. Marrach, J. Watson, R. Booth, G.D. Wetzel and R.W. Dutton. 1983. Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. J. Exp. Med. 158:822.

Tamaki, T., T. Yonejawa, Y. Kanakura, S. Katagiri, H. Mizutani, T. Kitani and S. Tarni. 1986. Miotogenic response of neoplstic B cells: comparison of reactivity of Staphylococcus aureus Cowan I and anti-immunoglobulin antibodies. Clin. Exp. Immunol. 64:181.

Teranishi, T., T. Hirano, N. Arima and K. Onove. 1982. Human helper T cell factor(s) (ThF). II. Induction of IgG production in B lymphoblastoid cell lines and identification of T cell-replacing factor (TRF) like factor(s). J. Immunol. 128:1903.

Teranishi, T., T. Hirano, B.H. Lin and K. Onove. 1984. Demonstration of the involvement of interleukin 2 in the differentiation of Staphylococcus aureus Cowan I-stimulated B cells. J. Immunol. 133:3062.

Tötterman, T.H., K. Nilsson and C. Sundström. 1980. Phorbol ester-induced differentiation of chronic lymphocytic leukemia cells. Nature 288:176.

Tsudo, M., T. Uchiyama and H. Uchino. 1984. Expression of Tac antigen on activated normal human B cells. J. Exp. Med. 160:612.

Tsujimoto, Y., J. Yunis, L. Onorato-Showe, J. Erickson, P.C. Nowell and C.M. Croce. 1984. Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science 224:1403.

Vahdeti, M., H. Graafland and J.M. Emberger. 1983. Karyotype analysis of B-lymphocytes transformed by Epstein-Barr virus in 21 patients with B cell chronic lymphocytic leukemia. Hum. Genet. 63:327.

Valinsky, J.E., T.G. Eoston and E. Reich. 1978. Microcyanin 540 as a fluorescent probe of membranes: Selective staining of leukemia and immature hemopoietic cells. Cell 13:487.

Van Den Bergh, H., C. Parloir, G. David, J. Michaux and G. Sokal. 1979. A new characteristic karyotypic anomaly in lympho-proliferative disorders. Cancer 44:188.

Vitale, B., M. Antica, B. Benkovic, B. Burek and B. Jaksic. 1985. The relationship between membrane characteristics functional reactivity of T-lymphocytes, and the progression rate of B-cell chronic lymphocytic leukemia. Cancer 56:1075.

Vitetta, E.S., J.C. Cambier, F.S. Ligler, J.R. Kettman and J.W. Uhr. 1977. B-cell tolerance. IV. Differential role of surface IgM and IgD in determining tolerance susceptibility of murine B cells. J. Exp. Med. 146:1804.

Vitetta, E.S., J. Ohara, C. Myers, J. Layton, P.H. Krammer and W.E. Paul. 1985. Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. J. Exp. Med. 162:1726.

Waldmann, T.A., S. Broden, R.M. Blaese, M. Durm, M. Blackman and W. Strober. 1974. Role of suppressor T cells in the pathogenesis of common variable hypogammaglobulinemia. Lancet 2:609.

Waldmann, T.A., C.K. Goldman, W.J. Leonard, J.M. Depper, R.J. Robb, S.J. Korsmeyer and W.C. Greene. 1984. Interleukin-2 receptors on activated malignant and normal B cells. Cur. Top. Microbiol. Immunol. 113:96.

Waldschmidt, T.J. and E.S. Vitetta. 1985. The use of haptenated-immunoglobulin molecules to induce tolerance in B cells from neonatal mice. J. Immunol. 134:1436.

Wang, C.Y., R.A. Good, P. Ammirati, G. Dymbort and R.L. Evans. 1980. Identification of a p69, 71 complex expressed on human T cells sharing determinants with B-type chronic lymphatic leukemic cells. J. Exp. Med. 151:139.

Winchester, R.J., S.M. Fu, P. Wernet, H.G. Kunkel, B. DuPont and C. Jersild. 1975. Recognition by pregnancy serums of non-HL-A alloantigens selectively expressed on B lymphocytes. J. Exp. Med. 141:924.

Wybran, J., S. Chantler and H. Fundenberg. 1973. Isolation of normal T cells in chronic lymphatic leukemia. Lancet 1, 126.

Yoshizaki, K., T. Nakagawa, T. Kaieda, A. Muraguchi, Y. Yamamura and T. Kishimoto. 1982. Induction of proliferation and Ig production in human B leukemic cells by anti-immunoglobulins and T cell factors. J. Immunol. 128:1296.

Yoshizaki, K., T. Nakegawa, Y. Fukumaga, T. Kaieda, S. Maruyama, S. Kishimoto, Y. Yamamura and T. Kishimoto. 1983. Characterization of human B cell growth factor (BCGF) from cloned T cells or mitogen-stimulated T cells. J. Immunol. 130:1241.

Yunis, J.J., C.D. Bloonfield and K. Ensrud. 1981. All patients with acute nonlymphocytic leukemia may have a chromosomal defect. N. Engl. J. Med. 305:135.

Yunis, J.J., M.M. Oken, M.E. Kaplan, K.M. Ensrud, R.R. Howe and A. Theologides. 1982. Distinctive chromosomal abnormalities in histological subtypes of non-Hodgkin's lymphoma. N. Engl. J. Med. 307:1231.

Zubler, R.H., J.W. Lowenthal, F. Erard, N. Hashimoto, R. Devos and H.R. MacDonald. 1984. Activated B cells express receptors for, and proliferate in response to, pure interleukin 2. J. Exp. Med. 160:1170.