Current Topics in Microbiology and Immunology

182

Mechanisms in B-Cell Neoplasia 1992

Workshop at the National Cancer Institute,
National Institutes of Health,
Bethesda, MD, USA, April 21–23, 1992

Edited by M. Potter and F. Melchers

With 188 Figures and 64 Tables

Springer-Verlag
Berlin Heidelberg New York
London Paris Tokyo
Hong Kong Barcelona
Budapest

MICHAEL POTTER, M.D
Chief
Laboratory of Genetics
Bldg. 37, Rm 2B04
National Cancer Institute
National Institutes of Health
Bethesda, MD 20892, USA

Prof. Dr. FRITZ MELCHERS
Director
Institute for Immunology
Grenzacherstr. 487
CH-4005 Basel

Cover illustration:
The cover shows the time schedule of actions, growth factors, oncogenes, and of cell cycle-related proteins in a B cell during the cycle from G1 through S, G2 and M, into the next cycle. The picture depicts an area of the germinal center next to the follicular mantle zone from human tonsil. Plasma cells in the germinal cells do not express the oncogene bcl-2 and stain red with a plasma cell-specific antibody. Conversely, plasma cells in the crypt epithelium are bcl-2-positive and are stained orange. Most of the green cells are B cells of the follicular mantle.
Picture by courtesy of Dr. I. C. M. MacLennan.

ISSN 0070-217X
ISBN-13: 978-3-642-77635-9 e-ISBN-13: 978-3-642-77633-5
DOI: 10.1007/978-3-642-77633-5

Softcover reprint of the hardcover 1st edition 1992

Library of Congress Catalog Card Number 15-12910

Offsetprinting: Saladruck, Berlin; Bookbinding: Lüderitz & Bauer, Berlin.

23/3020-5 4 3 2 1 0 – Printed on acid free paper.

Preface

The 1992 workshop on "Mechanisms in B-Cell Neoplasia", held in Bethesda, Maryland, April 21-23, was the tenth in this series. The meetings in Basel for the years 1983, 1985, 1987, 1989, and 1991 have all been published by Editiones Roche in Basel as edited transcripts of the presentations and discussions. On the alternate years the workshop was held in Bethesda, and the papers have been published in this series *(Current topics in microbiology and immunology,* vols. 113, 132, 141, and 166). This field has grown in interest and depth, and the discussions now can no longer cover the entire subject in a 3-day meeting, hence the selection of topics. Initially, in 1983 we hoped to bring about discussions between those interested in growth and development of normal B cells and tumor biologists interested in B-lineage tumors. In 1981 the pivotal discovery of Hayward, Neel and Astrin (Nature 209:475) implicated the activation of the c-*myc* protooncogene in the major B-cell neoplasm in avians, the bursal lymphoma. Then, almost as the first meeting was being planned, the fascinating story of the activation of c-*myc* by chromosomal translocations in mouse plasmacytomas and Burkitt lymphomas unfolded, and a third faction consisting of workers interested in oncogenes became an integral part of these discussions. In the more recent meetings there has been increasing participation by clinicians and epidemiologists. Epidemiological studies of multiple myeloma (G. I. Obrams and M. Potter, eds., Epidemiology and biology of multiple myeloma, Springer Verlag, 1991) and non-hodgkin lymphoma (a meeting report is to appear in *Cancer research,* 1992) have revealed an unexplained increased incidence of these neoplasms over the last four decades. The student of the B cell is much like a child at a five-ring circus where something exciting is happening simultaneously in all arenas. The great challenge for the B-cell biologist is to adapt this knowledge to the special complexity of the pathogenesis of B-cell tumors where different regulatory mechanisms operate during the successive stages of development.

The last decade has seen great technological progress in understanding B-cell growth. Specific B-cell growth factors can now be produced in large quantities. Methods for culturing B cells at various developmental stages have been developed. Model systems for each of the major forms of B-cell tumors in man have been constructed. Transgenic mouse systems have added a new dimension. More recently, we are beginning to see the application of the "knock-out" mouse that has been ingeniously constructed so that the function of a specific endogenous gene is inactivated. All of these spectacular developments have added to the depth of this field.

The development of the B cell has been a particularly intriguing area of research from two aspects: the complex immunoglobulin gene rearrangements that take place at the genetic level and the different cellular phenotypes which must accompany these determinative events. The field of modern immunology began with the beautiful hypothesis of F. M. Burnet which implicated "clones of lymphocytes" in antibody formation. A revolutionary idea in his paper was that circulating lymphocytes were precursors of antibody-forming cells.

We continue to see the development of new information on how B cells are formed with the dissection of the events that take place in germinal centers, in special populations such as the $Ly1^+$ B lymphocytes and in early B-cell development. The availability of the cytokine interleukin-7 and cooperative stromal cells from B-cell-generating organs such as bone marrow have allowed the development of sophisticated culture systems in which progenitor and precursor cells proliferate and differentiate. Immunoglobulin genes in germline configuration rearrange and the cells can undergo maturation. Not only can this be achieved in tissue culture, but transfer of these progenitor and precursor cells into SCID mice results in repopulation of the pre-B and B-cell compartments in a stable way for long periods of time (Melchers, Dorshkind, Paige, Nishikawa, Saffran, Morrow, Hardy). This opens exciting possibilities for studying various factors that influence neoplastic transformation, such as genes that determine susceptibility to B-cell tumors, environmental influences (stromal cells, T cells, cytokines, antigens), and exogenous agents (e. g., pristane).

Chromosomal translocations are putatively the key initiating mutational steps in the development of many clinical and experimental forms of B-cell tumors. In the B cell, these illegitimate exchanges appear to be related to *V-(D)-J* rearrangements and heavy chain isotope switching. There are fundamental unanswered questions about the mechanisms of illegi-

timate exchanges. First, despite the near location of translocation sites to the vicinity of immunoglobulin gene signal sequences most of the breaks are not in signal or coding joints per se but rather involve a considerable part of adjoining sequences. The situation is even less clear in the case of the switch region-associated translocations which are not site-specific rearrangements to begin with. The accusing finger suggests the mechanisms that make immunoglobulin chromatin accessible during a given stage of B-cell development may be involved. Various cytokines and DNA binding factors participate in accessibility, and several of these associated with switching are now being characterized. Possibly, accessible immunoglobulin DNA is more prone to pathological breaks and rejoinings and, thus, DNA repair enzymes may become implicated in both the normal and pathological processes. Second, we still do not have a clear explanation of the factors that bring segments from different chromosomes into alignment and proximity for recombination.

What is neoplasia? Is James Ewing's definition of a tumor as "an autonomous new growth of tissue" (J. Ewing, *Neoplastic diseases,* W. B. Saunders, Philadelphia, 1928) descriptive of the phenomenology we see in B-cell neoplasia? Clearly, the diversity of patterns of growth in common human B-cell tumors is unique and extraordinary. The tumor types range from the most benign forms such as MGUS, smoldering myeloma, B-CLL and follicular lymphoma, to intermediate forms such as mantle zone lymphoma (see Williams), to explosively growing acute blastic tumors such as Burkitt's lymphoma, the high-grade lymphomas associated with various forms of immunodeficiency including AIDS (see McGrath) and the acute B-cell leukemias. All of these B-cell tumors have some degree of autonomy, but many of them have curious patterns of growth. The indolence of follicular lymphomas, the seemingly regulated growth of MGUS, the regressions of some blastic lymphomas that occur after withdrawal of immunosuppressive drugs, and the apparent lack of proliferative activity in many of the cells that accumulate in multiple myeloma and B-CLL are unusual examples of tumor growth and proliferation.

In multiple myeloma large numbers of nondividing plasma cells accumulate in the bone marrow cavities. The source of these plasma cells is still being debated but it is now under intense investigation in several laboratories (see discussion here by Pilarski, Jensen, Witzig, Epstein, and McLennan). The existence of circulating B-cells expressing the same idiotype as found in the immunoglobulins secreted by the bone marrow

plasma cells is gaining general acceptance. It is not known if these circulating cells are myeloma stem cells that seed marrow cavities or "metastatic" derivatives of bone marrow myeloma cells. The cells are found in the peripheral blood in measurable levels but they apparently do not greatly expand, as might be expected of a leukemic cell, nor are they the same cell that is found in the late occurring plasma cell leukemic phase of myelomatosis. The circulating cells appear refractory to chemotherapy (Pilarski). The unsettled question is to explain the origin and relationship of the circulating cells to the bone marrow plasma cells that accumulate in such large numbers.

Many protooncogenes code for proteins that are components of signal transduction pathway which start at the cell surface and converge in the nucleus on the regulatory sequences of target genes, thereby controlling their transcription and translation. These highly complex, redundant, cross-circuiting mechanisms are far from understood. Very often these pathways have been worked out in some other cell type and require special evaluation to determine relevance to B-cell development and function. *C-myc* is still the most important single oncogene in B-cell neoplasia. The important discovery of Blackwell and Eisenman that Myc protein heterodimerizes with Max to form a specific DNA-binding protein complex has resulted in progress in understanding Myc's functions. While it is generally thought that Myc is a transcription factor, the target genes have not been defined. An exciting development, then, is identification of an ornithine decarboxylase gene as one of the genes positively regulated by c-*myc* (see Bello-Fernandez and Cleveland). Ornithine decarboxylase is a rate-limiting enzyme in polyamine biosynthesis that is required in the progression from G_1 to the S phase of the cell cycle.

The open discussions and exchanges of ideas in the "Mechanisms of B-Cell Neoplasia" workshops have been marked by enthusiasm and cooperation among the participants, and this has contributed to the progress in this field.

Acknowledgments

This meeting was sponsored by the DCDBC of the National Cancer Institute, and special thanks are due to Dr. Alan Rabson for his enthusiasm and encouragement in supporting this meeting as a function of the Division. This year we obtained additional financial support from private donors, and we wish to express our gratitude to them for making it possible to fund the travel of many of our foreign colleagues. Mr. Bruce Lorick of the National Cancer Association was vital in making the arrange-

ments with these anonymous donors at a time when we were in desperate need of help. Despite the contributions of the above-mentioned benefactors, many of the participants funded their own travel to the meeting. We could not have arranged such a meeting as this without the skilled and diplomatic assistance of Ms. Fran Oscar of Cygnus Corporation and Ms. Victoria Rogers of the Laboratory of Genetics, NCI. These two people made it easy for us to have this meeting and added a spirit of cordiality to the proceedings. Ms. Victoria Rogers has handled all of the correspondence and technical editing involved in producing this volume.

MICHAEL POTTER
FRITZ MELCHERS

Table of Contents

B-Cell Development

Multiple Myeloma, Plasmacytomas

Lymphomas:
B-CLL, Follicular Lymphomas BCL-2, BCL-1

List of Contributors

(Their addresses can be found at the beginning of their respective chapters)

B-Cell Development

Progenitor and Precursor B Lymphocytes of Mice. Proliferation and Differentiation In Vitro and Population, Differentiation and Turnover in SCID Mice In Vivo of Normal and Abnormal Cells

F. MELCHERS, D. HAASNER, H. KARASUYAMA, L. REININGER and A. ROLINK

Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel

1 Introduction

Mouse progenitor B cells (pro B cells*), with H and L chain genes in germline configuration, and precursor B cells with L chain gene loci in germline configuration and H chain gene loci in D_HJ_H-rearranged forms (pre B-I cells, ref. 1) can be grown from single cells of fetal liver, of neonatal liver, spleen, blood and bone marrow, and from adult bone marrow for several months in serum-substituted media on stromal cells in the presence of IL7. When IL7 is removed from the medium, more than 90% of the progenitors and precursors loose the capacity to proliferate on stromal cells in the presence of IL7 within 2 days. In these two days they rearrange their H and L chain gene loci in- and out-of-frame and thereby generate sIg^- and sIg^+ B cells which both die rapidly by apoptosis. The sIg^+ B cells can be activated by lipopolysaccharide (LPS), antigen and IL2 to a primary response of proliferation and maturation to IgM-secreting, plaque forming cells (PFC). Most of these findings have been published by Rolink et al. [7].

Early differentiation along the B lymphocytic pathway is marked by successive rearrangements first of D_H to J_H, then of V_H to D_HJ_H segments of the H chain locus, and finally of V_L to J_L segments of the L chain loci. It has been repeatedly suggested that expression of productive rearrangements of the H chain locus either as $D_HJ_HC\mu$-protein or as ($V_HD_HJ_H$-rearranged) μH chains on the surface signals the pre B cell to rearrange the next step along the differentiation pathway, thereby permitting only cells with productive rearrangements to proceed in their differentiation towards a B cell (see ref. [1]). The different forms of membrane-bound μH-chains can be deposited on the surface of the pre B cells because they associate with a surrogate L chain composed of V_{preB} and $\lambda5$ in which $\lambda5$ forms disulfide bonds (like L chains) with the μH-chains. It has been speculated that pro B cells express a surrogate L chain-containing complex with unknown polypeptides (called X) on the surface to signal the initiation and propagation of D_HJ_H-rearrangements [8]. Such polypeptide chains have now been identified on pro and

* Different research groups call different precursors of the B-lymphocytic lineage by different names. Osmond and his colleagues [2], Rajewsky and his colleagues [3], Nishikawa and his colleagues [4], Paige and his colleagues [5], and Hardy and his colleagues [6] call different parts of the precursor B cell pool "pro B cells" (see also their contributions to this volume). We call all cells with H and L chain loci in germline configuration (but committed to become B cells) "pro B cells", while we call cells with L chain loci in germline configuration, but H chain loci in D_HJ_H-rearranged forms (committed to become B cells) "pre B-I cells" - and all $V_HD_HJ_H$-rearranged cells with L chain gene loci in germline configuration "pre B-II cells".

Current Topics in Microbiology and Immunology, Vol. 182

pre B-I cells, and one of them, p64, is disulfide bonded to λ5 (Karasuyama et al. submitted). We will discuss experimental evidence for and against an involvement of these surface-located complexes with surrogate L chains in the control of the B cell differentiation pathways.

Mouse progenitor and precursor cells growing on stromal cells in the presence of IL7 can be transferred into lightly irradiated SCID mice. Within four to eight weeks this leads to a population of bone marrow (and also to some extent of spleen) with the transferred progenitors and precursors, to a low level (5% to 15% of normal) of sIg^+ B cells in spleen, but not in lymph nodes, and to a normal level of $CD5^+$ sIg^+ B cells in the peritoneum. This population of SCID mice with pro B, pre B and B cells is stable for several months. Nevertheless, pre B cells in bone marrow and B cells in spleen turn over rapidly, with half-lives of less than a week. The preB cell-populated SCID mice have normal levels of serum IgM, but a 10-fold lower concentration of IgA and no detectable $IgG_{1,2a,2b \text{ or } 3}$ in their serum. The splenic, donor-derived sIg^+ B cells can be stimulated by LPS to a response of IgM-PFC. Most of these findings, again, have been published by Rolink et al. [7].

This paper discusses some of the questions arising from recent findings in this system of study of pre B cell development, and their potential relevance to abnormal and malignant forms of this development.

2 Which Cells Proliferate "In Vitro", for How Long, and by Which Molecular Contacts is This Proliferation Regulated?

The cells which proliferate for many weeks on stromal cells in the presence of exogenously added IL7 are $B220^+$ or $^-$, $BP\text{-}1^+$ or $^-$, $PB76^+$, $RAG\text{-}1^+$, $RAG\text{-}2^+$, MHC class I^+, class II^-, $CD5^-$, $CD4^-$, $CD8^-$, $CD3^-$, $mb\text{-}1^+$, $c\text{-}kit^+$, $V_{preB}{}^+$, $\lambda5^+$ and react to IL7, i.e. are likely to be IL-7 receptor $(R)^+$. The majority of all fetal liver-derived cells are TdT^-. Their D_HJ_H-joints are, therefore, devoid of N region diversity, and the same holds true for the $V_HD_HJ_H$-joints of sIg^+ B cells, if they are generated in fetal liver. Since fetal liver-derived, D_HJ_H (N^-)-rearranged pre B-I cells can populate the bone marrow of SCID mice (see below) it is not yet clear whether V_H rearrangements to these D_HJ_H-rearranged H chain loci in bone marrow are N^+ or N^-. This is under investigation.

The majority of bone marrow-derived pre B-I cells are TdT^+, and their D_HJ_H-joints contain N-region diversity. The presence or absence of N-region diversity is, therefore, a sign for where the rearrangements have been made. $Ly1^+$ B-LL cells of the mouse are often N^-, while the majority of human B-CLL's are N^+ [9].

Fetal liver-derived and bone marrow-derived progenitors and precursors can also be distinguished by differential expression of a novel regulatory myosin light chain (PLRLC) in bone marrow [10] and a novel LiM-homeo domain in fetal liver-derived cells (Alt, personal communication).

Early in the development of fetal liver and of neonatal bone marrow some of the H chain loci of the stromal cell-IL7-reactive cells are still in germline configuration. In fact, normal frequencies of stromal cell/IL7-reactive cells are found in bone marrow of RAG-2-deficient mice [11] which have all H and L chain gene loci in germline configuration (Melchers et al., in preparation). During ontogeny "in vivo", and during continued proliferation "in vitro" the germline H chain loci, as well as the D_HJ_H-rearranged loci of the normal early pro B and pre B

points along the pathway of differentiation from a pluripotent hematopoietic stem cell to a committed stem cell of a given lineage of blood cells, as well as the possible plasticity of a state of differentiation along this pathway to reconcile these experimental differences. For abnormal and neoplastic development it is evident that deregulated states of stromal cells are prime environmental factors for abnormal, deregulating influences on early stages of B lymphocyte development. This should be particularly effective for the eventual development of B-ALLs and B-CLLs with chromosomal translocations involving the J_H and the V_H regions of the H chain locus to proto-oncogenic loci such as bcl-1, bcl-2 and bcl-3 (see papers of this volume). It will be rewarding to study the control of gene expression and differentiation not only of the Ig loci, but also of the proto-ontogenic loci by stromal cell-pro B/pre B/ B cell interactions to investigate what may go wrong (and how) in these premalignant transformations.

3 Which Cells Do Not Grow on Stromal Cells in the Presence of IL7?

When an Ig H chain gene locus is productively rearranged and expresses either $D_HJ_HC_\mu$-protein or $V_HD_HJ_H$-C_μ μH chain on the surface of pre B cells, long-term proliferation on stromal cells in the presence of IL7 ceases. The cells may continue to grow for a limited time period in IL7 alone [4, 7] but this growth is not maintained for more than a week. We have found three mouse strains that do not follow this rule. NZB, BXSB and Balb/cAn pre B cells will proliferate for more than one week in IL7 alone, and survive thereafter for weeks without further proliferation 'in vivo' (Melchers et al., in preparation). This may be of interest in view of a potential mutation making these strains susceptible to plasmacytogenesis induced by mineral oil (Potter et al., previous volumes of this series).

Experimental evidence for the termination of long-term proliferation of pre B cells by the surface membrane expression of different forms of μH chains comes from the finding that pre B cells of fetal liver, carrying D_HJ_H rearrangements without N region diversity in reading frame (rf) II, are suppressed in the repertoire of pre B cells [19]. Since nearly all D_H segments contain promoters at the 5' ends they can be expressed in pre B cells as protein, together with J_H Cμ if rearrangement of D_H to J_H occurs in rf II. When the DJCμ-protein cannot be expressed on the surface of pre B cells (i.e. in the μH-transmembrane protein-deficient mice [20] or in the λ5-deficient mice [21]) rf II is equally represented and not suppressed in the repertoire of H-chain loci of pre B cells (Haasner et al., in preparation).

The termination of the long-term proliferative capacity of pre B cells is also evident in H chain-transgenic mice ([22], Melchers et al., in preparation). A 20-100 fold decrease in the frequency of clonable pre B cells is observed in these mice. In μH-transmembrane-deficient and in λ5-deficient pre B cells, the productively rearranged μH chains cannot be transported and inserted into the surface membrane. One might expect (and that is tested at present) that pre B cells with the capacity of long-term proliferation on stromal cells in the presence of IL7 should include cells with productively D_HJ_H and $V_HD_HJ_H$-rearranged H chain loci, i.e. cells with intracytoplasmic μH chain expression, typical for pre B-II cells of normal mice.

Nevertheless, practically all pre B cells from normal or deficient animals proliferating on stromal cells in the presence of IL7 lose their capacity to do so within 2 to 3 days when IL7 is removed and differentiate to sIg^+ B cells. This argues strongly against a signalling role of the μH chain/surrogate L chain complex

cells appear to continuously rearrange D_H to J_H on both chromosomes until they either have reached the most 5' located D_H, or the most 3' located J_H on the H chain locus (Haasner et al., in preparation). A single pro B or Pre B-I cell can, thereby, give rise to a wide variety of D_HJ_H-rearranged pre B-I cells.

The tyrosine kinase c-kit, receptor for the steel-derived factor (SCF, stem cell factor), is involved in the regulation of proliferation of pro B and pre B-I cells on stromal cells in the presence of IL7. A monoclonal antibody specific for the extracellular portion of c-kit inhibits pre B-I cell proliferation ([12] see also paper by S.I. Nishikawa et al., this volume). Steel-derived SCF acts as a costimulator in precursor B cell growth [13]. Since SCF can be produced by stromal cells it is likely that SCF/c-kit-interactions are part of the proliferation-controlling molecular interactions in pre B-I cell growth that, furthermore, involve CD44 and hyaluronate, and VLA-4 and fibronectin [15,16,17]. It remains to be investigated whether the membrane-bound or the secreted form of SCF control the pre B-I proliferation, or how the two forms of SCF might modulate the reactions of pre B-I cells.

The high efficiency of plating of progenitors and precursors on stromal cells in the presence of IL7 has allowed cloning from the original organ (fetal liver, spleen and bone marrow) "ex vivo", and determination of the frequencies of cells with various surface marker expression patterns during the life of a mouse (Rolink et al. in preparation). A first wave of clonable cells in fetal liver, is followed by an initially high (1 in 50 to 1 in 100) frequency of clonable pre B-I cells in bone marrow early in life (between 2 and 4 weeks after birth), declining with age by factors between 10 and 20. Sorting for B220, c-kit and CD43 revealed high frequencies (1 in 5 to 1 in 15) of clonable pre B cells in the $B220^+$, $c\text{-}kit^+$, $CD43^+$ bone marrow cell population of both young and old mice. In young but <u>not</u> old mice, high frequencies (~1 in 50) were also found in the $B220^-$, $c\text{-}kit^+$ population. No clonable pre B cells are detectable in the $B220^-$, $c\text{-}kit^-$ and the $B220^+$, $c\text{-}kit^-$ populations. Therefore, the decline of clonable pre B cells in the aged bone marrow must be due to a decrease of the $B220^-$, $c\text{-}kit^+$ progenitors. The vast majority of pre B cells in the bone marrow are $B220^+$, $c\text{-}kit^-$, $CD43^-$, and are not capable of long-term proliferation on stromal cells in the presence of IL-7.

These findings suggest that the production of a relatively constant number of sIg^+ B cells throughout the life of the animal (estimated by Osmond [2] to around $1\text{-}3x10^6$ sIg^+ cells/day from $5x10^7$ cells/day generated "in toto") is maintained from precursors which have been formed early in life from $B220^-$, $c\text{-}kit^+$ progenitors. It is tempting to speculate that these $B220^-$, $c\text{-}kit^+$ progenitors are B-lineage-committed cells which have at least a part of their H chain loci in germline configuration.

Our culture conditions with PA-6 cells as stromal cells [17] and exogenously added IL7 in serum-substituted tissue culture media appear to favor the proliferation of B-lineage committed progenitors and precursors. Differentiation "in vitro" and "in vivo" of cells from normal mice usually do not generate detectable numbers of myeloid cells or T-lineage cells [7], although other types of cells exhibit limited growth potential but are usually lost after recloning of the pro B and pre B-I cells. However, others have observed differentiation from stromal cell/IL7-reactive progenitors to macrophages ([5] and references therein) and T-lymphocyte lineage cells [18]. Precursor B cells also are capable of growth on S17 stromal cells which do not produce IL7. Insulin-like growth factor-1 has been identified in these cultures to be a cytokine effecting pre B cell proliferation (see paper by Dorshkind et al., this volume). It is clear that we need to know more about the lineages of differentiation of stromal cells, their regulation of cytokine production and expression of cell contact molecules involved in regulating the various decision

in the induction of successive V_H to D_HJ_H- or V_L to J_L-rearrangements (see also below). The simple picture which emerges for B cell differentiation 'in vivo' expects the bone marrow (or fetal liver) to contain a finite number of stromal cell sites, on which early in life B220$^-$, c-kit$^+$ and B220$^+$, c-kit$^+$, CD43$^+$ and later in life only B220$^+$, c-kit$^+$, CD43$^+$ progenitor and precursor B cells can retain their state as cells with long-term proliferative capacity, in contact with stromal cells and under the influence of IL7. When they are pushed away from these sites, because bone marrow gets too crowded, they differentiate into cells rearranging V_H to D_HJ_H and V_L to J_L, generating sIg$^-$ and sIg$^+$ B cells no longer capable of further attachment to stromal cells and no longer reactive to IL7.

4 How Do B220$^+$, c-kit$^-$, CD43$^-$ Pre B Cells Accumulate in Bone Marrow?

The vast majority of pre B cells in the bone marrow are B220$^+$, c-kit$^-$, CD43$^-$, and are unreactive to stromal cells and IL-7. It is entirely possible that they just represent a transitory state to all sIg$^+$ B cells of no interactive capacity with bone marrow stromal cells. Alternatively, it may be that (different) stromal cells interact and retain these pre B cells, and regulate their further development, but no evidence for such stromal cells has yet been found. If these pre B cells, in fact, express μH chains on their surface (together with surrogate L chains) they could be selected via binding specificities of the V-regions. Again, there is no evidence for selection of the V_H-repertoire at this level of pre B cell development, but it is worth noting that the vast majority of all B lineage tumors express productively rearranged Ig-loci, while carrying the oncogenic translocations (to myc, bcl-2, bcl-1, bcl-3 etc.) on the second, nonproductive allele (see papers this volume). In the generation of B-ALLs and B-CLLs, positive selection for a productively rearranged H chain locus appears to dominate the development of malignant cells, which are expected to develop the successive oncogenic transformations involving more than one oncogene.

5 Is the Vpre/λ5 Surrogate L Chain Needed for B Cell Differentiation?

The VpreB/λ5 surrogate L chain is important for proper and timely development of B cells. This becomes evident in mice where the l_5 gene has been disrupted by targeted integration of a defective λ5 gene [21]. While the heterozygous litter mates appear to develop their B cells normally and have normal precursor pools, the homozygous λ5 deficient mice have a smaller precursor pool. Unlike in normal mice, the vast majority of these are B220$^+$, c-kit$^+$, CD43$^+$, thus the λ5 gene seems to play an important role in the transition from the earliest B220$^+$, c-kit$^+$, CD43$^+$ pre B cells that can proliferate on stromal cells, and IL-7 to B220$^+$, c-kit$^-$, CD43$^-$ pre B cells that lost this capacity. It is likely, as a consequence of this, that B cell development in these mice is delayed. While the CD5$^+$ B cell compartment in the peritoneum fills up within 4 weeks after birth, though with a reduced rate of accumulation of cells, the CD5$^-$ B cell compartment in spleen and lymph nodes, at 6

weeks of age, is 5% of normal, and is at best half of normal at half a year of age. The B cells generated in λ5-deficient mice appear normal, in that they are capable of T-independent and T-dependent B cell responses and in that they are allelically excluded.

For a possible explanation of this delayed B cell development we come back to our speculation that the different steps of Ig-gene rearrangements are effected in different cells, in which the product of the previous rearrangement controls the next step in development [8]. The products of the rearrangements, DJCμ-protein (possibly in association with a germline-transcribed V_H-fragment [24]), and μH chains are associated with the surrogate L chain in disulfide-bonds via the λ5 protein and complexed with the B29 and mb-1 proteins in the surface membrane. In progenitor cells with all Ig loci in germline configuration (but committed to the B lineage differentiation pathway), a precursor complex consisting of p64 disulphide-bounded to the surrogate L chain (Karasuyama et al., submitted) (called X in ref. [8]), is deposited on the surface. The three complexes, we have speculated, might recognize ligands (called S1, S2, S3) on cooperating stromal cells which bind to these Ig-like complexes on pre B cells to induce the next step of differentiation. Ligand S1, binding to p64/surrogate L would induce D_H to J_H, ligand S2, binding to $D_HJ_HC\mu$-protein/germline V_H/surrogate L would induce V_H to D_HJ_H, and ligand S3 (maybe a collection of antigens, see preceding paragraph) to μH chain/surrogate L would induce V_L to J_L rearrangements. Finally, the complete Ig molecules with μH chains and conventional L chains is likely to be monitored by antigens (self and nonself) for positive or negative selection into the peripheral pool of mature B cells [25, 26, 27].

However,this speculation is an apparent contrast to our "in vitro" experiments with pro and pre B-I cells. These cells can rearrange D_H to J_H, V_H to D_HJ_H and V_L to J_L simply when IL7 is omitted from culture [7]. Pre B-I cells unable to produce mH chains upon VDJ-rearrangement still rearrange V_L to J_L, though at lower rates (clone 18 in ref. [7], Haasner et al., in preparation). Pre B cells from μH-transmembrane-deficient mice unable to display μH chains on the surface still rearrange V_L to J_L, though at lower rates [23]. λ5-deficient mice produce B cells, though at reduced rates [21], and long-term proliferating pre B cells prepared from λ5 deficient mice are as efficiently stimulated by LPS to IgM secreting cells as those of normal mice (Rolink et al., in preparation). It should, however, be kept in mind that the function of the V_{preB}/λ5 surrogate L chain and their partners in the Ig-like complexes on the surface of different stages of pre B cells might not be properly testable in our "in vitro" experiments since we add excess amounts of IL7 to our cultures and since it is likely that we do not provide the proper ligands S1, S2 and S3 for interaction with these Ig-like complexes. We do not expect PA-6 or 3T3 preadipocytic fibroblast lines to express all the surface molecules of stromal cells of fetal liver or bone marrow, and we have evidence that heterogeneous stromal cells of normal fetal liver or bone marrow often do not support extensive proliferation even in the presence of IL7, while allowing ordered B cell differentiation [28]. The identification of possible ligands binding to the Ig-like complexes, and their expression on given types of bone marrow cells might clarify these issues.

Ig rearrangements can occur out of the sequential order, i.e. V_L to J_L before H-chain gene rearrangements, though probably at lower rates [29]. It could be that all Ig-gene rearrangements can occur "out of order" at a lower rate, and the lower rate may indicate an uncontrolled mechanism, maybe uncontrolled by ligand-receptor interactions. If so, this would allow accumulation of productively rearranged $V_HD_HJ_H$-H chain loci or productively rearranged V_LJ_L-L chain loci in λ5-deficient pre B cells proliferating on stromal cells in the presence of IL7. Whenever such a cell would have accumulated both a productive H and a productive L chain locus, it could express sIg, transit to become a B cell and be

selected to enter the peripheral pool of mature B cells. Since B cells of λ5-deficient mice appear allelically excluded, it could be that productive L chain rearrangements accumulate more effectively and faster than productive H chain rearrangements. An analysis of the rearrangements of the Ig loci in pre B cells of λ5-deficient mice is currently under way.

6. Which Pre B and B Cell Compartments are Populated in SCID Mice by the Transfer of Pre B-I Cells?

Long-term proliferating pre B-I cells from fetal liver can be transferred from the stromal cells/IL7-cultures into lightly irradiated SCID mice even after several weeks of culture to populate practically all of the available sites in bone marrow and give rise to a stable level of 5-15% of the normal number of sIg^+ B cells in spleen, and normal levels of $CD5^+$ IgM^+ $IgD^{+/-}$ B cells in the peritoneum in the apparent absence of T cells of donor or host origin [7]. The spleen cells are $Ig^+/IgD^{+/-}$ and might be $CD5^+$, but this remains to be clarified. These "in vitro"/"in vivo" conditions allow studies on factors which influence the development of $Ly1^+$ B lymphoma in mice.

Similarly, Hardy and Hayakawa ([30] see also this volume) have been able to populate the $CD5^+$ B cell compartment in the peritoneum of SCID mice by transfer of D_HJ_H-rearranged, $B220^+$, $CD43^+$, HSA^+ fetal liver pre B cells. The peritoneal compartment of $CD5^+$ B cells is stably populated for long periods of time, again in the apparent absence of T cells. However, while our experiments led to a stable, long-term population of bone marrow with pre B-I cells of donor origin, Hardy's and Hayakawa's pre B cells led to a wave of differentiation of $CD5^+$ B cells in the peritoneum, but pre B cells of donor origin could not be detected in the bone marrow. It appears that the two pre B cell populations transferred into SCID mice are different.

Turnover of our originally stromal cell/IL7-reactive pre B-I cells in the bone marrow of the SCID mice, and also of the sIg^+ B cells in spleen is rapid, with half-lives of less than a week (Rolink et al., in preparation). By these criteria they appear not be selected by ligands (see ref. [27]). Injections of T cell-independent antigens (such as TNP-Ficoll) lead to short, primary B cell responses with no detectable switching to IgG, nor to the development of measurable levels of $CD5^-$ IgM^+ IgD^{++} B cells.

It remains possible that $CD5^-$ IgM^+ IgD^{++} B cells could be generated from the $CD5^+$ IgM^{++} $IgD^{+/-}$ B cells by T cells and T cell-dependent antigens. We are in the process of testing the effects of transfer of T cell population with and without antigens on the development of the B cell compartment in these pre B-I-populated SCID mice.

Hardy and Hayakawa also transferred D_HJ_H-rearranged, $B220^+$, $CD43^+$, HSA^+ pre B cells of bone marrow into SCID mice and found that after three weeks (and as late as 5 months) $CD5^+$ B cells did not develop in these mice. Three weeks after transfer the spleen contained $CD5^-$ B cells. They concluded that $CD5^+$ and $CD5^-$ B cells are generated from different precursors. Our experience with bone marrow-derived pre B-I cells proliferating on stromal cells and IL7 is still limited. While they are capable of differentiation to sIg^+ B cells "in vitro" upon removal of IL7 from culture, their capacity to populate SCID mice for long terms with pre B

and B cells appears more limited. In agreement with Hardy's and Hayakawa's findings we find them not to populate the peritoneum with $CD5^+$ B cells.

Again, and as reasoned above, it might be that the development of stable pools of $CD5^-$ IgM^+ IgD^{++} B cells might be T cell dependent, and experiments to test this are under way.

7. Do SCID Mice Populated with Pre B Cells of Abnormal Strains of Mice Elicit these Abnormalities "In Vivo" in the Absence of T Cells and Other Donor-Derived Cooperating Cells?

(NZBxNZW)F_1 (B/WF_1) mice develop a systemic autoimmune disease resembling systemic lupus erythematosis (SLE). The disease development is controlled by several genetic loci, which can, in part, be genetically separated in recombinant inbred strains (RI) with different assortments of chromosomes for NZB and from a normal mouse (such as C58, Sm or B6) (for literature see ref. [31]).

Progenitor and precursor B cells from B/WF_1 mice, reactive to stromal cells and IL7, can be grown in long term tissue cultures. They can be transferred into SCID mice and elicit many parameters of the lupus-like disease in the SCID mice [31]. Most notably, they develop hyperplasia of IgM- and IgG-secreting cells in many lymphoid organs, elicit elevated levels of IgM, IgG_{2a} and IgG_3, in which autoantibodies to nuclear antigens (ANA), and to single and double-stranded DNA can be detected - all classical signs of the lupus-like disease. They do not develop gp70-retroviral envelope protein-specific antibodies and no lymphoid infiltrations, which occur in B/WF_1 mice in many organs during the disease. Like B/WF_1 mice, the B/WF_1 pre B-populated SCID mice develop glomerulonephritis, though les severe. These results show that genetic defects expressed in B-lineage cells can be expressed in SCID mice separately, and without the influence of T cell and other environmental factors of the B/WF_1 mice. They allow a distinction of those disease-eliciting genetic factors, which need this B/WF_1 environment, and other disease-suppressing factors which suppress the disease in crosses of NZB with normal mice. These enhancing and suppressing factors may be molecules or cells, acting directly or indirectly on B-lineage cells. This experimental system, therefore, allows a diagnosis of the genes, expressed as phenotypes, involved in the disease, in propagation as well as in suppression. Those genes expressed in B-lineage cells can be isolated from cDNA libraries of normal mice for a possible cure of the disease. Such an analysis can also be performed with pre B cells from immunodeficient mice (Kenny et al., this volume), and from mice with susceptibilities to tumor induction in the B lineage pathway of differentiation (Potter et al., previous volumes of this series). Again, the influence of factors environmental to the B cells (T cells, other cooperating cells, cytokines, antigens, etc.) appears amenable to investigations which might aid our eventual understanding of the origins and progression of autoimmune, immunodeficient and malignant cells of the B lymphocyte lineage.

Acknowledgments

The Basel Institute for Immunology was founded and is supported by F. Hoffmann- La Roche Ltd., Basel, Switzerland.

References

1. Rolink A, Melchers F (1991) Molecular and cellular origins of B lymphocyte diversity. Cell 66:1081-1094
2. Osmond DG (1991) Proliferation kinetics and the lifespan of B cells in central and peripheral lymphoid organs. Curr. Op. In Immunol. 3:179-185
3. Rajewsky K (1992) Early and late B-cell development in the mouse. Curr. Op. In Immunol. 4:171-176
4. Hayashi SI, Kunisada T, Ogawa M, Sudo T, Kodama H, Suda T, Nishikawa S, Nishikawa SI (1990) Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. J. Exp. Med. 171:1683-1695
5. Cumano A, Paige CJ, Iscove NN, Brady G (1992) Bipotential precursors of B cells and macrophages in murine fetal liver. Nature 356:612-615
6. Hardy RR (1992) Variable gene usage, physiology and development of $Ly1^+$ ($CD5^+$) B cells. Curr. Op. Immunol. 4:181-185
7. Rolink A, Kudo A, Karasuyama H, Kikuchi Y, Melchers F (1991) Long-term proliferating early pre B cell lines and clones with the potential to develop to surface Ig-positive mitogen-reactive B cells "in vitro" and "in vivo". EMBO J. 10:327-336
8. Melchers F, Strasser A, Bauer SR, Kudo A, Thalmann P, Rolink A (1989) Cellular stages and molecular steps of murine B-cell development. Cold Spring Harbor Symp. Quant. Biol. LIV:183-189
9. Gu H, Förster I, Rajewsky K (1990) Sequence homologies, N sequence insertion and J_H utilization in $V_HD_HJ_H$ joining: Implications for the joining mechanisms and the ontogenetic priming of Ly1 B cells and B-CLL progenitor generation. EMBO J. 9:2133-2140
10. Oltz EM, Yancopoulos GD, Morrow MA, Rolink A, Lee G, Wong F, Kaplan K, Ollies S, Melchers F, Alt FA (1992) A novel regulatory myosin light chain gene distinguishes pre B cell subsets and is IL-7 inducible. EMBO J. 11:2759-2767
11. Shinkai Y, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelson M, Charron J, Datta M, Young F, Stall AM, Alt FW (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to intiate (V(D)J) rearrangement. Cell 68:855-867
12. Rolink A, Streb M, Nishikawa SI, Melchers F (1991) The c-kit-encoded tyrosine kinase regulates the proliferation of early pre-B cells. Eur. J. Immunol. 21:2609-2612
13. McNiece IK, Langley KE, Zsebo KM (1991) The role of recombinant stem cell factor in early B cell development. J. Immunol. 156:3785-3790
14. Miyake K, Medina KL, Obo S, Hamaoka T, Kincade OW (1990a) Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. J. Exp. Med. 171:477-488
15. Miyake K, Underhill CB, Lesley J, Kincade PW (1990b) Halyuronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. J. Exp. Med. 172:69-75

16. Miyake K, Weissman IL, Greenberger JS, Kincade PW (1991) Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J. Exp. Med. 173:599-608
17. Kodama H, Sudo H, Koyama H, Kasai S, Yamamoto S (1984) In vitro hemopoiesis within a microenvironment created by MC3T3-G2.PA6 preadipocytes. J. Cell Physiol. 118:233-240
18. Takai Y, Sakata T, Iwagami S, Tai XG, Kita Y, Hamaoka T, Sakaguchi N, Yamagishi H, Tsuruta T, Teraoka H, Fujiwara H (1992) Identification of IL-7-dependent bone marrow-derived thy$^-$, B220$^-$, lymphoid cell clones that rearrange and express both Ig and T cell receptor genes. J. Immunol. 148:1329-1337
19. Gu H, Kitamura D, Rajewsky K (1991) B cell development regulated by gene rearrangement arrest of maturation by membrane-bound D_μ protein and selection of D_H element reading frames. Cell 65:47-54
20. Kitamura D, Roes J, Kühn R, Rajewsky K (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. Nature 350:423-426
21. Kitamura D, Kudo A, Schaal S, Müller W, Melchers F, Rajewsky K (1992) A critical role of λ5 protein in B cell development. Cell 69:823-831
22. Era T, Ogama DL, Nishikawa SI, Okamoto M, Honjo T, Akaji K, Miyasaki JI, Yamamura K (1991) Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J. 10:337-342
23. Kitamura D, Rajewsky K (1992) Targeted disruptioin of μ chain membrane exon causes loss of heavy-chain allelic exclusion. Nature 356:154-156
24. Yancopoulos GD, Alt FW (1985) Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. Cell 40:271-281
25. Nemazee DA, Bürki K (1989) Clonal deletion of B lymphocytes in a transgenic mouse bearing anti MHC class I antibody genes. Nature 337:562-566
26. Goodnow CC, Crosbie J, Jorgensen H, Brink RA, Basten A (1989) Induction of self-tolerance in mature peripheral B lymphocytes. Nature 342:385-391
27. Gu H, Tarlinton D, Müller W, Rajewsky K, Förster I (1991) Most peripheral B cells in mice are ligand-selected. J. Exp. Med. 173:1357-1371
28. Kubagawa H, Cooper MD, Carroll AJ, Burrows PD (1989) Light-chain gene expression before heavy-chain gene rearrangement in pre B cells transformed by Epstein-Barr-Virus. PNAS 86:2356-2360
29. Strasser A, Rolink A, Melchers F (1989) One synchronous wave of B cell development in mouse fetal liver changes at day 16 of gestation from dependence to independence of a stromal cell envrionment. J. Exp. Med. 170:1973-1986
30. Hardy RR, Hayakawa K (1991) A developmental switch in B lymphopoiesis. Proc. Natl. Acad. Sci. USA 88:11550-11554
31. Reininger L, Radaszkiewicz T, Kosco M, Melchers F, Rolink A (1992) Development of autoimmune disease in SCID mice populated with long-term "in vitro" proliferating (NZBxNZW) F1 pre B cells. J. Exp. Med., in press

Isolation and Characterization of a Stromal Cell Line

K. DORSHKIND[1], R. NARAYANAN[2], and K. S. LANDRETH[3]

[1] Division of Biomedical Sciences, University of California, Riverside, CA 92521,
[2] Department of Molecular Genetics, Hoffmann-La Roche Inc., Nutley, New Jersey 07110
[3] Department of Microbiology and Immunology and The Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV 26506

1. Introduction

Considerable progress in identifying cells and cytokines that regulate primary B cell differentiation has been made in the last few years, and it is now possible to present a model that identifies the stages at which various developmental signals act on B lineage cells. The formulation of such a scheme has been possible largely because of the use of various in vitro systems that allow the stromal cell dependent growth of B cells and their precursors and the application of molecular techniques to the analysis of cells in these cultures (Dorshkind 1990; Kincade et al. 1989).

In order to identify environmental components that regulate B lymphopoiesis, many laboratories have utilized cloned stromal cell lines derived from the adherent layer of long-term bone marrow cultures. One such population isolated in this laboratory, referred to as S17, has been studied extensively. In the following sections, the experiments that have been performed with this cell line and how the data obtained have contributed to the understanding of stromal cell regulation of B lymphopoiesis will be reviewed.

2. Isolation and Characterization of a Stromal Cell Line

2.1 Support of Myelopoiesis and Lymphopoiesis by Stromal Cells

The impetus for the derivation of stromal cell lines in our laboratory was to address the question of whether distinct stromal cell populations existed that could support myelopoiesis and lymphopoiesis or if one stromal cell type could support both lineages. Accordingly, the hemopoietic support capability of several stromal cell lines was tested under different long-term bone marrow culture conditions optimal for myelopoiesis and lymphopoiesis. These experiments were performed by seeding stromal cell adherent layers with adherent cell depleted bone marrow cells. One property of the S17 cells that made these experiments possible was that the line grows as a contact inhibited adherent layer, making it possible to seed cells onto it and not be concerned about stromal cell overgrowth of the cultures.

The results clearly demonstrated that the S17 line could support myelopoiesis, since the number of cells and myeloid colony-forming units had increased at least 10 fold over numbers seeded. After three weeks of growth under myeloid conditions, the reseeded S17 cultures were transferred to conditions optimal for B lymphopoiesis. Over the course of four weeks, the cultures gradually shifted from myelopoiesis to B lymphopoiesis, and a proportion of cells in the cultures expressed the 220,000 MW B220 antigen and surface IgM. Taken together, these results suggested that a single stromal cell population can support both myelopoiesis and B lymphopoiesis (Collins and Dorshkind 1987).

2.2 Stromal Cell Derived Soluble Mediators Support B Cell Differentiation

At the time the above observations with the S17 line were made, one of us (KSL) had been working on characterization of a pre-B cell differentiation factor(s) present in

Current Topics in Microbiology and Immunology, Vol. 182

the urine of patients with cyclic neutropenia (Landreth et al. 1985). The source of that activity was unknown, and a logical question was whether stromal cells produced similarly acting molecules. This was addressed by incubating freshly isolated bone marrow cells, from which B220 and Ig expressing cells had been rigorously depleted, for 24-48 hours with medium conditioned by the S17 line. The data demonstrated that S17 stroma secreted a protein(s) that could potentiate the acquisition of cytoplasmic Ig μ heavy chain protein and surface B220 expression in B cell progenitors that did not express these molecules. A surprising finding was that this differentiation occurred in the absence of detectable cell proliferation, raising the possibility that differentiation in the B cell lineage was not linked to cell growth (Landreth and Dorshkind 1988). Cumano and colleagues (1990) have also demonstrated that maturation in response to S17 factors occurs with little or no proliferation.

2.3 The S17 Activity Does Not Potentiate Expression of Surface Immunoglobulin

The above studies indicated that stromal derived signals exist which only allow development of cells to the stage at which cytoplasmic μ, but not light chain protein expression, occurs. On initial analysis, this observation seemed at odds with the findings in which S17 cells supported the generation of surface IgM expressing cells in long-term bone marrow cultures. However, the latter study could not distinguish if S17 cells directly supported the generation of surface IgM expressing B cells or other accessory cell populations, such as macrophages (Kincade et al. 1981), present in the cultures

That the S17 line does not support the development of Ig light chain expressing cells was substantiated by Henderson et al. (1990). In those studies, an S17 underlayer or conditioned medium from the line was used to potentiate the growth in semisolid medium of colonies composed of lymphoid cells. Phenotypic analysis of the cells present in the colonies indicated that cμ expressing cells were present but that presence of light chain protein was never observed. These results were consistent with the above findings using short-term in vitro differentiation assays and together suggest that the S17 stromal cell line is highly efficient at supporting the maturation of B220 negative, Ig negative B cell precursors to the pre-B cell stage of development. In this regard, the S17 line is similar to other stromal cell lines that also allow development to cμ but not light chain expression (Whitlock et al. 1987; Hunt et al. 1987).

More recently, Henderson and colleagues have attempted to establish a molecular basis for these observations and examined the status of kappa light chain gene rearrangements in the S17 potentiated pre-B cells using various polymerase chain reaction strategies. The results demonstrated that kappa light chain genes in cells from many of these colonies were rearranged, but the use of a reverse transcriptase PCR strategy failed to detect mature kappa transcripts (submitted for publication).

3. Identification of Soluble Mediators Produced by S17 Stroma

The observation that the S17 stromal cell line produced an activity that allowed the development of cμ expressing pre-B cells from pro-B cells initiated a series of studies aimed at identification of the factor. The initial characterization studies only provided information on which cytokines did not have the effect, including IL-1,2,3,4,5 and 6 and the colony stimulating factors (Landreth and Dorshkind 1988). With the identification of stromal cell derived IL-7 and c-*kit*-ligand (KL), an obvious question was whether or not these cytokines could explain the differentiation effect of S17 cells. Billips et al. (1992) addressed this issue by culturing bone marrow from which B220 and Ig expressing cells had been depleted with various concentrations of IL-7, KL, or combinations of the two factors. The data clearly demonstrated that pre-B cell formation from B220-, Ig- progenitor cells and the expression of μ heavy chain of immunoglobulin is dependent on the factor produced by the S17 cell line and cannot be reproduced with IL-7, KL, or both cytokines in combination. However, the data did demonstrate that once pre-B cell formation in response to S17 factors occurred, the cells then became responsive to the proliferation stimulating effects of IL-7 and KL.

3.1 Insulin-Like Growth Factor-I is a Pro-B Cell Differentiation Activity

That neither KL or IL-7 was responsible for the generation of pre-B cells from their precursors could have been predicted by comparing the molecular characteristics of the S17 derived differentiation activity to those factors. The molecular weights of IL-7 (Namen et al. 1988) and KL (reviewed in Witte 1990) are 14.9 and 20-30 kD, respectively, while fractions of S17 conditioned medium collected from a Superose 12 gel filtration column were found to have two peaks of activity associated with molecules of 40 to 60 kD and less than 10 kD (Landreth and Dorshkind 1988).

Recent findings from our laboratories now indicate that the pro-B cell activity produced by the S17 stromal cell line is insulin-like growth factor-I (IGF-I). This conclusion is supported by several experimental observations. First, like S17 conditioned medium recombinant IGF-I potentiates the expression of cytoplasmic μ heavy chain protein on B220-, Ig- bone marrow target cells in short term cultures and this occurs in the absence of detectable cell proliferation. Second, anti-IGF-I antibodies abrogate the activity present in S17 conditioned medium. Finally, pretreatment of S17 stromal cells with an anti-sense oligonucleotide for IGF-I suppresses the ability of the cells to produce conditioned medium that potentiates pre-B cell formation (Landreth et al. 1992). These findings provide convincing evidence that IGF-I plays a role in the differentiation of pro-B cells into pre-B cells.

The size of the low molecular weight activity secreted by S17 cells (<10 kD) is consistent with the 7.5 kD IGF-I molecule. A puzzling observation in our earlier characterization was the association of the differentiation activity with a 40-60 kD fraction when S17 stromal cell conditioned medium was eluted by gel filtration. However, a recent report demonstrated that stromal cell derived IGF-I is associated with a stromal cell derived IGF-I binding protein of 40 kD (Abboud et al. 1991). This observation suggests that the high molecular weight activity secreted by S17 cells may also be attributable to IGF-I as well.

3.2 IGF-I and IL-7 Interact To Stimulate Pre-B Cell Growth

Although no cell growth was detected in differentiation assays, conditioned medium from the S17 line stimulates B cell progenitors to proliferate and form pre-B cell colonies in semisolid medium (Dorshkind et al. 1989). Further experiments revealed that while IGF-I alone did not potentiate colony formation, it was involved in that process because treatment of S17 conditioned medium with an anti-IGF-I antibody removed up to 80% of the lymphoid colony stimulating activity. The actions of IGF-I in this system appear to be due to its ability to augment the effects of proliferation stimulating factors on pre-B cell growth. For example, pre-B cells proliferate in response to IL-7, and if IGF-I is also present, the degree of cell growth is significantly augmented. Recent ongoing studies which have tested the effect of IGF-I on pro-B and pre-B cell clones indicate that these effects of IGF-I on developing B lineage cells are direct (KSL, unpublished observations).

While S17 cells secrete IGF-I, the identity of the factor it produces that stimulates the proliferation of B lineage cells is unclear. No IL-7 transcripts are detected in S17 cells, but there are data that an anti-IL-7 antibody neutralizes the ability of S17 cells to support the growth of IL-7 dependent pre-B cells (P. Kincade, personal communication). Thus, S17 cells may produce enough IL-7 that, in association with factors such as IGF-I and KL, act to potentiate growth of pre-B cell colonies.

4. A Model of B Cell Development

Based on the above observations using the S17 stromal cell line, it is now possible to propose the following working model of primary B cell development (Fig. 1). Among the earliest events in that process is the differentiation of pro-B cells to B cell progenitors/pre-B cells that express the B220 antigen and cytoplasmic μ heavy chain protein, and the data indicate a role for IGF-I in this. Whether IGF-I acts to directly stimulate differentiation or as a survival factor that allows cells to progress through a stochastic program of differentiation is unclear. However, in support of the former

possibility are observations that have defined a paracrine role for IGF-I in differentiation of myoblasts (Florini et al. 1986; Schmid et al. 1984), adipocytes (Smith et al. 1988), neurons (Xue et al. 1988), and oligodendrocytes (McMorris et al. 1986).

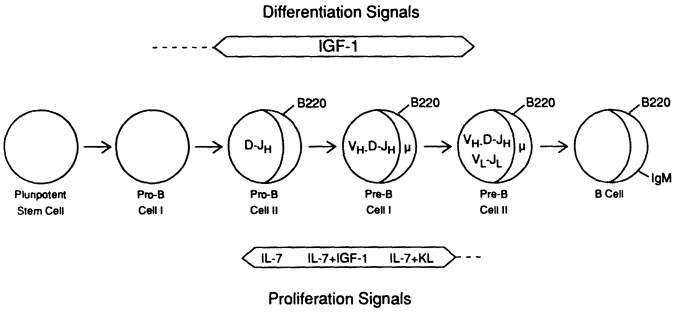


Fig. 1. A model of primary B cell development. Dashed lines indicate that the placement of developmental or proliferative signals is approximate. Ig gene rearrangement events are placed at the cellular stage at which they first occur. Whether IGF-I acts to directly stimulate differentiation or is a survival factor that allows cells to progress through a stochastic program of development is not clear.

A hallmark of B cell development is that there is extensive expansion of the progenitor cell pool, but IGF-I alone does not appear to mediate this activity. However, once pro-B cells have differentiated into progenitors/pre-B cells in response to IGF-I, they then become sensitive to specific growth promoting molecules such as IL-7. Recent evidence would suggest this event occurs at the stage of D-J_H rearrangements (Rolink et al. 1991; Hardy et al. 1991). It is possible for the proliferative effects of IL-7 to be augmented by two additional factors, KL (McNeice et al. 1991) and IGF-I, which alone have no growth promoting activity. This observation is also consistent with findings that IGF-I acts as a cofactor in association with specific proliferation factors. With regards to hemopoiesis, IGF-I acts with erythropoietin or G-CSF to potentiate the effects of those lineage specific mediators on formation of erythroid (Kurtz et al. 1985; Claustres et al. 1987) and granulocytic colonies (Merchav et al. 1988), respectively.

In vivo kinetic studies have revealed that the maturation of small pre-B cells into surface Ig expressing B lymphocytes occurs with minimal to no proliferation (Landreth et al. 1981). This observation would predict that cells would have lost responsiveness to IL-7, KL, and IGF-I by this stage of development. In support of this conclusion is the fact that cells in the pre-B cell colonies whose growth is potentiated by S17 supernatants no longer express receptors for IL-7 or KL (submitted for publication). Thus, by the time kappa light chain gene rearrangements have occurred, cells apparently no longer respond to these factors.

5. Negative Regulators of B Cell Development

Although the S17 stromal cell activity which potentiates pre-B cell formation from pro-B cells appears to be IGF-I, multiple cell types, including fibroblasts, make IGF-I but nevertheless do not function in many hemopoietic assays. This point supports the idea that the hemopoietic support capacity of stromal cells, and by inference hemopoietic regulation within the hemopoietic microenvironment in vivo, must be considered in the context of the sum total of positive and negative regulatory signals present. For example, high levels of colony stimulating factors can be inhibitory for B lymphopoiesis in vitro and in vivo (Dorshkind 1991), but in most passages of S17 cells, G- and GM-CSF transcripts

are not detected by reverse transcriptase PCR. Thus, while many cell types may produce IGF-I, the activity may be masked by high levels of other inhibitory and/or competitive cytokines.

These points raise the issue of whether the particular cytokine profile of S17 cells is similar to what occurs in stromal cells in vivo. This may in fact be the case. However, one can not exclude the possibility that the characteristics of the line that have made it so useful are just a fortuitous adaptation of this particular cell population to long-term in vitro growth.

References

Abboud SL, Bethel CR, Aron DC (1991) Secretion of insulinlike growth factor I and insulinlike growth factor-binding proteins by murine bone marrow stromal cells. J Clin Invest 88:470-475

Billips LG, Pettite D, Dorshkind K, Narayanan R, Chiu CP, Landreth KS (1992) Differential roles of stromal cells, interleukin-7, and kit-ligand in the regulation of B lymphopoiesis. Blood 79:1185-1192

Claustres M, Chatelain P, Sultan C (1987) Insulin-like growth factor I stimulates human erythroid colony formation in vitro. J Clin Endocrinol Metab 65:78-82

Collins LS, Dorshkind K (1987) A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. J Immunol 138:1082-1087

Cumano A, Dorshkind K, Gillis S, Paige CJ (1990) The influence of S17 stromal cells and interleukin 7 on B cell development. Eur J Immunol 20:2183-2189

Dorshkind K (1990) Regulation of hemopoiesis by bone marrow stromal cells and their products. Annu Rev Immunol 8:111-137

Dorshkind K (1991) In vivo administration of recombinant granulocyte-macrophage colony-stimulating factor results in a reversible inhibition of primary B lymphopoiesis. J Immunol 146:4202-4208

Dorshkind K, Johnson A, Harrison Y, Landreth KS (1989) A colony assay system that detects B cell progenitors in fresh and cultured bone marrow. J Immunol Methods 123:93-101

Florini JR, Ewton DZ, Falen SL, van Wyk JJ (1986) Biphasic concentration dependency of stimulation of myoblast differentiation by somatomedins. Am J Physiol 250:C771-778

Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med 173:1213-1225

Henderson AJ, Johnson A, Dorshkind K (1990) Functional characterization of two stromal cell lines that support B lymphopoiesis. J Immunol 145:423-428

Hunt P, Robertson D, Weiss D, Rennick D, Lee F, Witte ON (1987) A single bone marrow-derived stromal cell type supports the in vitro growth of early lymphoid and myeloid cells Cell 48:997-1007

Kincade PW, Lee G, Paige CJ, Schied MP (1981) Cellular interactions afecting the maturation of murine B lymphocyte precursors in vitro. J Immunol 127:255-260

Kincade PW, Lee G, Pietrangeli CE, Hayashi SI, Gimble JM (1989) Cells and molecules that regulate B lymphopoiesis in bone marrow. Annu Rev Immunol 8:111-143

Kurtz A, Hartl W, Jelkman W, Zapt J, Baber C (1985) Activity in fetal bovine serum that stimulates erythroid colony formation in fetal mouse liver is insulinlike growth factor I. J Clin Invest 76:1643-1648

Landreth KS, Rosse C, Clagett J (1981) Myelogenous production and maturation of B lymphocytes in the mouse. J Immunol 127:2027-2034

Landreth KS, Engelhard D, Beare MH, Kincade PW, Kapoor N, Good RA (1985) Regulation of human B lymphopoiesis: effect of a urinary activity associated with cyclic neutropenia. J Immunol 134:2305-2309

Landreth KS, Dorshkind K (1988) Pre-B cell generation potentiated by soluble factors from a bone marrow stromal cell line. J Immunol 140:845-852

Landreth KS, Narayanan R, Dorshkind K (1992) Insulin-like growth factor I (IGF-I) regulates pro-B cell differentiation. Blood In press

McMorris FA, Smith TM, DeSalvo S, Fulanetto RW (1986) Insulin-like growth factor I/somatomedin C: a potent inducer of oligodendrocyte development. Proc Natl Acad Sci USA 83:822-826

McNeice IK, Langley KE, Zsebo KM (1991) The role of recombinant stem cell factor in early B cell development. J Immunol 146:3785-3790

Merchav S, Tatarsky S, Hochberg Z (1988) Enhancement of human granulopoiesis in vitro by biosynthetic insulin-like growth factor I/somatomedin C and human growth hormone. J Clin Invest 81:791-797

Namen AE, Lupton S, Hjerrild K, Wignall J, Mocizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, Gillis S, Cosman D, Goodwin RG (1988) Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 333:571-573

Rolink A, Kudo A, Karasuyama H, Kikuchi Y, Melchers F (1991) Long-term proliferating early pre-B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J 10:327-336

Schmid C, Steiner T, Froesch ER (1984) Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF-I and IGF-II) in primary cultures of chicken embryonic cells. FEBS Lett 173:48-52

Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS (1988) Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J Biol Chem 263:9402-9408

Whitlock CA, Tidmarsh GF, Muller-Sieburg C, Weissman IL (1987) Bone marrow stromal cell lines with lymphopoietic activity express high levels of a pre-B neoplasia-associated molecule. Cell 48:1009-1021

Witte ON (1990) Steel locus defines new multipotent growth factor. Cell 63:5-6

Xue ZG, Le Douarin NM, Smith J (1988) Insulin and insulin-like growth factor-I can trigger the differentiation of catechoaminergic precursors in cultures of dorsal root ganglion. Cell Differ Dev 25:1-10

Growth and Differentiation of a Bipotent Precursor of B Cells and Macrophages

C. J. PAIGE, A. CUMANO, G. BRADY and N. N. ISCOVE

Ontario Cancer Institute, The Wellesley Hospital Research Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, Toronto, Canada

Introduction

Progress in single cell analysis is yielding important new insights concerning the process of B cell development. Single cell cloning assays which distinguish early members of the hemopoietic pathway, some of which are prior to the lymphoid/myeloid branch point, provide the starting material to determine which genetic events are associated with lineage commitment [1,2]. In this report, we describe a cell which is at or near the time of commitment to the B cell pathway. This cell has not yet undergone immunoglobulin gene rearrangement and still retains the potential to develop into macrophages as well as B lineage cells [2]. In addition to progress in cellular cloning assays, technical innovations now allow the creation of cDNA libraries from genes expressed in single progenitors [3]. A strategy to identify the genetic events which lead to the commitment process in these cells is presented.

Single Cell Analysis

Colony Forming Assays

A key element in our experiments is the development of single cell cloning assays which selectively allow the growth and differentiation of B lineage progenitor cells. Single cell cloning assays have been utilized for a number of years [Reviewed in 4,5]. They offer several advantages including: 1) frequency determination; 2) unequivocal identification of differentiated progeny; and 3) the ability to distinguish between direct and indirect effects. We have utilized both semi-solid medium based assays (agar or methyl cellulose) as well as limiting dilution assays in 96 well plates. Progress in this area is based on parallel developments in growth factor identification and cell selection techniques.

Modification of the agar-based CFU-B assay of Kincade, allowed the growth and differentiation of B cell progenitors as colonies [6]. Additional manipulation of the progeny which developed in these cultures allowed us to analyze routinely the immunoglobulin produced by cells in the resultant colonies at both the protein and

Current Topics in Microbiology and Immunology, Vol. 182

RNA levels [7,8]. Analysis of Ig heavy chain allotypes and Ig light chain isotypes within single colonies led to the distinction of cells before and after commitment to Ig heavy and light chain expression [9]. Although a variety of primitive B cell progenitors could be studied in this assay, the growth factor requirements remained poorly defined and optimal stimulation relied on the presence of freshly prepared fetal liver adherent cells. Two important developments in the last few years have spurred progress in this field: 1) the discovery of IL-7 and its action on B cell precursors provided access to cells that previously had been difficult to study; and 2) the identification of stromal cell lines which could substitute for the heterogeneous adherent cells previously used [10-18].

One significant limitation of the semisolid assay is that the length of time cells can be cultured is limited by medium exhaustion. It is also difficult to modify the culture conditions to meet the changing requirements of differentiating cells. Both of these difficulties are largely overcome in liquid-based limiting dilution cultures. We currently use a two step procedure [1]. In the first step progenitor cells are placed in 96-well costar plates which contain adherent irradiated S17 stromal cells [15] and relevant growth factors. The cells are seeded at limiting dilution. The accuracy of this method is routinely validated by visual inspection. In some cases single cells were micromanipulated under direct microscopic observation to ensure clonality [2]. After 10-14 days the culture conditions are modified by transferring clones to fresh wells containing either LPS and S17 for the production of Ig secreting B cells or CSF-1 for the growth of pure macrophage colonies. Cells placed in the second culture system can also be seeded by limiting dilution if frequencies of a particular subset within a developing clone are of interest.

Several diagnostic techniques can be applied to determine the commitment stage of the founder cell. For example, determination of the allotype and isotype of secreted immunoglobulin yields information regarding the state of commitment to immunoglobulin gene expression. Analysis of mRNA that encodes immunoglobulin variable regions with probes that detect V gene families allows access to more detailed information on V gene selection. In some cases the retention of immunoglobulin gene segments in germ line configuration, detectable in large clones by standard Southern blotting techniques or by quantitative PCR in smaller samples, shows that the founder cell was not rearranged at the initiation of the culture period. Each of these methods, applied to the developing clone, yield a retrospective snapshot of the state of the founding cell. A more innovative method relies on sibling analysis of single cells, some of which are used to generate cDNA, while others are allowed to express their developmental potential in culture.

Representative Amplification of cDNA of Single Cells

We have previously described a PCR method, known as *polyA PCR* which can be used to amplify all of the polyadenylated message in a single cell [3,19]. Briefly, this involves creation of representative short cDNA molecules each bracketed by known sequences complementary to the PCR primer. The known sequences are

added in two steps. In step one the 3' end is defined in a cDNA reaction using reverse transcriptase and an oligo (dT) primer, which will prime via the poly (A) tail present at the 3' end of most mRNA molecules. In the second step a homopolymer (dA) tract is added to the 3' end of each newly created cDNA molecule using terminal transferase. Since the PCR reaction is most efficient for relatively short sequences, amplification of full-length cDNA would result in disproportionate amplification of smaller cDNAs. This bias is deliberately avoided in the *polyA PCR* protocol by limiting the length of the initial cDNA strand to around 100-700 bases regardless of the size of the original RNA template.

PCR amplification of the (dA)/(dT) bracketed cDNA is carried out using a single oligonucleotide, which consists of a 5' sequence containing a restriction enzyme site and a 3' (dT) stretch. Priming of the cDNA in the PCR reaction is initiated via annealing of the (dT) region of the oligo to the homopolymer (dA) regions present at the termini of the cDNA molecules. The unique 5' sequence provides a convenient means of cloning the amplified product using the included restriction enzyme site, and increases stability and precision of annealing during PCR.

Purification steps have been avoided to minimize losses of starting material and opportunities for contamination. The final protocol is carried out in a single tube and involves only three pippeting steps prior to PCR amplification. The amplified product can be cloned into bacterial vectors and renewed indefinitely through further rounds of PCR. From samples as small as a single cell, *polyA PCR* produces microgram amounts of representative cDNA, which is infinitely renewable and can be used as a substrate for conventional molecular techniques such as filter hybridisation, cloning and sequencing.

Progenitor cells are rare in hematopoietic tissues, are available in only small numbers, and are heterogeneous in terms of their differentiative and proliferative potential. These features have effectively blocked comprehensive analysis of the gene expression programs in the early stages of normal precursor development. The ability to amplify messenger RNA globally from single cells represents an important breakthrough, solving the problems of progenitor quantity as well as progenitor purity. The approach is most useful in circumstances in which the biological potential of individually processed cells is known with a high degree of likelihood, for example, when applied to members of highly purified populations. The resulting amplified material can be repeatedly probed to determine expression of a variety of genes of interest. The material also provides an inexhaustible source of nucleic acid for subtractive analyses aimed at identifying genes that are differentially expressed during progenitor cell differentiation and that might play roles in steering the differentiation process.

Cell Surface Phenotype

The most primitive progenitor cells are often among the least common cells found in a particular site. Methods to enrich these cells are, therefore, essential for any direct analytical approaches. Cell separation techniques based on the physical attributes of cells have been effectively used in many instances. For the work

reported here, we rely on the differential expression of cell surface markers recognized by antibodies.

We have utilized four monoclonal antibodies to enrich progenitor cells in our recent experiments. These include AA4.1, B220, Mac1, and Ly6a. AA4.1 was derived from a series of rat anti-mouse lymphoma monoclonal antibodies developed by McKearn and colleagues [20]. It was shown to detect a variety of hemopoietic progenitors. More recently Jordan et al. have demonstrated, using retrovirus marked cells in an in vivo reconstitution assay, that fetal liver derived AA4.1$^+$ cells included long-term reconstituting units [21]. B220, the B cell form of CD45, is a phosphoprotein phosphatase which is involved in signal transduction. Our experiments use the 14.8 antibody developed by Kincade and colleagues [22]. The B220 antigen has been known to be a marker of committed B cell progenitors for many years. Recent data from Hardy and colleagues shows that some bone marrow derived B220$^+$ B cell precursors have not yet rearranged the Ig locus [23]. Mac1 is a member of the integrin family and was originally identified as an antigen found on macrophages [24]. It has also been reported to be expressed on some B cell subsets, notably the CD5 population [25]. The Ly6 alloantigens are small cell surface proteins encoded by a cluster of about 20 genes [26]. Originally identified as T cell activation markers, subsequent studies have shown that they have a broad tissue distribution. Weissman and colleagues, using the SCA1 antibody, have shown that Ly6A is expressed on primitive hemopoietic progenitors [27].

These monoclonal antibodies have been utilized in a panning protocol that yields highly enriched populations of cells suitable for subsequent clonal analysis [1,2]. The panning technique has the disadvantage of not quantifying the degree of cell surface expression of a particular antigen. Nonetheless, this method has proved in our hands to be superior to other cell selection methods when single cell, functional, assays are the desired endpoint. We adopt the convention of designating cells that fail to adhere to a plate coated with a particular antibody as "negative" for that marker, although such cells may in fact express antigens in amounts insufficient for removal.

Bipotential B Cell/Macrophage Progenitors in 12 Day Fetal Liver

B cell progenitors in the developing fetal liver are heterogeneous. They differ from each other in cell surface phenotype, degree of commitment to Ig expression, responsiveness to growth and differentiation stimuli, and in other respects. In most cases a set of traits such as Ig commitment level and a particular cell surface phenotype are found associated with each other, although causal relationships have not yet been established. Nonetheless, it is useful to attempt characterization based on the criteria of cell surface markers, growth properties, and commitment level.

Based on the criteria described below, at least one progenitor can be described which maintains the capacity to develop into either B cells or macrophages.

The Detection of the Bipotent Progenitors.
The bipotent progenitors were first observed in the course of an investigation focused on B lymphopoiesis. In those initial studies we observed the clonal expansion of small, non-adherent cells, which became IL-7 responsive and $B220^+$. Such clones generally gave rise to cells which could respond to B cell mitogens and secret immunoglobulin. In almost every case the Ig secreted from single clones indicated the utilization of both light chain isotypes and both heavy chain allotypes. This finding demonstrated that the founder cells of such clones were not yet committed to the expression of a particular immunoglobulin molecule and suggested that the rearrangement process was still active in the developing cells. This was confirmed by additional experiments in which we demonstrated that a substantial number of clones contained members that retained the Ig locus in the germ line configuration. Those clones generally came from progenitors that were selected for the expression of AA4.1 and depleted of cells which expressed B220. Moreover, occasionally such clones also contained adherent cells which were morphologically distinct from the developing B lineage cells.

Further enrichment of the progenitor which gave rise to both adherent and B lineage cells was achieved (see below). The B lineage cells derived from progenitors found in this population were now invariably accompanied by adherent cells. The designation of these adherent cells as macrophages was based on the expression of lysozyme, non-specific esterase, and their response to the macrophage growth factor, CSF-1.

Enrichment for Bipotent Progenitors and Confirmation of the Single Cell Origin
The enrichment protocol which allowed us to isolate the bipotent cell consisted of a three stage panning procedure [2]. Fetal liver derived cells which adhere to plates coated with AA4.1 were depleted of cells which were retained on plates coated with anti-B220/anti-Mac1, and subsequently selected on plates coated with anti-Ly6a. This population represented approximately 0.1% of the fetal liver. One in three of the $AA4^+$ $(B220,Mac\text{-}1)^-$ $Ly6a^+$ cells was able to found a colony consisting of both B cells and macrophages whether tested by limiting dilution or single cell manipulation. Limiting dilution analysis was carried out under conditions which yield an average of 3, 2, or 1 cell/10 wells. Under these conditions, the frequency of colonies which contained both macrophages and B cells was far higher than predicted based on the likelihood of coincidence of two committed unipotent progenitors. Moreover, visual inspection of the cultures was used to assure that doublets were excluded. The single cell origin was confirmed by micromanipulation in which single cells were picked from enriched populations and deposited in a well. A second micropipette was then used to transfer such cells to the culture plate. This double transfer procedure excluded the transfer of a potentially unobserved second cell sticking to the pipette.

Differentiation Capacity of the Bipotent Progenitor

The 12 day fetal liver population that contained bipotent progenitors also contained progenitors which gave rise to macrophages alone, and to mixed myeloid colonies. Colonies containing B cells alone were not found. Moreover, B cell containing colonies never contained cell types other than macrophages. The possibility that inhibition of B cell growth by a second cell type was responsible for this result, was excluded by seeding cells at densities expected to yield two or more distinct progenitors/well. Under these conditions the expected number of B/Macrophage clones was observed, but now these were frequently accompanied by other members of the myeloid lineage. A number of experiments were also carried out with other growth/differentiation factors such as IL-1, IL-3, kit-ligand, and M-CSF, but we have not yet observed other lineages developing from founder cells with B cell potential. A further measure of the differentiation potential of the bipotential progenitor was assessed in a six month erythroid in vivo reconstitution assay. We showed that the population which contained nearly all bipotent progenitors completely lacked the ability to sustain erythropoiesis in this assay. This reconstitution assay shows a strong correlation to the long-term reconstituting multipotent stem cell, these data demonstrate that the bipotent progenitor is distinct from this stem cell.

The immunoglobulin locus of the bipotent cell was in germ line configuration. This was demonstrated by quantitative PCR-based cDNA amplification of macrophage samples. We found that both alleles were germline, confirming that the founder cell had not yet begun to rearrange its Ig locus. To further characterize the enriched population, cDNA was amplified from 10^3 cells and examined for the presence of various expressed genes. We found that this population contained cells expressing RAG-2, c-fms, and cμ [2]. We have not yet determined whether the bipotent cell itself expresses these genes.

Growth and Differentiation Factor Requirements

The bipotent cell requires exposure to an adherent stromal cell for the initial part of the assay. We use the S17 cell line which has proven effective for cells already committed to B cell differentiation. We do not yet know if other stromal lines also support differentiation of the bipotent cell. After several days of culture on S17 stroma, non-adherent IL-7 responsive B cell progenitors arise. At the same time, CSF-1 dependent adherent cells are seen. The IL-7 responsive cells are >98% $B220^+$, and within 10-12 days such cells can be transferred to fresh plates which contain additional S17 stromal cells and the B cell mitogen, LPS. Under these conditions they begin to secrete immunoglobulin after several days. We have not yet determined which stromal cell derived factors are needed to support growth and differentiation in this assay system.

Acknowledgements: This work was supported by the Medical Research Council of Canada and by the National Cancer Institute of Canada. AC is a Fellow of the Medical Research Council. GB is a Special Fellow of the Leukemia Society of America. We thank K. Dorshkind, F. Melchers, D. Schatz, Immunex, and Genetics Institute for reagents.

References

1. Cumano A, Paige CJ (1992) Enrichment and characterization of uncommitted B cell precursors from fetal liver at 12 days of gestation. EMBO J 11: 593-601
2. Cumano A, Paige CJ, Iscove NN, Brady G (1992) Bipotential precursors of B cells and macrophages identified in murine fetal liver at day 12 of gestation. Nature 356: 612-615
3. Brady G, Barbara M, and Iscove NN (1990) Representative in vitro cDNA amplification from individual hemopoietic cells and colonies. Meth Mol Cell Biol 2:17-25
4. Kincade PW (1987) Experimental models for understanding B lymphocyte formation. Adv Immunol 41:181-267
5. Rolink A, Melchers F (1991) Molecular and cellular origins of B lymphocyte diversity. Cell: 66 1081-1094
6. Paige CJ (1983) Surface Ig-negative B cell precursors detected by formation of antibody-secreting colonies in agar. Nature 302: 711-713
7. Sauter H, Paige CJ (1987) Detection of normal B cell precursors that give rise to colonies producing both κ and α light immunoglobulin chains. Proc Natl Acad Sci USA 84:4989-4993
8. Wu GE, Paige CJ (1988) Vh gene family utilization is regulated by a locus outside of the Vh region. J Exp Med 167:1499-1504
9. Sauter H, Paige CJ (1988) B cell progenitors have different growth requirements before and after immunoglobulin heavy chain commitment. J Exp Med 168:1511-1516
10. Namen AE, Lupton S, Hjerrild K, Wignall J, Mochizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, Gillis S, Cosman D, Goodwin RD (1988) Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 333:571-573
11. Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW (1984) Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β. J Immunol 142:3875-3883
12. Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi SI, Ogawa M, Sakai K, Nishikawa S, Nishikawa SI (1989) Interleukin 7 production and function in stromal cell-dependent B cell development. J Exp Med 170:333-338
13. Cumano A, Dorshkind K, Gillis S, Paige CJ (1990) The influence of S17 Stromal cells and interleukin 7 on B cell development. Eur J Immunol 20:2183-2189
14. Hayashi SI, Kunisada T, Ogawa M, Sudo T, Kodama H, Suda T, Nishikawa S, Nishikawa SI (1990) Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. J Exp Med 171:1683-1695
15. Collins LS, Dorshkind K (1987) A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoisis. J Immunol 138:1082-1087
16. Kincade PW, Witte PL, Landreth K (1987) Stromal cell and factor dependent B lymphopoiesis in culture. Curr Top Microbiol 135:1-21
17. Kincade PW (1991) Molecular interactions between stromal cells and B lymphocyte precursors. Sem Immunol 3:379-390
18. Rolink A, Kudo A, Karasuyama H, Kikuchi Y, Melchers F (1991) Long-term proliferating early pre-B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J 10:327-336
19. Brady G, Iscove NN (1992) Amplified representative cDNA libraries from single cells. Methods in Enzymology, Wassarman PM and DePamphilis ML (eds), in press
20. McKearn JP, Baum C, Davie JM (1984) Cell surface antigens expressed by subsets of pre-B cells and B cells. J Immunol 132:332
21. Jordan CT, McKearn JP, Lemischka R (1990) Cellular and developmental properties of fetal hematopoietic stem cells. Cell 61:953-963
22. Kincade PW, Lee G, Watanabe T, Sun L, Scheid MP (1981) Antigens displayed on murine B lymphocyte precursors. J Immunol 127:2262

23. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K (1991) Resolution and Characterization of Pro-B and Pre-Pro-B Cell Stages in Normal Mouse Bone Marrow. J Exp Med 173:1213-1225
24. Springer T, Galfré G, Secher DS, Milstein C (1979) Mac-1: A macrophage differentiation antigen identifed by monoclonal antibody. Eur J Immunol 9:301-306
25. Herzenberg LA, Stall AM, Braun J, Weaver D, Baltimore D, Herzenberg LA, Grosschedl R (1987) Depletion of the predominant B-cell population in immunoglobulin μ heavy-chain transgenic mice. Nature 329:71
26. LeClair KP, Palfree RGE, Flood PM, Hammerling U, Bothwell A (1986) Isolation of a murine Ly-6 cDNA reveals a new multigene family. EMBO J 12:3227-3234
27. Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58-62

Control of Intramarrow B-Cell Genesis by Stromal Cell-Derived Molecules

S. I. NISHIKAWA, T. ERA, M. OGAWA, S. NISHIKAWA, N. OHNO, S. I. HAYASHI and T. KUNISADA

Department of Morphogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860, Japan

A remarkable progress has been made in the investigation of B cell-genesis since Whitlock and Witte have reported a long-term culture system of bone marrow B cells (Whitlock and Witte 1982). Although some more stromal cell molecules are yet to be cloned before concluding that we know all molecules involved in the intramarrow B cell-genesis, the basic framework of the molecular requirements for B cell-genesis has been understood to a considerable extent. As a result of this progress, a numbers of methods which use stromal cell lines and recombinant cytokines to control B cell-genesis *in vitro* are presently available (for review see Kincade et al. 1989; Rolink and Melchers 1991, Kunisada et al. 1992). It is also true, however, that some inconsistency is still present among the previous results on the growth signal requirement of B precursors. Given that control of cell proliferation in higher organisms might be achieved in a redundant and fail-safe manner, this redundancy could be a source of the inconsistency among the previous results. Thus, in this article, we would like to re-examine three key propositions which we have been stated in the previous studies and discuss them in detail in light of the previous results of ours and other groups.

IL-7 is an absolute requirement for B cell-genesis

We made this statement on the basis of our results that a stromal clone, PA6 which does not express IL-7 is unable to support B cell-genesis, whereas B cell-genesis starts by adding of recombinant IL-7 in this culture (Sudo et al. 1989; Hayashi et al. 1990). This result is further confirmed by our recent study that transfection of IL-7 gene into PA6 renders this stromal cell clone capable of supporting B cell-genesis (Kunisada et al. 1992). Furthermore, similar results have been reported from another group using PA6 as stromal cell clone for culture (Rolink et al. 1991). Taken together, as far as PA6 is used in the culture, IL-7 is an absolute requirement for B cell-genesis. The strongest evidence against this proposition, however, has also been presented in the culture system using S17 stromal cell clone (Collins and Dorshkind 1987). As shown in the previous paper,

S17 does not express IL-7, while it can support B cell-genesis (Billips et al. 1992). A morestriking observation which is described in this issue by D.Saffran is that pre-culturing bone marrow cells on S17 produces $B220^+$ cells which do not respond to IL-7 (see Doug Saffran et al. : Growth of enriched population of B lymphoid progenitor cells is stromal cell dependent and does not require SLP or IL-7, in this issue). The implication of these results is that B cell-genesis occurs in the complete absence of IL-7. If one must accept both results, the simplest possibility would be to assume existence of an IL-7-equivalent factor which is expressed in S17 . Obviously, this putative factor should not be expressed in PA6, because PA6 is not able to support B cell-genesis in the absence of IL-7. In fact, previous results suggests that other cytokines could work as growth factor for B cell-genesis under certain but probably unusual circumstances(Palacios et al 1984, Paige et al. 1985). Of note in this context is an observation of ours and other groups that a population of B precursor cells in the normal bone marrow is able to proliferate in response to IL-7 alone and form colonies in semi-solid culture systems (Lee et al. 1989; Suda et al. 1989). However, cytokines with such an activity as IL-7 has not been identified in any stromal cell clones. Thus, if S17 expresses an IL-7-equivalent factor, it would be interesting to know whether or not it has pre-B colony inducing ability.

Although sound paradoxically, another point which we would like to emphasize is our notion that IL-7 is not the absolute requirement for B cell differentiation. Instead, we think that most processes of B lineage differentiation might be by and large cell autonomous rather than being directed by stromal cell molecules. Thus, it may be possible that some stem cells undergoes complete differentiation into mature B cells without IL-7, if a minimum level of cell proliferation is maintained by other stromal cell factors. Actually, although extremely low in number, $B220^+$ cells are always included in the bone marrow cells proliferating on the PA6 layer in the absence of IL-7. However, to maintain a constant and extensive B cell-genesis as observed in normal bone marrow (for review see Gallagher and Osmond 1991), additional growth factors like IL-7 are required. In conclusion, although not proven yet, we prefer to think that IL-7 as well as other stromal cell derived factors play a role as pure growth factors which make B cell-genesis enough efficient so as to be able to produce $5x10^7$ cells everyday. Thus, any factors, that can touch the machinery for B cell proliferation and increase its efficiency, could promote B cell-genesis .

C-kit and its ligand *Steel factor* (SLF) play a functional role in B cell-genesis

Since the role of SLF in B cell-genesis is rather new issue, there might not be a firm argument against this statement yet. Previous studies using the recombinant SLF have

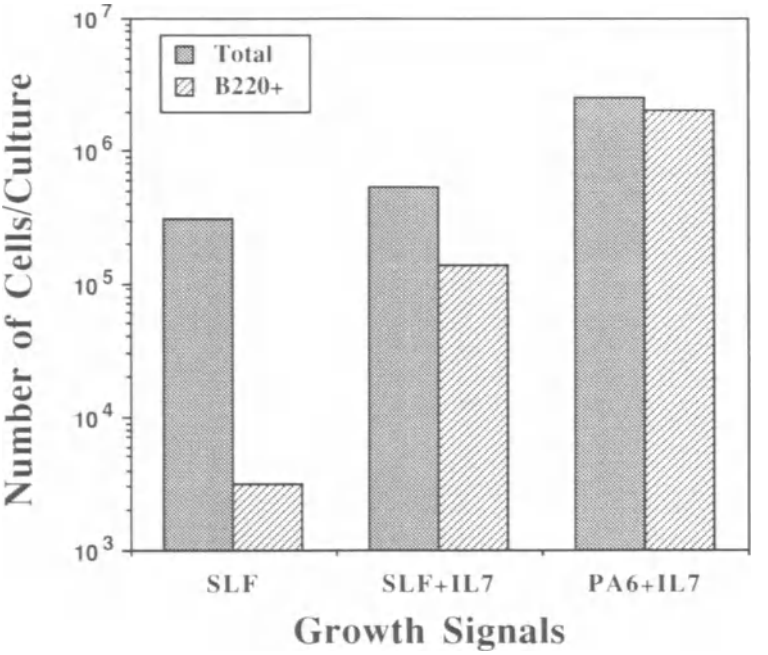


Figure 1. B cell-genesis from C-kit$^+$lineage$^-$ cells under various culture conditions. C-kit$^+$lineage$^-$ cells were sorted by FACScan, and 10^4 of them were cultured in the presence of either SLF alone, SLF+IL-7 or PA6+IL-7. Recoveries of total cells and B220$^+$ cells were counted 8 days after culture

provided positive evidence for the role of SLF in B cell-genesis (McNeice et al. 1991). Among all, the most remarkable is a report by Hirayama et al that a single IL-3 induced blast colony can give rise to B lineage cells in the culture containing IL-7 and SLF (Hirayama et al. 1992). Our result shown in Fig.1 is consistent with their observation. In this experiment, we purified c-kit$^+$lin$^-$ cells from normal bone marrow and cultured in the presence of SLF, SLF+IL7 and PA6+IL7. Our result shows clearly that B cell-genesis from the early B220$^-$ precursors is supported by SLF+IL7, although involvement of other factors present in fetal calf serum could not be excluded. In contrast to these results, a recent paper by Billips et al. (1992) showed that SLF+IL7 is not enough to support B cell-genesis from B220$^-$ stem cells. More contrasting is an observation of Saffran described in this issue that B progenitors harvested from the bone marrow culture on S17 do not respond to IL-7 nor SLF (Saffran et al. in this issue). In fact, previous studies on *W* or *Sl* mutant mice demonstrated that B cell compartment remains rather intact in these mice (Landreth et al. 1984). Moreover, we failed to block B cell-genesis in normal bone marrow by injecting an anti-c-kit mAb which antagonize c-kit function and

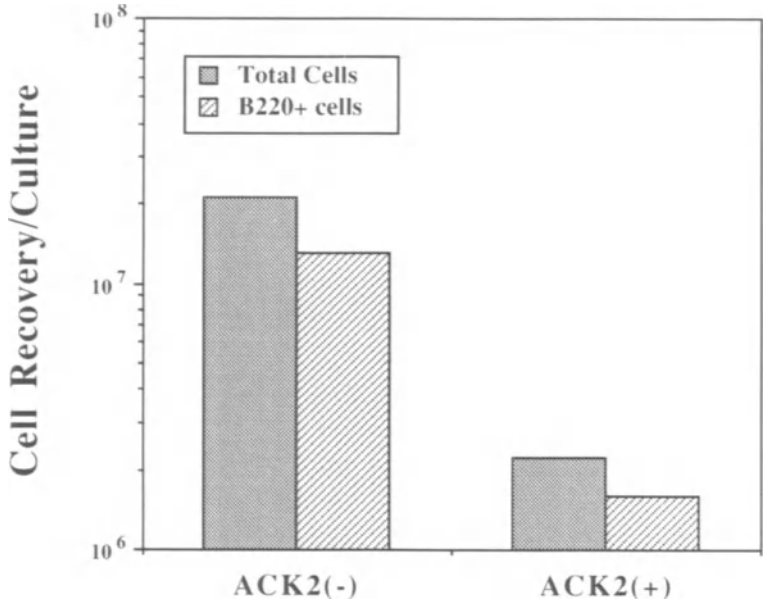


Figure 2. Effect of an anti-c-kit mAb, ACK2 on B cell-genesis in stromal cell-dependent culture. 10^6 bone marrow cells precultured on PA6 for two weeks were cultured in the presence of PA6+IL-7. 10μg/ml ACK2 was added to inhibit c-kit function. Cell recovery was estimated 9 days after culture

blocks in vivo myelopoiesis and erythropoiesis (Ogawa et al. 1991). All these results indicate that SLF does not play a principal role in actual B cell-genesis, although it may have some effect on *in vitro* B lymphopoiesis.

Notwithstanding these facts, we believe that SLF plays a functional role in B cell-genesis. First, in vivo B cell-genesis is blocked by the same anti-*c-kit* mAb, if the mAb is injected into such a mouse as κ-chain transgenic mouse in which the frequency of the IL-7-CFCs is low. Thus, we think that our failure to block B cell-genesis in normal mice by anti-*c-kit* mAb is because overgrowth of pre-B cells which are IL-7-dependent but independent from other stromal cell-derived molecules compensate for the decrease of earlier B precursors. Second, B cell-genesis from $B220^-$ cells in the culture containing IL-7 and PA6 is severely suppressed by the addition of this mAb (Fig 2). It is interesting to note, however, the effect of the mAb is not complete and about 10% of B cell progenitors remain proliferative and give rise to mature B cells even in the presence of an excess dose of the mAb. In agreement with this observation, the ability of SLF+IL-7 to support in vitro B cell-genesis is far less than that obtained by PA6+IL7 (Figure 1). Thus, SLF+IL-7 can support the proliferation of B progenitors to some extent, thereby

increasing an efficiency of B cell-genesis. However, presence of some additional but yet unknown stromal cell-derived molecules appears to induce much higher level of B cell-genesis.

B cell differentiation is directed by expression of immunoglobulin

This statement (Era et al. 1991), although having been the most controversial one, is now being accepted by most B cell biologists because emerging evidence on various gene-knock-out mice indicates that the expression of a transmembrane form of μ-chain by itself is the most important signal which triggers the differentiation of B cells (Kitamura et al. 1991). Therefore, I do not have much difficulty in stating the process of B cell differentiation includes a change of growth signal requirements, namely differentiation from PA6+IL-7 dependent stage into IL-7 alone dependent stage. In fact, in the last B cell neoplasia meeting, K.Rajewsky mentioned that no IL-7 colonies are present in the μMT/μMT mouse.

One additional statement which we would like to make in this context is that the outcome of μ-chain expression is merely an acquisition of a new signalling machinery which has not existed in the earlier differentiation stage. Thus, if other signalling pathways which share some downstream parts with the μ-chain-derived signalling pathway is activated, one might be able to induce the similar B cell differentiation by bypassing the μ-chain expression. For example, while no IL-7-CFC are present in fresh bone marrow of *scid* mice or the μMT/μMT mouse, it is possible to obtain cell lines which proliferate in response to IL-7 alone without expressing μ-chain proteins. Therefore, any exceptions from this statement would not surprise us, because we are aware that the signalling pathways in a given B progenitor are complex and redundant.

Conclusion and Prospect

Figure 3 illustrates our current view on signalling pathways involved in B cell-genesis. Our previous studies showed that *c-kit* is expressed in pluripotent stem cells and plays a functional role for their selfrenewal (Ogawa et al. 1991; Okada et al. 1991). Recent studies of other group demonstrated that other cytokines are additionally required for supporting the efficient self-renewal of the stem cells (for review see Scadden et al. 1991). Taken together, hematopoietic stem cells at the earliest part of their differentiation require at least two signalling pathways; one from tyrosine kinase receptor system and the other from cytokine receptor family molecules. Even after commitment into B cell lineage, the basic properties of their growth requirements remains unchanged. Namely, both cytokine receptor family molecules (IL-7R) and tyrosine kinase receptor molecules(c-kit) are required for cell proliferation. It is of important to note that c-kit

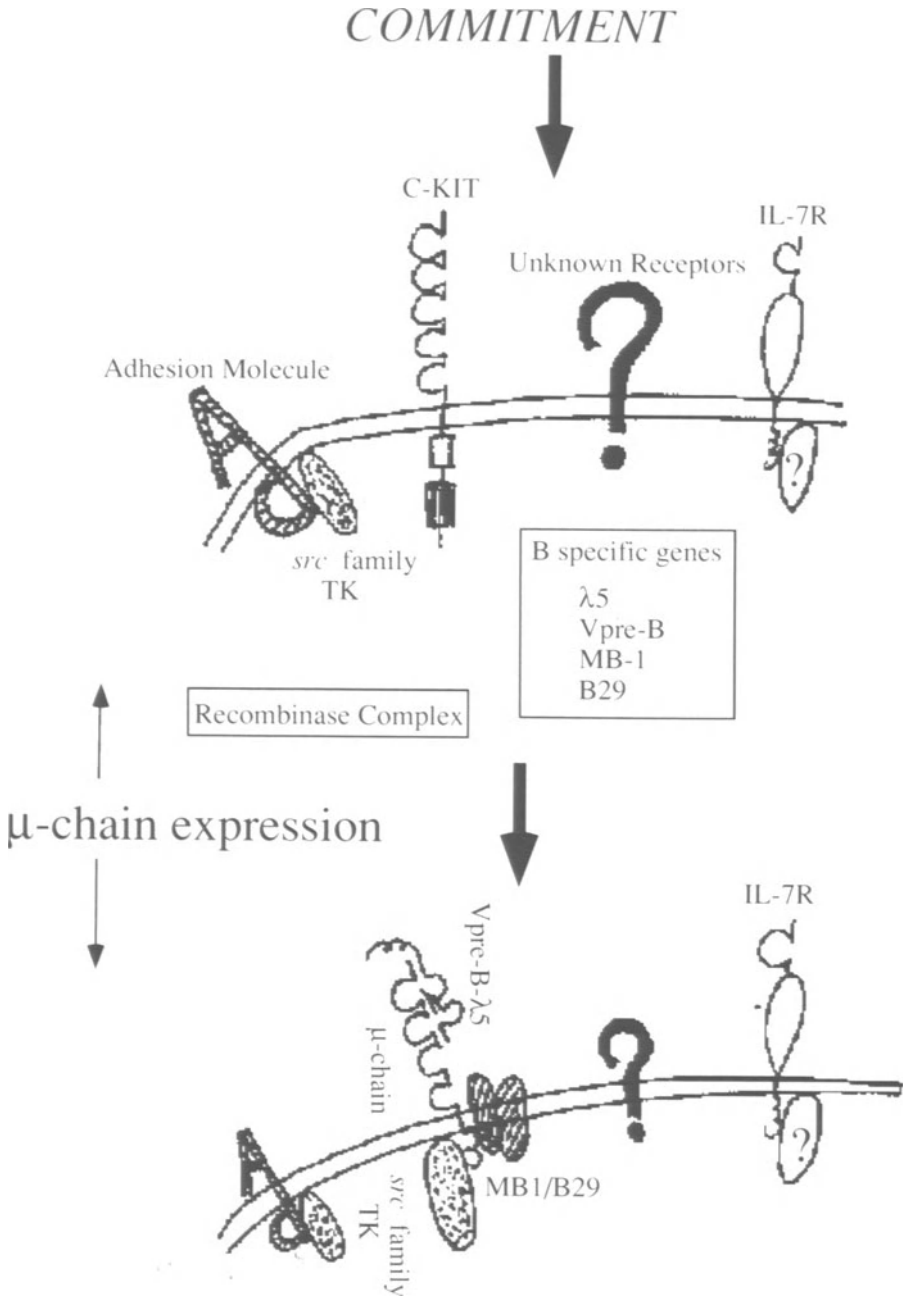


Figure 3. Signalling machinery involved in B cell-genesis. For explanation see text

and IL-7R are not the whole signalling machinery available in B cells. In fact our result in Figures 1, 2 and previous studies on S17 derived signals suggests the presence of other signalling pathways, although the molecular nature of these ligands and receptors are yet to be determined.

Previous studies demonstrated that the initial events taking place after the commitment are activation of immunoglobulin gene rearrangement and expression of a number of "B cell specific molecules"(for review see Rolink and Melchers 1991). Since expression of an immunoglobulin molecule is an ultimate destination of B cell differentiation, it would be reasonable that the molecules regulating VDJ recombination are activated upon commitment into B cell lineage. Then, what is the functional role of "B cell specific molecules" which are expressed before expression of μ-chain? In this respect, it is interesting to note that virtually all of the "B cell specific genes" obtained by subtraction cloning method are μ-chain associative molecules. Although it might be possible that these molecules can function by themselves without μ-chains (see a paper by F.Melchers in this issue), they albeit most efficiently when assembled on μ-chains. Taken together, we propose that major function of "B cell-specific molecules" is to make a signalling complex together with μ-chains, thereby allowing pre-B cells to know whether or not they have succeeded to express μ-chains. If one compares B cell differentiation with erythrocyte differentiation, the most important difference resides in the fact that not all B precursors succeeded to express immunoglobulin genes, while all erythroid precursors are able to express hemoglobulin genes. This is because V gene rearrangement per se is an error prone process. Obviously, by developing such an imprecise way to undergo V gene rearrangement which produces an enormous diversity at junctional sites of V genes, the size of antibody repertoire increases remarkably. Thus, despite the cost to generate non-functional B cells during the differentiation process, this impreciseness would be important for immune system. Nevertheless, this cost seems to be overcome beautifully by developing a group of molecules which can convert μ-chain to a signal for differentiation. Thus, next questions to be addressed is what kind of signalling molecules exist in the downstream of this complex of μ-chains and B cell-specific molecules and what kind of effects are produced by this signalling pathway. In this respect, one point which we want to make here is that *c-kit* -independence of B progenitors is acquired at this stage, because pre-B cells which proliferate in response to IL-7 alone appears upon expression of μ-chains. Thus, we speculate that the signalling pathway from μ-chains may overlap to some extent with the signalling pathway downstream of the receptor tyrosine kinase, c-kit, thereby allowing $c\mu^+$ pre-B cells proliferate in the absence of SLF.

Finally, since we expect that next issue of B cell development will be focused upon the intracellular signalling molecules for the differentiation, various model mice whose B cell differentiation is arrested due to the blockade of the most upstream of the signalling

pathway would be useful to identify the downstream molecules capable of complementing this defect.

Acknowledgement

This work is supported by grants from The Japanese Ministry of Education, Science and Culture, from The Institute of Physical Chemistry (RIKEN) and from the Mochida Foundation. We thank Drs. T. Sudo, Biomaterial Research Institute, H. Kodama, Ohu University, J. Nakao and T. Imamura, Chemo-Sero-Therapeutic Research Institute for reagents.

References

Billips LG, Petitte D, Dorshkind K, Narayanan R, Chiu CP, Landreth KS (1992) Differential roles of stromal cells, interleukin-7, and kit-ligand in the regulation of B lymphopoiesis. Blood 79: 1185-1192.

Collins LS, Dorshkind K (1987) A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. J Immunol. 138: 1082-1087.

Era T, Ogawa M, Nishikawa SI, Okamoto M, Honjo T, Akagi K, Miyazaki JI, Yamamura K (1991) Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J 10:337-342.

Gallagher RB, Osmond DG (1991) To B, or not to B: that is the question. Immunol Today 12: 1-3.

Hayashi SI, Kunisada T, Ogawa M, Sudo T, Kodama H, Suda T, Nishikawa S, Nishikawa SI (1990) Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. J Exp Med 171: 1683-1695.

Hirayama F, Shih JP, Warr GW, Clark SC, Ogawa M (1992) Clonal proliferation of murine lymphohemopoietic progenitors in culture. Proc Natl Acad Sci USA in press.

Kincade PW, Lee G, Pietrangeli CE, Hayashi SI, Gimble JM (1989) Cells and Molecules that regulate B lymphopoiesis in bone marrow. Annu Rev Immunol 7: 111-144.

Kitamura D, Roes J, Kühn R, Rajewsky K (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. Nature 350: 423-426

Kunisada T, Ogawa M, Hayashi SI, Era T, Nishikawa SI (1992) Interleukin-7 and B lymphopoiesis. Chemical Immunol 51: 205-235.

Landreth KS, Kincade PW, Lee G, Harrison DE (1984) B lymphocyte precursors in embryonic and adult W anemic mice. J Immunol 132: 2724-2730

Lee G, Namen AE, Gillis S, Ellingworth LR, Kincade PW (1989) Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-β J Immunol 142 3875-3883.

McNiece IK, Langley KE, Zsebo KM (1991) The role of recombinant stem cell factor in early B cell development. J Immunol 146: 3785-3790.

Ogawa M, Matsuzaki Y, Nishikawa S, Hayashi SI, Kunisada T, Sudo T, Kina T, Nakauchi H, Nishikawa SI (1991) Expression and function of c-kit in hemopoietic progenitor cells. J Exp Med 174: 63-71.

Okada S, Nakauchi H, Nagayoshi K, Nishikawa S, Nishikawa SI, Miura Y, Suda T (1991) Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. Blood 78: 1706-1712.

Paige CJ, Giseler RH, McKearn JP, Iscove NN (1984) Differentiation of murine B cell precursors in agar culture. Frequency, surface marker analysis and requirement for growth of clonable pre-B cells. Eur J Immunol 14: 979-987.

Palacios R, Henson G, Steimetz M, McKearn JP (1984) Interleukin 3 supports growth of mouse pre-B cells clones in vitro. Nature 309: 126-131.

Rolink A, Kudo A, Kayasuyama H, Kikuchi Y, Melchers F (1991) Long-term proliferating early pre-B cell lines and clones with the potential to develop to surface Ig-positive, mitogen reactive B cells in vitro and in vivo. EMBO J 10: 327-336.

Rolink A, Melchers F (1991) Molecular and Cellular origins of B lymphocyte diversity. Cell 66: 1081-1094.

Scadden DT, Feder D, Groopman JE (1991) Scratching the surface. Curr Biol. 1: 301-303.

Suda T, Okada S, Suda J, Miura Y, Ito M, Sudo T, Hayashi SI, Nishikawa SI, Nakauchi H (1989) A stimulatory effect of recombinant murine interleukin-7 on B cell colony formation and inhibitory effect of IL-1a Blood 74: 1936-1941.

Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi SI, Ogawa M, Sakai K, Nishikawa S, Nishikawa SI (1989) Interleukin 7 production and function in stromal cell-dependent B cell development. J Exp Med 170: 333-338.

Whitlock CA, Witte ON (1982) Long term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci USA 79: 3608-3612.

Establishment of a Reproducible Culture Technique for the Selective Growth of B-Cell Progenitors

D. C. SAFFRAN[1,2], E. A. FAUST[2] and O. N. WITTE[1,2]

[1]Howard Hughes Medical Institute, University of California, Los Angeles, CA 90024
[2]Department of Microbiology and Molecular Genetics, University of California, Los Angeles, CA 90024

We describe a culture system which permits growth of enriched populations of progenitor cells of the B-lymphocyte lineage. In this technique bone marrow cells are plated on the stromal line S17, which preferentially selects for growth of B-cell progenitors. After 3-4 weeks in culture the cells display size scatter profiles and morphological appearance characteristic of small to medium size lymphocytes. The majority of cells express the CD45R (B220) antigen, while a small percentage express cytoplasmic mu chain. Surface IgM, as well as stem cell, T-cell, and myeloid antigens are not expressed. Molecular analysis reveals germline configuration of the IgH chain locus and expression of genes associated with early stages of B-cell development. Progenitor cell populations can differentiate into mature B-cells after injection into CB.17 SCID mice. This culture technique represents a reproducible method to obtain enriched progenitor B-cells that can be used to identify genes and growth factors which regulate early B-cell development.

Our goal is to determine what signals are required for growth of early progenitors of the B-cell lineage. The development of B-cells involves discrete stages characterized by commitment of a stem cell to a progenitor (pro-) B cell which lacks lineage-specific antigens, with the possible exception of CD45R (B220) and low levels of Thy 1.2, and retains Ig heavy (H) chain genes in germline configuration (reviewed in 1,2). Pro-B cells differentiate to pre-B cells which express additional surface antigens and specific genes (ie. BP-1, VpreB, lambda5, TdT) and have rearranged IgH chain genes. Upon successful rearrangement at both the IgH and IgL loci, pre-B cells become mature B-cells which express surface Ig and are exported to the periphery. The use of the Whitlock-Witte culture system and transformation of pre-B cells by Abelson murine leukemia virus have permitted detailed analysis of the pre-B to B-cell transition including specific growth requirements and molecular mechanisms which result in Ig gene rearrangement and diversity of the antigen receptor (2-5). In contrast little is known about the early stages of B-cell development due to the inability to obtain pure or enriched populations of B-cell progenitors for study.

We report here a reproducible technique to obtain representative populations of B-cell progenitors. Enriched populations of B-cell progenitors selectively grow after plating BM cells on the stromal cell line S17 (6). We refer to this technique as the lymphoid progenitor cell (LPC) culture system. This system was previously used in our lab to show

that expression of the human chimeric oncogene BCR/ABL in mouse BM cells could stimulate growth of clonal lines of B-cell progenitors (7). In addition, several labs have used alternative techniques to grow out populations and clones of pro- and pre-B cells, which typically requires exposure to high doses of growth factors such as IL-3 or IL-7, and in some cases combination with stromal cells (8-11). The LPC culture system we describe here results in growth of B-cell progenitors which resemble those found in situ in BM, have had no exposure to exogenous growth factors, and have not been selected for clonal bias which may skew growth characteristics. We have used this system to demonstrate that steel locus factor, SLF, which is the ligand for c-kit, and IL-7 are not sufficient signals for growth of progenitor B-cells (E.A.Faust, D.C.Saffran, and O.N.Witte, manuscript in preparation).

In the LPC culture system BM cells are plated on the stromal cell line S17. This contrasts to Whitlock-Witte cultures, in which BM cells themselves give rise to a complex stromal layer which eventually supports several stages of B-lymphopoiesis. The LPC cultures are fed 3 days after initiation, and 4 days later by removal of 75% of spent medium and addition of fresh medium. This procedure is repeated weekly. Within 1-2 weeks most myeloid elements die, followed by selective growth of a more uniform population of cells with lymphoid morphology by weeks 2-3 of culture. Wrights stained cytospin preparations demonstrate that these cells exhibit a monomorphic cellular morphology characteristic of small and medium size lymphocytes (Figure 1). This is confirmed by the scatter profiles showing that cells from LPC cultures are less varied in size and complexity as compared to BM, but similar to cells from Whitlock-Witte cultures.

We evaluated the maturational status of the cells from LPC culture by staining them with fluorochrome-conjugated antibodies specific for early and late stages of B-cell development. The flow cytometry data in Figure 2 represent individual staining profiles we normally obtain with LPC cultures. These data show that LPC cultures are enriched for CD45R (B220+) cells, with a small percentage of cells which express low levels of cytoplasmic mu chain. However none of the cells express IgM on their surface. In addition, we have also stained the cells with a series of antibodies directed against other lineage-specific markers which shows that the cells do not express myeloid, T-cell, or the stem cell antigen FALL-3. However a small percentage of cells do express a low level of Thy 1.2, which indicates that a sub-population resembles B220+, Thy 1.2(lo) cells which have been proposed to represent the earliest stage of B-cell development (12). The staining data shows that the cells from LPC culture are committed to the B-lineage but lack cytoplasmic and surface Ig expression. This suggests that these represent cells at a differentiation stage prior to the characteristic pre-B cells that are normally derived by the Whitlock-Witte culture system.

To further identify the specific stage of B-cell development the LPC cultures represent, we performed molecular analysis of DNA and RNA from the cells. A summary is presented here, but the primary data will be reported elsewhere (E.A.Faust, D.C.Saffran, and O.N.Witte, manuscript in preparation). A hallmark of B-cell differentiation is rearrangement of Ig heavy (IgH) chain genes. Southern blot analysis of several LPC cultures revealed that the cells retained IgH genes in the germline configuration with a small proportion of D-J rearrangements. This is in contrast to Whitlock-Witte cultures where IgH D-J rearrangements have occurred among the majority of cells. We also examined expression of genes associated with early B-cell development by Northern analysis. Cells from LPC culture express transcripts for sterile mu, TdT, and B29, however transcripts for the RAG-1 and RAG-2 genes are present only at low levels.

PROTOCOL FOR THE GROWTH OF LYMPHOID PROGENITOR CELLS (LPC)

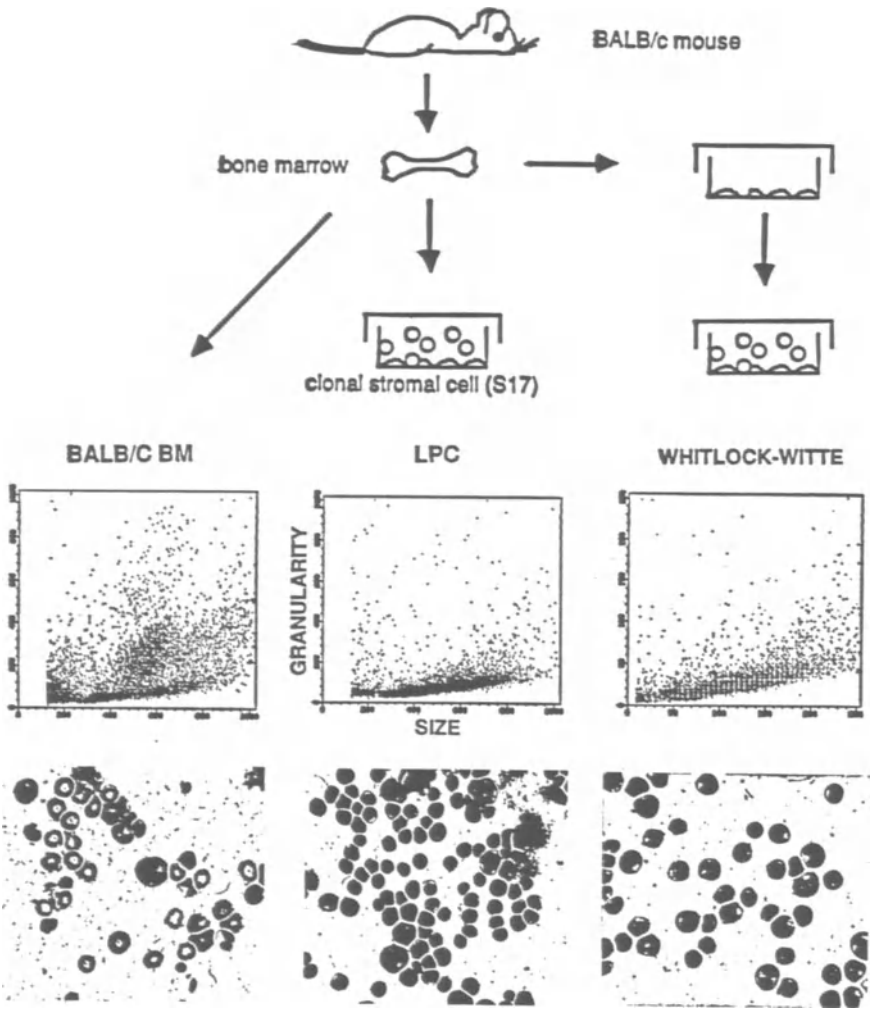


Fig. 1. This scheme represents the culture technique for the growth of B-cell progenitors as compared to the Whitlock-Witte culture system. Note the differences between the cultured cells and bone marrow (BM) with respect to size scatter profiles and general morphology.

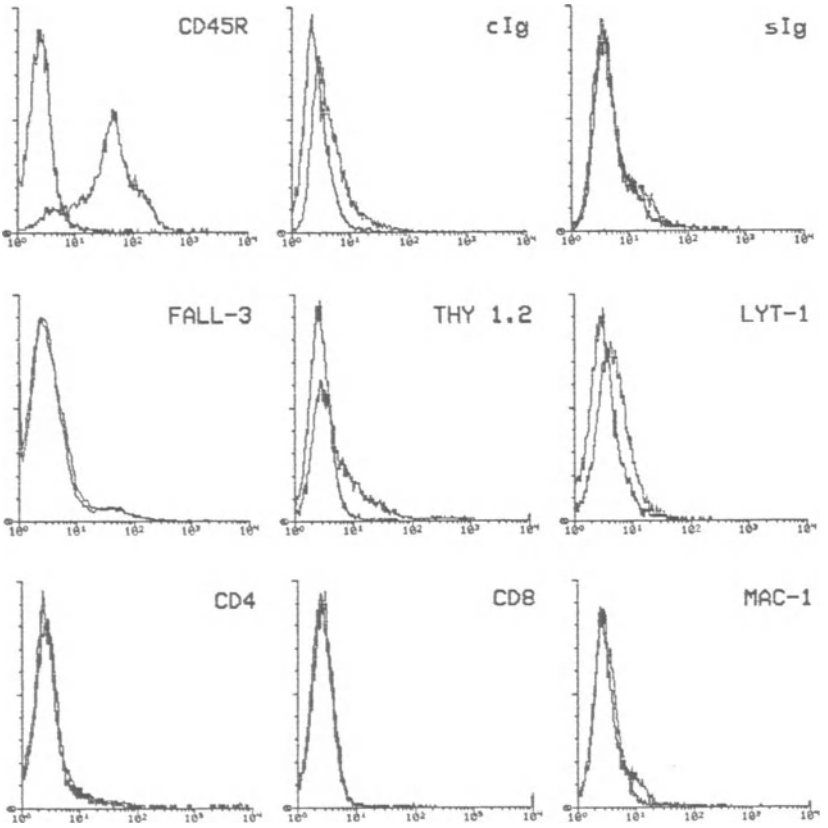


Fig. 2. FACScan analysis of cells from LPC cultures stained with specific fluorochrome-conjugated antibodies. Each panel illustrates the representative staining profile of cells stained with the indicated antibody. In all cases the cells are stained with the appropiate isotype-matched control antibody as shown.

These data demonstrate that low levels of RAG gene expression in LPC cultures correlates with germline configuration of IgH genes (E.A.Faust, D.C.Saffran, and O.N.Witte, manuscript in preparation). Although similar B-lymphoid specific genes are expressed in both LPC and Whitlock-Witte cultures, the low frequency of IgH gene rearrangements in LPC cultures clearly distinguishes the two systems.

To determine the ability of the cells from LPC culture to complete differentiation and become mature B-cells, we utilized severe combined immune deficient mice (CB.17 SCID) as a host to assess B-cell reconstitution. Sub-lethally irradiated CB.17 SCID mice were injected intravenously with 1-3 X 10^6 LPC from 3-4 week old cultures. After 8-10 weeks animals were sacrificed and reconstitution of the B-lymphocyte population was determined

by the presence of surface IgM positive cells in the spleen and soluble IgM in serum. FACS profiles in Figure 3 show that sIgM positive cells are present in comparable levels in the spleens of mice reconstituted with either cells from LPC culture or equal numbers of fresh BM cells. A selected experiment presented in Figure 4 compares data from several reconstituted animals. In this case injection of LPC results in a mean of 25% surface IgM positive cells in the spleen. In addition the same animals contain IgM in the serum, although levels are significantly high as compared to BM reconstituted animals. This may possibly represent deregulation of Ig production at the clonal level, which is currently under investigation.

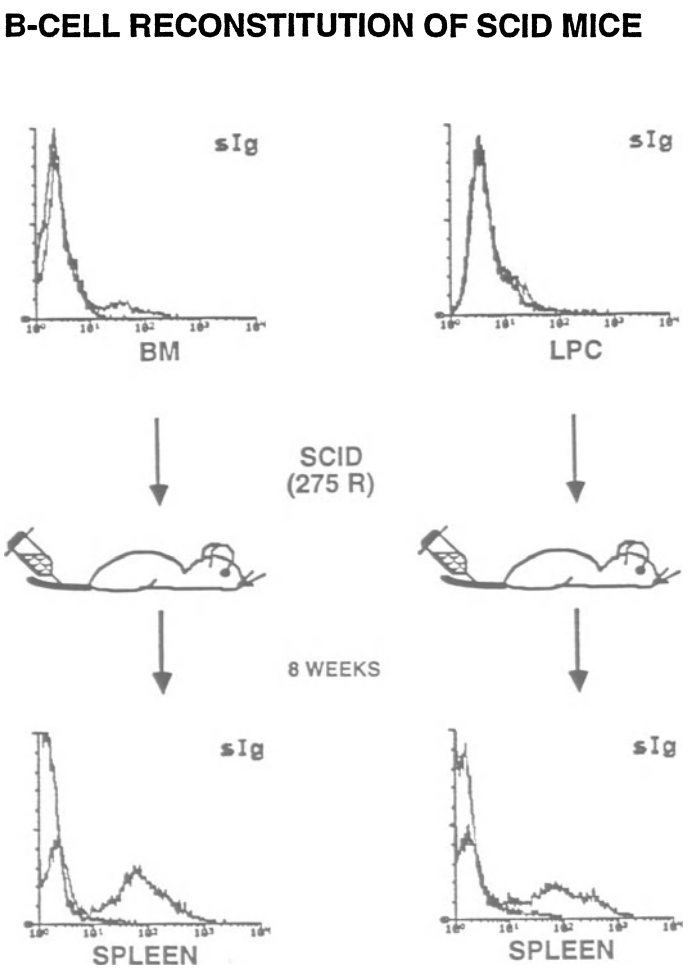


Fig. 3. CB.17 SCID mice were sub-lethally irradiated and injected with either Hanks BSS, Balb/c BM, or cells derived from LPC culture. After approximately 8 weeks the animals were sacrificed and analyzed for expression of sIgM positive cells in the spleen and the presence of IgM in the serum.

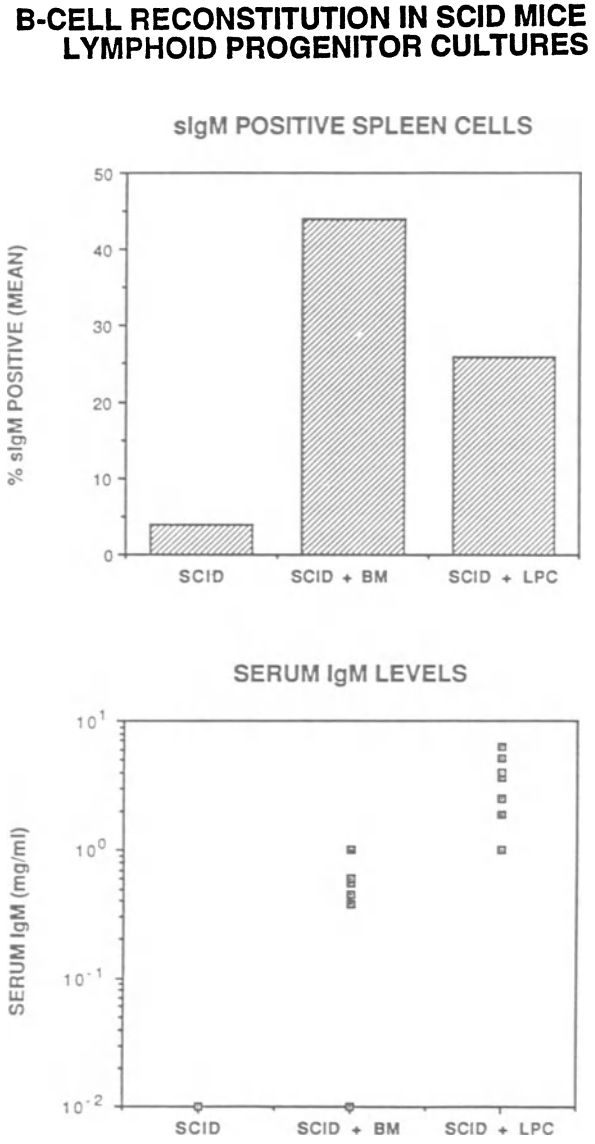


Fig. 4. The results represent a selected experiment in which reconstituted CB.17 mice were analyzed for the presence of B-cells in the spleen and IgM in serum.

Hardy and colleagues (13) have recently shown that B220+, HSA+, CD43+ sorted BM cells exhibit D-J rearrangements as determined by PCR analysis. The LPC cultures we describe likely represent cells one stage prior to this, since they have committed to expression of B220 but not Ig gene rearrangement. According to Osmond's scheme of B-cell development (14), these cells would be considered early to intermediate pro-B cells

based on expression of B220 and TdT, and lack of Ig heavy chain gene rearrangement. The establishment of the LPC culture system provides a reproducible source of enriched pro-B cells that may be used to define growth and differentiation signals required early in B-cell development. Several groups have reported that steel locus factor, SLF, and/or IL-7 could support growth of either pro-B cells (10, 15,16), pre-B cells (17), or both (18). The differences in growth responsiveness to SLF and IL-7 are likely due to the source of cells used in the different systems and their ambiguous definition as either pro- or pre-B cells. We have used the enriched populations from LPC cultures to demonstrate that SLF and IL-7 alone or in combination are not sufficient to maintain growth of pro-B cells (E.A.Faust, D.C.Saffran, and O.N.Witte, manuscript in preparation). Our findings suggest that stromal cells such as S17 produce a signal which acts independently or in concert with SLF and IL-7, and may come in the form of a growth factor(s) or specific adhesion molecules. Alternatively B-cell progenitors may lack the necessary downstream signalling components to respond to SLF and IL-7 at that specific stage of development. The LPC cultures provide a useful system to examine these issues as well as the role of known or novel genes and molecules on B-cell development.

Acknowledgements

We thank Mikhail Gishizky for critical reading of the manuscript. We also thank Numan Parada for assistance with photography and illustrations. D.C.S. is a Research Associate of the Howard Hughes Medical Institute. E.A.F. is a recipient of a Clinical and Fundamental Immunology Institutional Training Grant, AI-07126. O.N.W. is an Investigator of the Howard Hughes Medical Institute.

References

1) Kincade, P.W. (1987) Adv. Immunol. 41:181.
2) Rolink, A. and Melchers, F. (1991) Cell 66:1081.
3) Whitlock, C.A. et.al. (1985) Ann. Rev. Immunol. 3:213.
4) Alt, F.W. et.al. (1981) Cell 27:381.
5) Alt, F.W. et.al. (1987) Science 238:1079.
6) Collins, L. and Dorshkind, K. (1987) J. Immunol. 138:1082.
7) Scherle, P.A.S. et.al. (1990) P.N.A.S. 87:1908.
8) McKearn, J.P. et.al. (1985) P.N.A.S. 82:7414.
9) Kinashi, T. et.al. (1988) P.N.A.S. 85:4437.
10) Rolink, A. et.al. (1991) EMBO J. 10:327.
11) Palacios, R. et.al. (1987) EMBO J. 6:3687.
12) Tidmarsh, G.F. et.al. (1989) Mol. Cell. Biol. 9:2665.
13) Hardy, R.R. and Hayakawa, K. (1991) P.N.A.S. 88:11550.
14) Osmond, D.G. (1991) Mechanisms of B Cell Neoplasia, Eds. F. Melchers and M. Potter, pg.1.
15) Palacios, R. and Samaridis, J. (1992) Mol. Cell. Biol. 12:518.
16) Hayashi, S-I. et.al. (1990) J. Exp. Med. 171:1683.
17) Billips, L.G. et.al. (1992) Blood 79:1185.
18) McNiece, I.K. et.al. (1991) J. Immunol. 146:3785.

Multiple Steps in the Regulation and Dysregulation of B-Cell Precursors in the Bone Marrow: *c-myc* Expression, In Vivo Effects of Interleukins 1 and 7, and Stromal Cell Interactions

D. G. OSMOND, K. JACOBSEN, L. FAUTEUX, H. VALENZONA and S. RICO-VARGAS

Department of Anatomy and Cell Biology, McGill University, 3640 University Street, Montreal, Quebec, Canada H3A 2B2.

INTRODUCTION

The generation and selection of B lymphocytes in the bone marrow involves a complex series of differentiation steps, molecular interactions with local stromal cells and the influences of both short range and systemic growth factors (Osmond 1986,1990; Kincade 1989,1991). Normal regulation of B lymphopoiesis requires a balance between these various processes. Dysregulations, possibly predisposing to oncogenesis, may result from perturbations at a number of different points in the chain of regulatory events.

Differentiating precursor B cells in mouse bone marrow sequentially express characteristic molecules (Park & Osmond 1987,1989; Osmond 1990). Pro-B cells, before the expression of immunoglobulin (Ig) chains, transiently express intranuclear terminal deoxynucleotidyl transferase (TdT) while they are in the process of rearranging Ig heavy chain variable region (V_H) genes. B220 glycoprotein is then coexpressed on the surface membrane of some TdT^+ cells, and persists throughout the rest of the B cell lineage. Subsequently, μ heavy chains are assembled in the cytoplasm, the cell now being termed a pre-B cell. Finally, whole IgM molecules are assembled and inserted into the surface membrane, constituting the mature B lymphocyte. A series of cell divisions by pro-B cells and large pre-B cells results in much clonal expansion of the precursor B cell pool. This is followed, and possibly accompanied, by a massive degree of cell loss (Opstelten & Osmond 1983; Deenan et al 1990; Osmond 1990). Many precursor B cells appear to undergo a programmed cell death (apoptosis) and are rapidly ingested by resident macrophages (Jacobsen & Osmond 1990,1991). This process may represent an important screening mechanism to cull aberrant B lineage cells, including those that may have sustained a potentially oncogenic dysregulation.

Precursor B cells proliferate in intimate association with stromal reticular cells both in vivo (Jacobsen & Osmond 1990) and in vitro (Kincade et al 1989). Adhesion molecules retain B lineage cells on the stromal framework of the bone marrow, as an essential requirement for their development, until they reach the appropriate maturation stage to be released into the blood circulation. The same stromal cells provide similar services to other hemopoietic precursor cells. The molecular bases on which stromal cells may recognize and discriminate between various hemopoietic lineages and may prevent the dissemination of immature or dysregulated cells are unknown.

Stromal cell-derived growth factors, notably interleukin (IL)-7, are necessary for B lymphopoiesis. IL-7 stimulates pre-B cell proliferation (Namen et al 1988; Subo et al 1989; Kincade et al

Current Topics in Microbiology and Immunology, Vol. 182

1989,1990) and expression of the c-myc protooncogene (Morrow et al 1992). It remains to determine whether the target cells for this cytokine also include pro-B cells, a differentiation stage which is particularly vulnerable to genetic errors and dysregulation during V_H gene recombination events.

Systemic cytokines also appear to influence B lymphopoiesis in the bone marrow. The proliferation of pro-B cells and pre-B cells can be stimulated by administering various foreign agents in vivo, an effect mediated by the activity of macrophages in the spleen (Park & Osmond 1991). Pathological processes associated with intense macrophage activation, notably murine malaria and pristane oil-induced granulomata, also produce sustained stimulation of pro-B cells (Osmond, Priddle & Rico-Vargas 1990). These findings have suggested that the local stromal cell-dependent mechanisms regulating B cell genesis in the bone marrow may be susceptible to perturbations in the levels of certain circulating cytokines which could act on precursor B cells either directly or indirectly by inducing the production of stromal cell factors. Among the many cytokines produced by activated macrophages, IL-1 is released systemically but its possible in vivo effects on precursor B cells are not clear. Largely depressive effects have been observed in vitro (Dorshkind 1988). Identification of cytokines capable of mediating systemic stimulation of precursor B cell proliferation would be important to identify factors which may predispose to oncogenesis and to devise possible blocking strategies.

The present work has concerned three levels of the control of B cell production and loss in the bone marrow (1) intrinsic deregulation of the myc protooncogene (2) in vivo actions of candidate local and systemic cytokines, IL-7 and IL-1 (3) the nature of the molecular interface between stromal cells and B cell precursors.

PRODUCTION AND DEATH OF B CELL PRECURSORS IN Eμ-MYC TRANSGENIC MICE

Transgenic mice which carry a c-myc oncogene coupled to the immunoglobulin heavy chain enhancer all develop malignant clonal B lineage lymphomas and leukemias (Adams et al 1985; Harris et al 1988; Sidman, Marshall & Harris 1988). During a pretumorous period of several months which varies in length between individual mice, an initial burst of large proliferating B lineage cells in the blood is followed by a prolonged period in which blood B cells are essentially normal. In due course, however, a dysregulated cell invariably evades normal homeostatic protective mechanisms to produce a malignant clone. To investigate the cellular events leading up to tumorigenesis we have examined the perturbations in the proliferative dynamics and microenvironmental associations of B cell precursors produced by c-myc deregulation during the pretumorous phase.

In collaboration with Dr. Charles L. Sidman, Jackson Laboratories, Maine, precursor B cells were examined in bone marrow suspensions from C57BL/6J Smn-Eμ-myc (Eμ-myc) mice identified as being in the pretumorous phase by flow cytometry of blood lymphocytes, compared with cells of congenic C57BL/6J Smn (B6) mice. The population sizes of pro-B cells, pre-B cells and B lymphocytes, as well as the production rate of proliferating pro-B and pre-B cells were determined using double immunofluorescence labeling and mitotic arrest techniques, as described (Opstelten & Osmond 1983; Park & Osmond 1987,1989). The in situ organisation and cellular associations of $B220^+$ B lineage cells were examined by the binding of intravenously administered ^{125}I-labeled monoclonal antibody (mAb) 14.8 (Kincade 1989), detected by electron microscope radioautography, as described (Jacobsen & Osmond 1990,1991).

Despite the apparent normality of peripheral blood cells in Eμ-myc mice, marked perturbations of proliferation of precursor B cells were evident compared with B6 controls (Table 1).

Table 1. Number and proliferation of B lineage cells in bone marrow of Eμ-myc B6 transgenic mice[a]

Cell population[b]	Cells/femur ($x10^5$)[c]		Mitoses/femur ($x10^4$)[d]	
	Control	Eμ-myc	Control	Eμ-myc
Pro-B cells	8.6	35.4	15.2	40.1
Pre-B cells	51.0	78.0	18.0	54.6
B lymphocytes	22.3	14.6	-	-

[a] Each value was derived from a pool of three mice.
[b] Pro-B cells consist of 3 populations negative for μ chains (TdT^+B220^-; TdT^+B220^+; TdT^-B220^+); pre-B cells ($c\mu^+s\mu^-$); B lymphocytes ($s\mu^+$).
[c] Calculated from the incidence of cells of each phenotype relative to nucleated cells and total cellularity of the bone marrow.
[d] Calculated from the incidence of cells of each phenotype and percentage of cells of each phenotype in metaphase (metaphase index) 2h 40min after injecting vincristine sulfate i.p.

Pro-B cells in Eμ-myc mice were increased threefold in both population size and production rate, measured as the number of cells flowing through mitosis per unit time (Table 1). This included an increased production rate of TdT^+ intermediate pro-B cells as well as increased numbers and production rate of late pro-B cells. Pre-B cells also increased threefold in production rate, representing the subset of large dividing pre-B cells. Paradoxically, the population size of pre-B cells was increased only 1.5 fold, and the number of B lymphocytes was actually reduced to only 65% of control values (Table 1). The number of B lymphocytes in the spleen closely resembled control values (not shown).

Thus, c-myc deregulation in the B cell lineage increases the number of pro-B and pre-B cells dividing per unit time, yet their more mature progeny, small pre-B cells and B lymphocytes, are reduced in numbers. In the absence of evidence of accelerated migration of newly-formed B lineage cells from the bone marrow to the peripheral lymphoid tissues, these findings imply that the expanded proliferation of B precursors is associated with a greatly exaggerated degree of cell loss.

Electron microscope radioautography of bone marrow revealed prominent $B220^+$ cells, often in mitosis and in clusters which were larger and more numerous than usual (Fig. 1). Many of the $B220^+$ cells showed morphological evidence of programmed cell death (Wyllie et al 1984), including irregularly shaped nuclei with dense chromatin condensation, cytoplasmic electron density and vacuolization (Fig. 1). The apoptotic cells were more numerous and the degree of apoptotic morphology was more extreme than that normally seen. $B220^+$ cells were often associated with the extensive processes of macrophages which often surrounded apoptotic B lineage cells and had many ingested bodies within their cytoplasm (Fig. 1). However, some small $B220^+$ cells of normal morphology were also observed within the lumen of bone marrow sinusoids (not shown).

Thus, constitutive c-myc expression in the B cell lineage of Eμ-myc mice produces two opposing effects, increasing both the proliferation of precursor B cells and their tendency to undergo a programmed cell death and ingestion by macrophages. To become frankly neo-

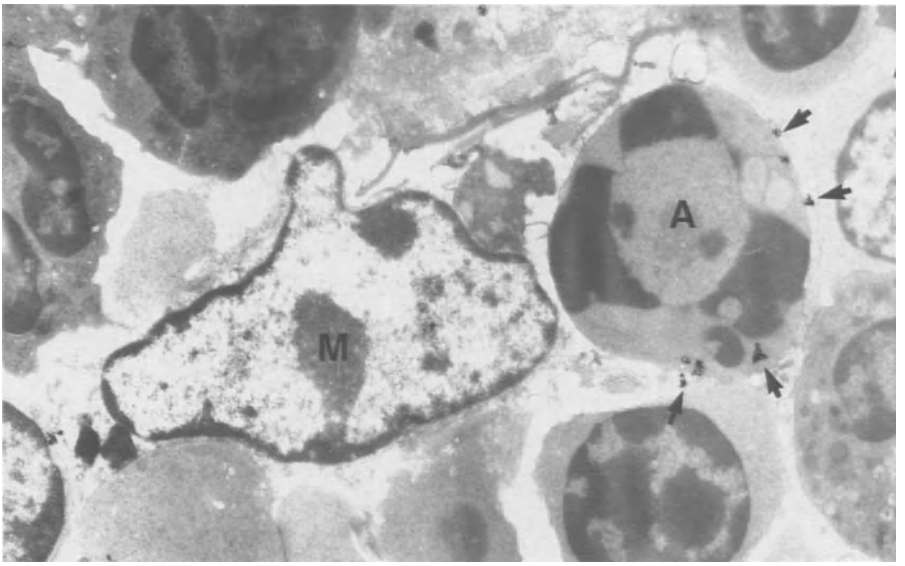


Figure 1. Bone marrow of Eμ-myc mouse. A macrophage (M, nucleus) with cytoplasmic phagosomes and crystalloid inclusions surrounds an apoptotic cell (A) with radioautographic labeling (arrows) of B220 glycoprotein (X 6250)

plastic the B lineage cells apparently must undergo further changes to acquire mechanisms to evade these processes of negative selection.

IN VIVO EFFECTS OF RECOMBINANT INTERLEUKINS 1 AND 7 ON PROLIFERATIVE DYNAMICS OF B CELL PRECURSORS

To examine their capacity to stimulate precursor cells at each phenotypic stage of B cell differentiation in vivo we have administered recombinant IL-1 and IL-7 provided by Dr. Anthony E. Namen, Immunex Corporation, Seattle, by subcutaneously implanted microosmotic minipump in 8-10 wk C3H/HeJ mice. The cytokines were delivered at a constant rate in mouse serum albumin (MSA) of low lipopolysaccharide activity. Control mice received an equivalent dose of the MSA vehicle alone.

Systemic IL-1 produced effects on B precursors in the bone marrow in a dose dependent fashion (Table 2). At a low dose rate (20 ng/d) IL-1 had stimulatory effects. The numbers and proliferative activity of pre-B cells were increased at 6d (Table 2). The TdT^+ subset of pro-B cells more than doubled in proliferative activity (not shown). In contrast, high dose rates (400ng/d), though still stimulating proliferation of cells at the large pre-B cell stage, severely depressed all other B lineage cell populations to less than half control population sizes and production rates (Table 2).

Table 2. Number and proliferation of B lineage cells in bone marrow of IL-1 treated C3H/HeJ mice[a]

	20 ng/day				400 ng/day			
	Cells[c]		Mitoses[d]		Cells[c]		Mitoses[d]	
Cell population[b]	Ctl	IL-1	Ctl	IL-1	Ctl	IL-1	Ctl	IL-1
Pro-B cells	6.8	4.1	4.0	1.3	5.8	3.1	3.9	2.2
Pre-B cells	11.8	15.3	3.3	4.1	16.0	11.1	9.5	14.0
B lymphocytes	9.8	9.8	-	-	8.2	3.4	-	-

[a] Each value was derived from a pool of three mice after 6 days of continuous perfusion of IL-1 by a subcutaneous micropump.
[bcd] See Table 1.

Systemic IL-7 (1000 ng/d) produced a well marked stimulation of proliferation of both pro-B cells and pre-B cells, as well as increased numbers of B lineage cells at all stages of differentiation (Table 3). The number and proliferative rate of the TdT^+ subset of pro-B cells increased fourfold relative to controls (not shown).

Table 3. Number and proliferation of B lineage cells in bone marrow of IL-7 treated C3H/HeJ mice[a]

	Cells/femur ($x10^5$)[c]		Mitoses/femur ($x10^4$)[d]	
Cell population[b]	Control	Eμ-myc	Control	Eμ-myc
Pro-B cells	4.9	7.9	18.2	34.0
Pre-B cells	12.3	31.1	17.9	46.0
B lymphocytes	13.0	28.5	-	-

[a] Each value was derived from a pool of three mice after continuous infusion of IL-7 (1000 ng/d) for 4 days.
[bcd] See Table 1.

The findings demonstrate that circulating IL-1 can stimulate B cell precursors in the bone marrow. Both IL-1 and IL-7 administered in vivo can enhance the proliferation of both pro-B cells at the stage of V_H gene rearrangement as well as pre-B cells.

MOLECULAR INTERFACE BETWEEN STROMAL CELLS AND LYMPHO-HEMOPOIETIC PRECURSORS

To examine the molecular interactions between precursor B cells and associated stromal cells in the bone marrow, we have compared the in vivo binding of two new mAbs raised against cultured bone marrow stromal cells by Drs. Paul W. Kincade and Kensuke Miyake, Oklahoma Medical Research Foundation, Oklahoma City. mAb MK/2 recognizes VCAM-1 and can disrupt established B cell-stromal cell cultures (Kincade 1991, Miyake et al 1991). mAb KMI6 recognizes a 110kDa protein which has yet to be more fully characterized. Radiolabeled mAbs were injected intravenously and their binding sites in bone marrow sections were detected by electron microscope radioautography.

Both M/K(VCAM) and KMI6 determinants were revealed on normal bone marrow stromal cells in vivo but there were interesting differences in distribution. VCAM-1 was expressed heavily in a uniform manner along the length of stromal cell processes which were often associated with more than one lineage of hemopoietic precursor cells (Fig. 2a). KMI6 labeling was also heavy over the plasma membrane of certain stromal cells, but was localized only to the part of the membrane which was in contact with cells having an undifferentiated lymphoid morphology. Other areas of the same stromal cell in contact with other distinguishable hemopoietic cells did not express KMI6 (Fig. 2b).

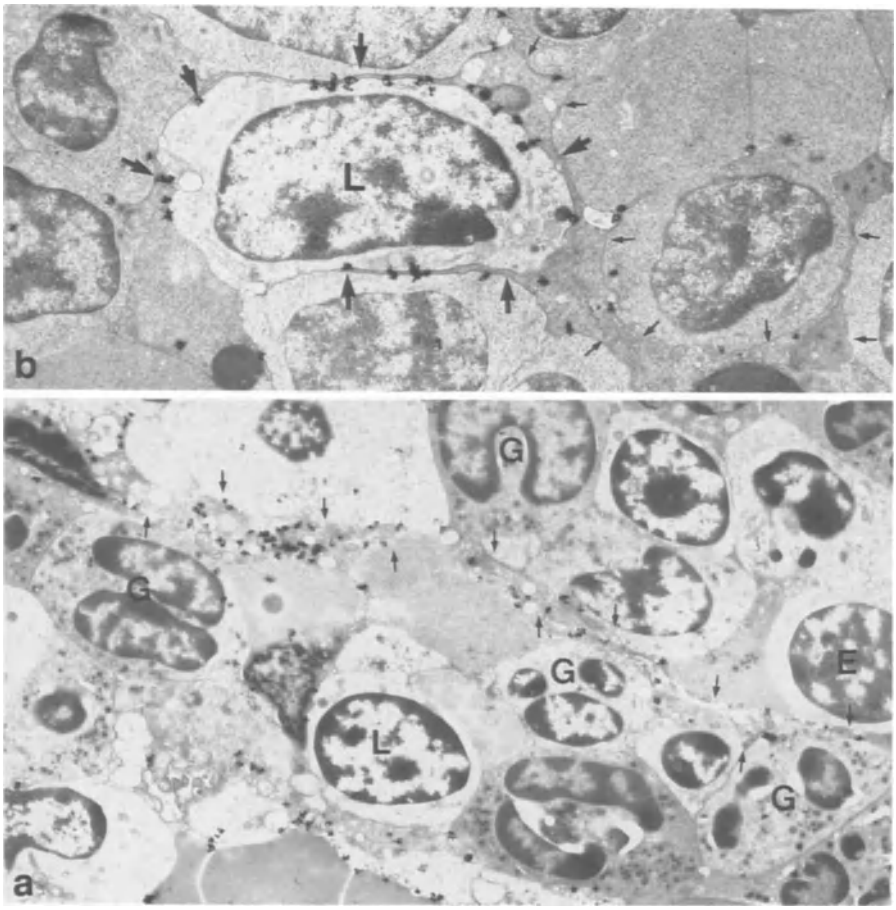


Figure 2.a) A stromal cell process in bone marrow (arrows) showing radioautographic labeling for VCAM-1 is associated with granulocytic (G), erythroid (E) and lymphoid (L) cells (X 4600). b) A large lymphoid cell (L) in bone marrow is almost surrounded by KMI6-labeled processes of a stromal cell (large arrows): processes not adjoining the lymphoid cell show little or no KMI6 label (small arrows) (X 4900).

After depletion of lymphoid and hemopoietic cells by lethal whole body γ-irradiation, the stromal cells continued to express both determinants, but whereas VCAM-1 expression was elevated, KMI6 was diffuse and reduced in intensity (not shown). Single cell suspensions of bone marrow cells, lacking stromal cells, showed little or no labeling for VCAM-1 or KMI6.

Interactions between VCAM-1 and its ligand VLA-4 appear to be widely involved in mediating adhesion between lymphoid and hemopoietic precursor cells and stromal cells in the bone marrow. The interaction may be responsible in part for retaining differentiating cells and exposing them to regulatory signals in the bone marrow until they either reach an appropriate stage of maturation for their release into the blood stream or start the apoptotic pathway when they are released for ingestion by macrophages. KMI6, on the other hand, appears to be the first example of a molecular determinant highly restricted to a particular region of the stromal cell membrane interacting only with lymphoid cells.

DISCUSSION

Oncogenesis in the B cell lineage must meet two main requirements; first, a deregulation of cell cycle control and, second, the evasion of culling mechanisms leading to apoptosis and elimination by macrophages. The present work emphasizes that various levels of control may be involved in the initiation and progression of B cell neoplasia.

Expression of the c-myc oncogene is central to normal B cell genesis and is deregulated in many B cell tumors. Yet, the substantial time lag before Eμ-myc mice develop clonal B lineage malignancies indicates that c-myc deregulation alone is not sufficient for oncogenesis. The present work shows that the constitutive expression of c-myc throughout the differentiation of precursor B cells in Eμ-myc mice produces substantial increases in the levels of production of both pro-B cells and pre-B cells. This is consistent with the known effects of c-myc in cell proliferation by promoting the transition from G_1 to S phase of the mitotic cell cycle (Heikkila et al 1987). To what extent the observed effects in Eμ-myc mice reflect an acceleration of the cell cycle due to a shortening of G_1 or the completion of additional cell cycles during precursor B cell differentiation remains to be determined. The increased precursor B cell proliferation is followed, however, by a considerable elevation of the normally high fraction of cells undergoing apoptosis and macrophage elimination. During the prolonged pretumorous phase of life, the deregulated cells in the bone marrow appear to be negatively selected. The cells become apoptotic at the late pre-B cell stage when the c-myc gene would normally be down regulated and cell divisions would cease. This observation is consistent with findings that c-myc expression induces apoptosis when combined with other conditions that would block progression through the cell cycle (Askew et al 1991; Evan et al 1992). Thus, persisting c-myc expression in differentiating precursor B cells in Eμ-myc mice may induce apoptosis when the cells reach a stage when they are no longer responsive to IL-7 or other factors necessary for cell cycle progression, either because the factors are no longer available or the differentiating cells have become refractory to their effect. The apoptotic effect of c-myc thus has a protective function in preventing the persistence of cells which could otherwise be tumorigenic (Evan et al 1992). Not all defective B lineage cells necessarily undergo apoptosis, yet they may still be rapidly recognized and eliminated by bone marrow macrophages, as in scid mice (Osmond et al 1992). Oncogenesis in

Eμ-myc mice therefore requires further transforming events eventually to produce a B lineage cell which not only has a deregulated cell cycle but is also capable of avoiding the apoptotic pathway and ingestion by macrophages. Somatic mutations are most likely to occur in dividing cells. Precursor B cells are particularly susceptible to genetic errors when dividing during the pro-B cell stage of differentiation, due to mistakes in DNA recombination. Any conditions associated with increased levels of pro-B cell proliferation may thus be particularly effective in increasing the probability of errors. In Eμ-myc mice some such errors, favoured by the c-myc-induced stimulation of pro-B cells and pre-B cells, could confer a survival advantage. In normal mice, some errors, including Ig:c-myc translocation, could perturb the cell cycle while others, including bcl-2 deregulation, could block apoptosis to initiate a potentially neoplastic clone.

Growth factors capable of stimulating proliferation of pro-B cells as well as pre-B cells are now shown to include IL-7 and, under suitable conditions, IL-1. IL-7, a required growth factor normally acting at short range from associated stromal cells, also acts when administered systemically at high dose levels. IL-1 stimulates some pro-B cells and pre-B cells when given in lower dose levels, calculated to augment physiological circulating levels. It is thus a candidate cytokine for mediating, at least in part, the stimulatory effects of macrophage activation on B cell genesis. An initial lag in its stimulatory effect (not shown) suggests that apart from a direct effect on the precursor B cells, possible including an inhibition of apoptosis (McConkey et al 1989), IL-1 may act indirectly by inducing bone marrow stromal cells to produce various growth factors (Kincade 1991). IL-7 may thus constitute a final common pathway both in normal regulation and in a variety of systemic conditions associated with precursor B cell hyper-stimulation and oncogenesis, eg. malaria and pristane-induced granuloma. It may be significant that the latter two conditions also show a characteristic combination of apparently contradictory effects on precursor B cells in the bone marrow, enhanced proliferation of pro-B cells followed by enhanced cell loss (Osmond, Priddle & Rico-Vargas 1990).

The highly restricted expression of KMI6 determinants on bone marrow stromal cells raises the possibility that these cells may be polarized so that their products are vectorially directed to lineage-specific "niches" of their plasma membrane. In this way, small amounts of stromal cell factors could influence lymphoid cell development without affecting adjacent hemopoietic cells. This could explain how individual stromal cells may simultaneously regulate more than one hemopoietic lineage, and how stimulation of one lineage may commonly occur at the expense of another. The stimulated precursor B cells may induce a more extensive area of B lineage-specific polarization of the associated stromal cell membrane with a corresponding reduction of the area of surface membrane available for other cell lineages.

The foregoing findings and speculations concerning their possible implications serve to reinforce the view that the primary genesis and survival of B cells are susceptible to regulatory influences at many different levels and that therefore a number of diverse mechanisms may be relevant to the etiology or prophylaxis of B cell neoplasias in the bone marrow.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada. The following collaborations are gratefully acknowledged. The studies of Eμ-myc transgenic mice, in collaboration with Dr. Charles L. Sidman, were conducted in part at the Jackson Laboratory, Bar Harbor, ME 04609. The work on bone marrow stromal cell determinants was performed using monoclonal antibodies raised and provided by Dr. Paul W. Kincade and Dr. Kensuke Miyake, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104. Recombinant IL-1 and IL-7 was provided by Dr. Anthony E. Namen, Immunex Corporation, Seattle, WN.

REFERENCES

Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD and Brinster RL (1985) The c-myc gene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318:533

Askew DS, Ashmun RA, Simmons BC and Cleveland JL (1991) Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 6:1915-1922

Deenan GJ, van Balen I and Opstelten D (1990) In rat B lymphocyte genesis sixty percent is lost at the transition of non-dividing pre-B cell to $sIgM^+$ B lymphocyte, the stage of Ig light chain gene expression. Eur J Immunol 20:557-564

Dorshkind K (1988) IL-1 inhibits B cell differentiation in long term bone marrow cultures. J Immunol 141:531-538

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119-128

Heikkila R, Schwab G, Wickstrom E, Loke SL, Pluznik DH, Watt R and Neckers LM (1987) A c-myc antisense oligodeoxynucleotide inhibits entry in S phase but not progress from G0 to G1. Nature 328:445-449

Jacobsen K and Osmond DG (1990) Microenvironmental organization and stromal cell associations of B lymphocyte precursor cells in mouse bone marrow. Eur J Immunol 20:2395-2404

Jacobsen K and Osmond DG (1991) Cell deletion in B-cell genesis and the role of bone marrow macrophages. In: Imhof BA, Berrih-Aknin S and Ezine S (eds) Lymphatic tissues and in vivo immune responses Marcel Dekker, New York, pp 343-353

Kincade PW (1991) Molecular interactions between stromal cells and B lymphocyte precursors. Sem Immunol 3:

Kincade PW, Lee G, Pietrangeli CE, Hayashi S-I and Gimble JM (1989) Cells and molecules which regulate B lymphopoiesis in bone marrow. Ann Rev Immunol 7:111-143

Lee G, Namen AE, Gillis S, Ellingsworth LR and Kincade PW (1989) Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-B. J Immunol 142:3875-3883

McConkey DJ, Hartzell P, Chow SC, Orrenius S and Jondal M (1989) Interleukin I inhibits T cell receptor-mediated apoptosis in immature thymocytes. J Biol Chem 265:3009-3011

Miyake K, Weissman IL, Greenberger JS and Kincade PW (1991) Evidence for a role of the integrin VLA-4 in lymphohemopoiesis. J Exp Med 173:599-607

Morrow MA, Lee G, Gillis S, Yancopoulos GD and Alt FW (1992) Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes. Genes Devel 6:61-70

Namen AE, Lupton S, Hjerrild K, Wignall J, Mochizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, Gillis S, Cosman D and Goodwin RG (1988) Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 333:571-573

Opstelten D and Osmond DG (1983) Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic μ chain bearing cells in normal mice. J. Immunol 131:2635-2640

Osmond DG (1986) Population dynamics of bone marrow B lymphocytes. Immunol Rev 93:103-124

Osmond DG (1990) B cell development in the bone marrow. Sem. Immunol 2: 173-180

Osmond DG, Kim N, Manoukian R, Phillips RA, Rico-Vargas SA and Jacobsen K (1992) Dynamics and localization of early B-lymphocyte precursor cells (Pro-B cells) in the bone marrow of scid mice. Blood 79:1695-1703

Park YH and Osmond DG (1987) Phenotype and proliferation of early B lymphocyte precursor cells in mouse bone marrow. J. Exp Med 165:44-458

Park YH and Osmond DG (1989) Dynamics of early B lymphocyte precursor cells in mouse bone marrow: proliferation of cells containing terminal deoxynucleotidyl transferase. Eur J Immunol 19:2139-2144

Park YH and Osmond DG (1991) Regulation of early precursor B cell proliferation in mouse bone marrow: Stimulation by exogenous agents mediated by macrophages in the spleen. Cell Immunol 135:168-183

Sidman CL, Marshall JD and Harris AW (1988) Genetic studies on Eμ-myc transgenic mice. Curr Top Microbiol Immunol 141:94

Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi S-I, Ogawa M, Sakai, Nishikawa S and Nishikawa S-I (1989) Interleukin 7 production and function in stromal cell-dependent B cell development. J Exp Med 170:333-338

Wyllie AH, Morris RG, Smith AL and Dunlop D (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Path 142:67-77

The Purification of B-Cell Precursors From Mouse Fetal Liver

T. MORROW[1] and M. SCHLISSEL[1,2]

[1]Department of Medicine and
[2]Department of Molecular Biology and Genetics
John Hopkins University School of Medicine
Baltimore, Maryland 21205

Lymphocyte progenitor cells isolated on sequential days of gestation from mouse fetal liver represent distinct stages in B cell development. We have utilized polymerase chain reaction (PCR)-based assays to detect immunoglobulin (Ig) gene rearrangement and flow cytometry to assay cell surface markers following fractionation based on the differential expression of the B cell-specific phosphatase CD45 (B220). The purification of $B220^+$ cells from day 17 fetal liver resulted in a 10-fold enrichment of cells which had undergone gene rearrangement events. We have also shown that day 13 fetal liver cells activate successive Ig gene rearrangements during short-term culture in the presence of fetal calf serum (FCS), interleukin-3 (IL3), and interleukin-7 (IL7). However, partially purified lymphocyte precursors fail to activate Ig gene rearrangement in culture unless they are cultured in the presence of a stromal cell line.

Introduction

During gestation, murine B cell development occurs in the yolk sac and fetal liver. B cell progenitors are first detected in fetal liver on day 12 of gestation and are thought to undergo development as a synchronous wave progressing through characteristic stages on specific days [1]. The assembly of genes encoding the Ig molecule involves the highly regulated

joining of variable (V_H), diversity (D_H), and joining (J_H) gene-segments to generate an Ig heavy-chain gene and variable (V_L) and joining (J_L) gene segments to generate an Ig light-chain gene. The temporal order of these events has been characterized using Abelson-Murine Leukemia Virus (A-MuLV) transformed cell lines, with heavy-chain gene D_H-to-J_H rearrangement preceding V_H-to-DJ_H rearrangement. Light-chain gene V_L-to-J_L rearrangement most often occurs next, giving a cell the potential to produce surface Igs [2].

The regulation of this complex series of events remains unclear. Ig heavy-chain (μ) protein is thought to be a critical regulator of this process. In addition, it has been proposed that transcription of individual unrearranged Ig loci (germline transcription) targets each in turn for rearrangement by making them accessible to the recombinase machinery [3]. In support of this notion, LPS treatment, which activates germline κ light-chain gene transcription, also activates Vκ-to-Jκ gene rearrangement [4]. Additionally, overexpression of the heavy-chain enhancer binding transcription factor E47 in a pre-T cell line activates germline heavy-chain gene transcription and D-to-J_H gene rearrangement [5]. These results provide evidence that transcription and rearrangement are closely related.

Another poorly understood phenomenon is allelic exclusion. A single B cell expresses only one functional heavy chain and one functional light chain despite having two heavy-chain and six light-chain loci [6]. One hypothesis is that expression of heavy-chain protein on the cell surface halts further heavy-chain gene rearrangement. Mice transgenic for a rearranged heavy-chain (μ) gene exhibit marked decreases in their endogenous heavy-chain gene rearrangement, suggesting that the presence of one productively rearranged heavy-chain gene inhibits rearrangement of the other allele (reviewed in [7]).

In order to begin to characterize the molecular mechanisms which regulate the progression of early B cell precursors through development, we have devised methods to purify B cell progenitors from the fetal liver. We have fractionated cells based on their expression of the B cell specific phosphatase CD45 (B220) and have determined that this method enriches for cells which have undergone gene rearrangement. Additionally, we have developed a short-term

culture system which recapitulates the in vivo pattern of Ig gene rearrangement.

Materials and Methods

Cell Preparation and Cell Culture

Balb/c mice were purchased from NCI (Bethesda, MD) and mated. The presence of a vaginal plug was taken as day 0 of gestation, and pregnant females were sacrificed on specific days thereafter. For FACS and PCR analyses, fetal livers were homogenized in 1x PBS with 10mM Hepes pH 7.2, passed through nylon mesh to remove aggregates, and spun through ficoll-paque (Pharmacia) to purify lymphocytes. Cells were then washed 3 times in 1x PBS. For panning, 1 x 10^7 cells were plated on petri dishes (Primaria) which were coated with 10μg antibody per plate. After 1 hr at 4° C, nonadherent and adherent cells were collected. The preparation of cells for culture involved disrupting livers in IMDM supplemented with 5% FCS, antibiotics, and 50μM mercaptoethanol (Sigma), and plating in 24 or 6 well plates.

Reagents and FACS Analysis

Unconjugated mab to B220 (clone RA3-6B2, Pharmingen) was used for panning, and biotinylated anti-B220 followed by AVPE (Caltag) was used for FACS analysis. Biotinylated CD4 (clone MT4) was a gift from Dr. Drew Pardoll. Single and two color FACS was conducted on a Becton Dickinson FACS flow cytometer. Nonviable cells were excluded from analysis by forward and side angle light scatter. The stromal cell line S17 was kindly provided by Dr. Ken Dorshkind.

Preparation of DNA for PCR

DNA was prepared for PCR by resuspending 1 x 10^6 trypan blue-excluding cells in 200μl PCR lysis buffer as previously described [8]. This cell lysate was used directly for DNA PCR.

DNA PCR

DNA PCR was performed as described [8] with one modification - 30 cycles consisting of 94°C for 1 min and 66°C

for 2.5 min were used. 2µl aliquots of cell lysate (10,000 cell-equivalents) was used in each assay. Quantitative standards were generated by dilution into PCR lysis buffer and run with each series of PCR assays. PCR products were separated by electrophoresis on 1.2% agarose gels, blotted to Zetabind (Cuno), and analysed by Southern blot hybridization as previously described.

Results

DNA PCR to Detect Gene Rearrangement

We have utilized sensitive PCR techniques to detect cells which have undergone Ig gene rearrangement following fractionation of fetal liver based on expression of B220. The assays all use one unique primer specific for the J-gene region

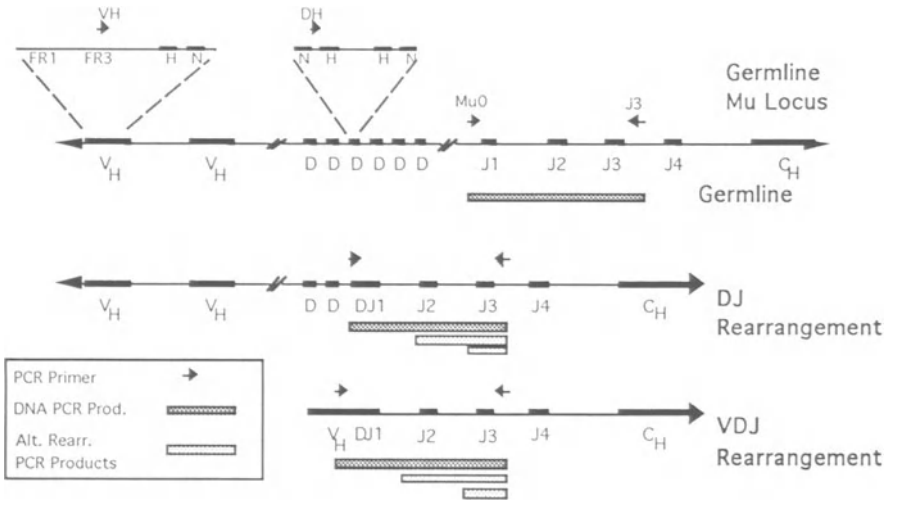


Fig. 1. Ig heavy-chain locus gene rearrangement PCR assays. The arrows denote the locations of the various primers used in assays to detect gene rearrangement. In each case, three PCR products can be generated depending on which of the three J_H genes is involved in the gene rearrangement event. The 5' V_H primer is a degenerate oligonucleotide mixture homologous to conserved sequences in the FR3 regions of the variable genes present in most of the V_H gene families and the 5' D_H primer is a degenerate oligonucleotide mixture homologous to a conserved portion of the recombination signal sequence 5' to 10 of the 11 known D_H genes (excluding DQ52). H and N indicate the locations of the heptamer and nonamer signal sequences

(either J_H or $J\kappa$) and one degenerate primer population which recognizes most V_H, $V\kappa$, or D_H gene segments (Fig.1). When the Ig locus is in germline configuration, the primers are spaced too far apart to amplify DNA. Thus, amplified products result only when a gene rearrangement event has occurred to bring the primers into close proximity. Figure 1 is a diagram showing the location of the primers and the amplified products formed after D_H-to-J_H and V_H-to-DJ_H rearrangement on the Ig heavy-chain locus. The different sized PCR products are detected and quantified by Southern blot hybridization with a heavy-chain J-region probe. By limiting the concentration of input DNA and the number of PCR cycles, and by comparison to serially diluted standard template reactions, measuring the intensity of the PCR bands yields quantitative information.

Since Ig gene rearrangement is restricted to lymphoid lineage cells in the fetal liver, we used this assay system to test cells harvested from fetal livers on different days of gestation and fractionated by a variety of means for the presence of progenitor B cells.

Purification of B Cell Progenitors by B220 Panning

We fractionated cells based on cell surface B220 which is expressed during B cell development [9]. After ficoll purification, lymphoid cells from day 17 fetal liver were plated on anti-B220 coated dishes. The nonadherent population was collected, and, following extensive washing to remove any cells adhering nonspecifically, the $B220^+$ population was removed. Figure 2 shows the analysis of cell populations before and after fractionation. FACS analysis demonstrates that a small fraction of ficoll-enriched day 17 fetal liver cells express B220 (<5%) and that following panning, the adherent population contains 30% $B220^+$ cells. In addition, DNA PCR shows that this procedure enriches for cells which have undergone gene rearrangement. The first five lanes show the DJ_H, VDJ_H, and $VJ\kappa$ rearrangement signals from crude and fractionated day 17 fetal liver. Control PCRs were done using primers to amplify a DNA fragment from a non-rearranging locus to show that the same amount of input DNA was used in each assay (data not shown). The last five lanes show the rearrangement signals from the indicated number of splenocytes.

Ficoll purification gave an approximately 2-fold enrichment of DJ_H-, VDJ_H-, and $VJ\kappa$- containing cells. The ficoll

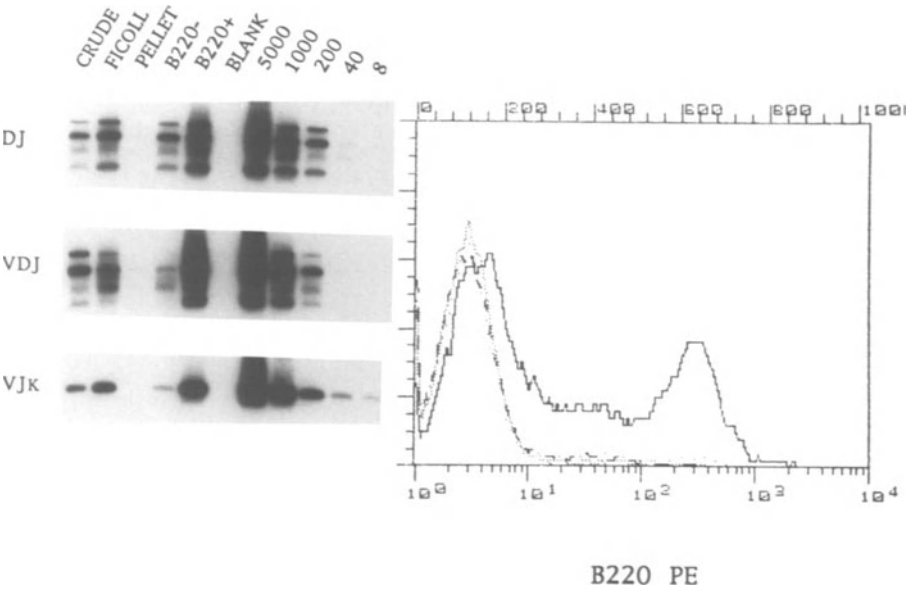


Fig 2. Purification of B cell progenitors from day 17 fetal liver. The left panel shows the PCR analysis of gene rearrangement in crude, ficoll-purified (interface and pellet), and B220- and B220+ populations following B220 panning from day 17 fetal liver. The indicated number of control DJ_H, VDJ_H, and VJκ alleles were also subjected to PCR analysis. An autoradiogram of the Southern blot is shown. The right panel shows FACS analysis of day 17 fetal liver before and after B220 panning. The solid line represents B220+ adherent cells, the dotted line the unfractionated population, the closely spaced dotted line the B220- population, and the dashed line unfractionated cells stained with an isotype matched control antibody. These fractions were collected and used for the PCR analyses shown on the left

pellet contained few cells which had undergone gene rearrangement. Enriching for $B220^+$ populations by panning resulted in a 10-fold purification of B cell precursors as compared to the crude cell lysate. The $B220^-$ population also contained cells which had undergone gene rearrangement. This might represent cells with low levels of B220 which don't adhere well to antibody-coated plates or cells which have undergone gene rearrangement which don't yet express surface B220.

We next examined the pattern of gene rearrangement from $B220^+$ fetal liver cells sorted by FACS from developmental days 15 and 18. These populations contained greater than 90% $B220^+$ cells by FACS analysis (data not shown). The rearrangement signals from the $B220^+$ populations isolated on both days showed that more than 50% of the cells had

undergone gene rearrangement, although we are unable to estimate the purity of these populations as our undiluted standard contained only 50% rearranged alleles.

Dispersed Fetal Liver Lymphocytes Activate Gene Rearrangement During Culture

We placed unfractionated day 13 fetal liver cells into culture for 4 days and assayed the effects of IL3 and IL7 on gene rearrangement. The starting day 13 fetal liver preparation contained few cells which had undergone gene rearrangement as detected by DNA PCR (Fig. 3). However, the cultured B cell progenitors activated gene rearrangement during 4 days of culture in the presence of IL7 alone or with both IL7 and IL3. This is similar to the pattern of gene rearrangement in vivo as represented by the rearrangement signals detected in crude and ficoll-purified day 17 fetal liver.

Interestingly, ficoll-purified fetal liver cells failed to activate gene rearrangement when cultured in the presence of both IL7 and IL3 (Fig. 3, culture - stroma). However, culturing these cells in the presence of a cloned stromal cell line derived from fetal liver reconstituted their ability to undergo gene rearrangement.

Discussion

The resolution of B-lineage cells into definitive populations representing specific stages of development is required in order to elucidate the mechanisms involved in regulating Ig gene rearrangement. We have used DNA PCR assays to track B cell progenitors from the fetal liver during cell fractionation. We have found that sorting cells from different days of development on the basis of B220 expression enriches for cells which have undergone gene rearrangement, and therefore provides a method for purifying developing B cells.

B220 is one of the first B cell lineage surface antigens to be expressed during development [9]. A recent study showed that $B220^{+}$ IgM^{-} cells isolated from the bone marrow could be sorted into 4 fractions based upon 3 differentially expressed

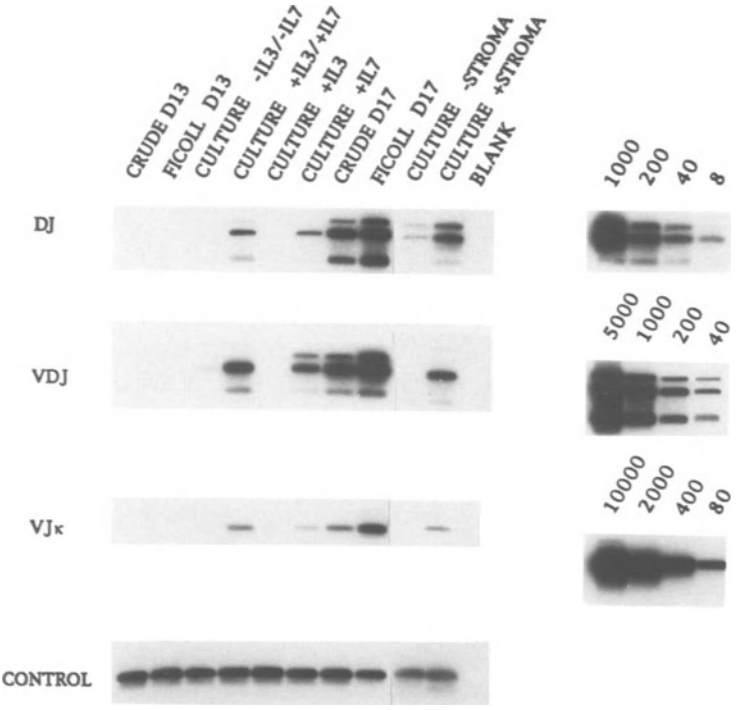


Fig. 3. In vitro culture of day 13 fetal liver supports gene rearrangement. Lanes labeled crude and ficoll show the patterns of Ig gene rearrangement seen in unfractionated and ficoll-purified cells obtained from day 13 or 17 fetal liver. Cells used to generate the signals in lanes labeled culture were from disrupted day 13 fetal livers assayed after 4 days in culture in the presence of the indicated interleukins. The lanes labeled culture + or - stroma reflect the patterns of Ig gene rearrangement seen in ficoll-purified cells from day 13 fetal livers assayed after 4 days of culture in the presence of both IL3 and IL7 with or without the stromal cell line S17. The control represents the signal from each sample following PCR using primers specific for a non-rearranging locus. The panels in the right half of the figure show quantitative control PCR assays

cell surface antigens (S7, BP-1, and 30F1 or heat stable antigen) and their ability to undergo gene rearrangement in a stromal cell culture system [10]. Changes in surface expression of these antigens during early B cell differentiation correlated with Ig gene rearrangement patterns characteristic of different stages of development. The most immature of these fractions expressed B220 and had the Ig loci in germline configuration.

These data were obtained from bone marrow and differ from another study which found that the earliest B cell

precursors in fetal liver contained unrearranged Ig loci but did not express B220 [11]. This population expressed an as yet uncharacterized antigen designated AA4.1 which is detected on both lymphoid and myeloid lineage cells. This discrepancy may stem from differences in B cell development during different stages in ontogeny or from differences in detection systems.

The roles which each of these surface antigens play during development is unclear. However, since they are expressed during or before gene rearrangement is activated in developing B cells, it is important to understand how they function in lymphoid development. We have developed culture conditions using dispersed fetal liver which support patterns of Ig gene rearrangement similar to that seen in vivo. Optimal conditions for culturing B cell precursors include the presence of FCS, IL3, and IL7. Additionally, early fetal liver cells were found to be dependent upon the presence of stromal cells for growth and differentiation as few viable cells were recovered from cultures initiated with ficoll-purified cells alone. Mixing of this population with the stromal cell line S17 allowed for activation of gene rearrangement.

Other investigators have reported that early B cell progenitors from fetal liver are absolutely dependent on contact with stromal layers for their development into mature surface Ig positive B cells [10, 12]. B cell progenitors which had already undergone gene rearrangement showed modest or no dependence on stromal cell contact.

We hope to test the involvement of various cells surface molecules and growth factors reported to function in B cell development by culturing early B cell precursors in the presence of blocking or crosslinking antibodies and assaying for the effect of these perturbations on the activation of gene rearrangement. We will also use antisense oligonucleotides to disrupt the expression of genes thought to be involved in regulating development in the B cell lineage.

References

1. A. Strasser, A. Rolink, F. Melchers (1989) One synchronous wave of B cell development in mouse fetal liver changes at day 16 of gestation from dependence to independence of a stromal cell environment. J. Exp. Med.: 170, 1973-1986.
2. F. Alt, et al. (1984) Ordered rearragnement of immunoglobulin heavy chain variable region segments. EMBO: 3, 1209-1919.
3. F. Alt, T. Blackwell, G. Yancopoulos (1987) Development of the primary antibody repertoire. Science: 238, 1079-1087.
4. M. Schlissel, D. Baltimore (1989) Activation of immunoglobulin kappa gene rearrangement with induction of germline kappa gene transcription. Cell: 58, 1001-1007.
5. M. S. Schlissel, A. Voronova, D. Baltimore (1991) Helix-loop-helix transcription factor activates immunoglobulin D-to-J rearrangement in a pre-T cell line. Genes & Devel.: in the press,
6. B. G. Pernis, G. Chiappino, A. S. Kelus, P. G. H. Gell (1965) Cellular localization of immunoglobulins with different allotype specificities in rabbit lymphoid tissue. J. Exp. Med.: 122, 853-875.
7. U. Storb (1989) Immunoglobulin gene analysis in transgenic mice. Immunoglobulin Genes (Academic Press) pp 304-326.
8. M. S. Schlissel, L. M. Corcoran, D. Baltimore (1991) Virally-transformed pre-B cells show ordered activation but not inactivation of immunoglobin gene rearrangement and transcription. J. Exp. Med.: 173, 711-720.
9. P. W. Kincade (1981) Formation of B lymphocytes in fetal and adult life. Adv. Immun.: 31, 177-245.
10. R. R. Hardy, C. E. Carmack, S. A. Shinton, J. D. Kemp, K. Hayakawa (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J. Exp. Med.: 173, 1213-1225.
11. A. Cumano, C. J. Paige (1992) Enrichment and characterization of uncommitted B-cell precursors from fetal liver at day 12 of gestation. EMBO J.: 11, 593-601.
12. P. C. Kierny, K. Dorshkind (1987) B lymphocyte precursors and myeloid progenitors survive in diffusion chamber culture but B cell differentiation requires close association with stromal cells. Blood: 70, 1418-1424.

BCR/ABL Enhances Growth of Multipotent Progenitor Cells But Does Not Block Their Differentiation Potential In Vitro

M. L. GISHIZKY and O. N. WITTE*

Department of Microbiology and Molecular Genetics,
*Howard Hughes Medical Institute, University of California, Los Angeles, 90024

We have developed an in vitro system for investigating the direct effect of the BCR/ABL oncogene on the growth and differentiation of single multipotent progenitor cells (MPPC). Using this system we demonstrate that MPPC expressing P210 BCR/ABL have an enhanced ability to grow in the presence of very low concentrations of IL-3 or steel locus factor (SLF). MPPC's expressing P210 BCR/ABL do not exhibit a block in differentiation and can give rise to mast cell, macrophage, granulocyte and B-lymphoid cell lines. Differentiation of the B-lymphoid cell lines can progress to the pre B-cell stage, marked by expression of CD45R (B220) and rearrangements of both heavy chain immunoglobulin gene alleles. Transformation of MPPC's, initiated by BCR/ABL progresses through defined stages that can be monitored by obvious changes in growth characteristics. These studies demonstrate that BCR/ABL may enhance the growth of MPPC's without abrogating their differentiation potential. This system should prove useful in evaluating the effect of specific genetic events on leukemogenesis and identifying the molecular signals which regulate normal hematopoietic development.

A major goal in the study of hematopoietic development is to identify the molecular signals which direct pluripotent and multipotent progenitor cells to proliferate vs. differentiate. One approach to elucidate these signals has been to study the molecular mechanisms involved in leukemogenesis. The rational being that by understanding the events involved in leukemogenesis we will gain insight into the signals directing normal hematopoietic development. A dominant theme emerging from this research is that leukemias result from the progressive accumulation of genetic lesions within a cell. These genetic alterations can occur at any stage during hematopoietic development as pluripotent stem cells differentiate into lineage committed cells. A major contribution to this field would be establishment of a model system that could follow the effect of a single

genetic lesion on the growth and development of a single target cell. Criteria for such a system must include: 1) ability to evaluate subtle alterations in cell growth and phenotype, 2) ability to control the external environment and 3) identification of discrete stages in the progression of the transformed phenotype. Using such a system we present a model which describes the progressive transformation of multipotent hematopoietic stem cells (MPPC) initiated by the BCR/ABL oncogene (**Figure 4**).

One of the most extensively studied leukemias associated with a specific genetic lesion is human chronic myelogenous leukemia (CML; **reviewed in Kurzrock 1989**). The cellular origin of CML has been traced to a clonal expansion of a multi-potent or pluripotent hematopoietic progenitor cell. The hallmark of this disease is the presence of the Philadelphia chromosome (Ph^1). A molecular consequence of this translocation is the formation of the chimeric BCR/ABL oncogene. The product of the BCR/ABL gene (P210) is an activated form of the c-ABL protein tyrosine kinase. The presence of the P210 BCR/ABL protein in cells during both chronic phase and acute blast crisis suggests that BCR/ABL expression plays an important role in the etiology of CML. BCR/ABL like v-ABL can transform pre B-cells in vitro. The BCR/ABL oncogene can also induce a myeloproliferative disease in mice, similar to that observed in patients with CML (**Daley 1990, Kelliher 1990, Gishizky manuscript in preparation**). This demonstrates that P210 BCR/ABL is a critical factor in the pathogenesis of the CML. However, the question remains whether the BCR/ABL oncogene initiates the disease, by deregulating the growth of MPPC's, or functions as a necessary cofactor which deregulates the growth of committed progenitor cells.

To investigate the direct effect of BCR/ABL expression on growth and development of MPPC's, we developed a single step in vitro assay that selects for cells which express the oncogene based on their ability to grow in the presence of low concentrations of cytokines. Prior work demonstrated that BCR/ABL expression stimulated the growth of lineage committed B-lymphoid progenitor cells in vitro without blocking their ability to differentiate (**Scherle 1990**). Other studies showed that expression of BCR/ABL in cytokine dependent cell lines can induce factor independent growth (**Harikarian 1988, Young 1991**). These observations suggested that BCR/ABL may stimulate growth by altering the cells sensitivity to specific factors. We reasoned that incubating BCR/ABL containing progenitor cells in the presence of cytokines, at concentrations below the threshold amounts required to stimulate the growth of normal bone marrow cells, could positively select for the effect of the oncogene.

The experimental protocol used in these studies is schematically depicted in figure 1. The details of this procedure have been previously reported (**Gishizky 1992**). In brief, murine bone marrow enriched for progenitor cells, by pretreatment with 5-fluorouracil (5-FU), was infected with helper free retroviruses expressing either the BCR/ABL oncogene or the TK-neo gene

(mock). Following infection cells were seeded in a soft agar colony forming assay in the absence or presence of decreasing concentrations of cytokines (murine IL-3, rat steel locus factor [SLF], murine GM-CSF and human G-CSF). The cultures were analyzed at 8-10 days post seeding for colony number, cellular morphology of individual colonies as determined by Wright-Giemsa stain and presence of the BCR/ABL gene as determined by BCR/ABL specific polymerase chain reaction (PCR) gene amplification.

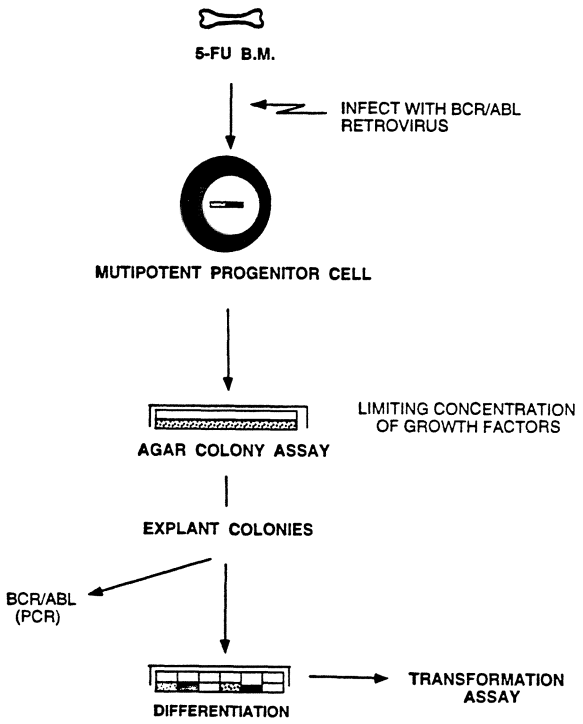


FIGURE 1: Experimental Protocol

In analyzing these cultures we observed that at low concentrations of IL-3 (10-100 pg/ml) there was a striking difference in the number of colonies growing in wells seeded with BCR/ABL infected cells vs. mock (**Fig 2**). The concentration of cytokine at which the greatest difference in colony growth between BCR/ABL vs. mock infected cells was observed varied between individual experiments. However, we consistently observed a 2.5 - 10 fold greater number of agar colonies in wells containing BCR/ABL infected bone marrow when compared to mock infected controls at the same concentration of cytokine within each experiment. Using PCR and oligonucleotides specific for BCR/ABL we determined up to 90% of the small colonies (range 65 - 90%) contained the BCR/ABL oncogene (**Gishizky 1992**). Enhanced growth of BCR/ABL infected bone marrow cells was also observed at low concentrations of SLF (0.3-30

ng/ml). However, in the presence of GM-CSF and G-CSF we observed no difference in colony growth between BCR/ABL or mock infected 5-FU bone marrow. This indicates that the ability of BCR/ABL to enhance growth is not a general growth stimulatory phenomenon, but rather, may work through specific growth factor pathways.

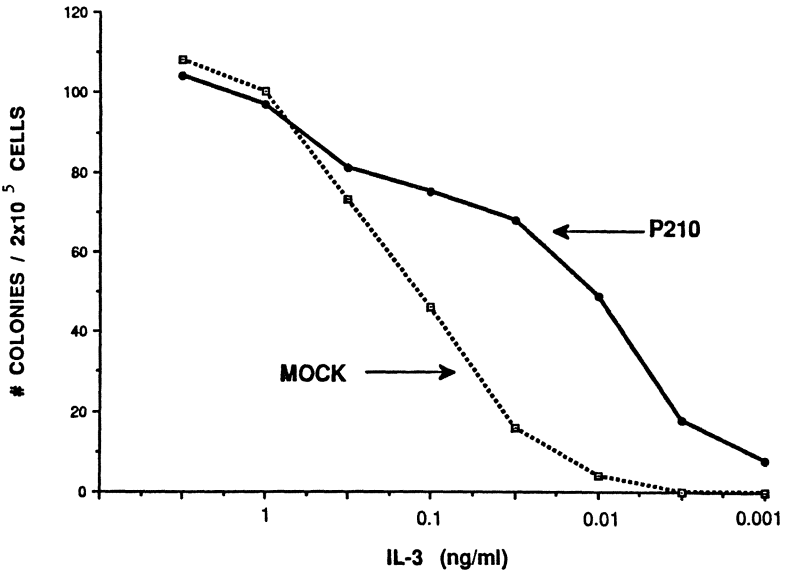


FIGURE 2: BCR/ABL stimulates hematopoietic cell growth in an IL-3 supplemented soft agar colony assay. Following infection cells were washed and resuspended at 1 x 10^5 cells/ml. 2 ml of cell suspension was seeded per well in soft agar supplemented with IL-3. The threshold concentration of growth factor necessary to stimulate agar colony formation varied between individual experiments. In each experiment the optimal concentration of growth factor necessary to preferentially support the growth of BCR/ABL expressing cells was determined by supplementing cultures with serial dilutions of either murine IL-3 (1-500 pg/ml) or rat SLF (0.3-100 ng/ml). Triplicate cultures were established at each concentration of growth factor and colony number determined between days 8-10 post seeding. Each experiment also included cultures which contained no exogenously added cytokines. The reported number of colonies for each experiment represents the mean number of colonies counted in three wells

As previously reported, the agar colonies containing the BCR/ABL gene could be segregated into several classes based on size and cellular morphology (**Gishizky 1992**). The most interesting colony type was a small (<0.4mm), rare (represented approximately 2% of all colonies) colony that contained cells from different hematopoietic lineages. The presence of lymphoid, mast cell, macrophage and myeloid cells with segmented nuclei within the same colony suggested that these "mixed phenotype" colonies originated from or may still contain MPPC's.

To demonstrate that cells in the mixed phenotype colonies contained MPPC's it was necessary to derive clonal cultures of different hematopoietic lineages and show that they all originated from the same BCR/ABL retroviral infected progenitor cell. Cells from individual colonies were subcultured in liquid media and also in methyl-cellulose, in the presence of specific growth factors including IL-3, IL-4, IL-6, IL-7, erythropoietin, SLF, GM-CSF and G-CSF.

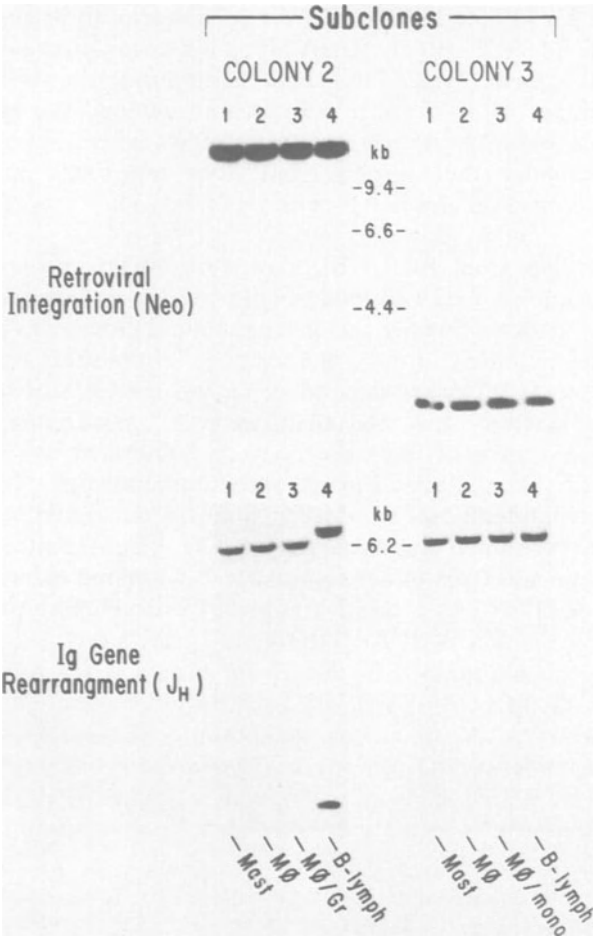


Figure 3: Molecular characterization of differentiated subcultures originating from single BCR/ABL expressing multi-lineage progenitor cells in vitro. 12 ug of DNA from each culture was digested and analyzed as described in the legend for figure 1B. Phenotypic designations of each culture were assigned based on the expression of lineage specific antigens analyzed by flow cytometry, mast - mast cell, M0 - macrophage, Gr - granulocyte and B-lymph - B-lymphocyte. At the time of analysis the B-lymphoid cells derived from agar colony 3 expressed the B-lymphoid specific marker B-220 and most cells retained the Ig gene in the germline configuration.

In three independent experiments we derived clonal outgrowths of B-lymphocytes and cells of different myeloid lineages (mast cells, macrophages, granulocytes) from the same mixed phenotype colony. Cellular phenotype was confirmed by flow cytometry analysis using lineage specific markers (Mac-1, Gr-1, B220, **Gishizky 1992**). To determine the origin of individual cultures genomic DNA was prepared and analyzed for the presence of a unique BCR/ABL retroviral integration site (**Figure 3**). Identical proviral integration sites were present in all cell lines derived from the same agar colony. Two examples are shown in figure 3. This confirmed that the subcultures all originated from the same BCR/ABL infected MPPC. DNA blot analysis to detect immunoglobulin (Ig) gene rearrangement revealed that most B-lymphoid cultures had rearranged both Ig gene alleles while the myeloid cultures retained the Ig genes in the germline configuration. These data demonstrate that expression of BCR/ABL in MPPC's can enhance their growth, but does not block their ability to differentiate into myeloid and pre B-cells.

Cultures established from BCR/ABL expressing MPPC progressed through stages as they acquired a transformed phenotype (**Fig 4**). Two criteria used to assess cellular transformation are factor independent growth in vitro and ability to cause tumors in mice. Initially the cultures established from BCR/ABL containing murine MPPC were dependent on the presence of specific growth factors and grew at a very slow rate (**Gishizky 1992**). With extended passage in vitro (> 6 weeks), most of the cultures were dominated by growth factor-independent cells that exhibited lineage specific phenotypes. To test whether these factor-independent cell lines were tumorigenic, groups of sublethally irradiated severe combined immunodeficient (CB.17 SCID) mice were injected intravenously with cells from either myeloid or B-lymphoid cultures established from the same MPPC. As a positive control for developing pathology other mice were injected with a BCR/ABL expressing growth factor-independent pre B-cell line that was maintained in culture for over a year. Surprisingly, only mice injected with the control cell line have developed tumors. None of the other mice show any pathology at 5 months post inoculation. This demonstrates that factor independence and tumorigenicity represent different stages in the development of BCR/ABL induced pathogenicity (**Figure 4**) and that additional genetic alterations are necessary for the progression toward a malignant phenotype.

The experimental strategy described in these studies can evaluate the kinetic progression of leukemia from a single multi-potent progenitor cell. This paradigm can also identify discrete stages in the transformation pathway leading toward malignancy. Using this system it should be possible to follow the molecular changes that are initiated by a single genetic event (such as expression of BCR/ABL) in the appropriate target cell (MPPC) which lead to the development of CML and other leukemias. This experimental paradigm and the BCR/ABL oncogene may also be useful in establishing culture systems which promote expansion of MPPC's without inducing their differentiation.

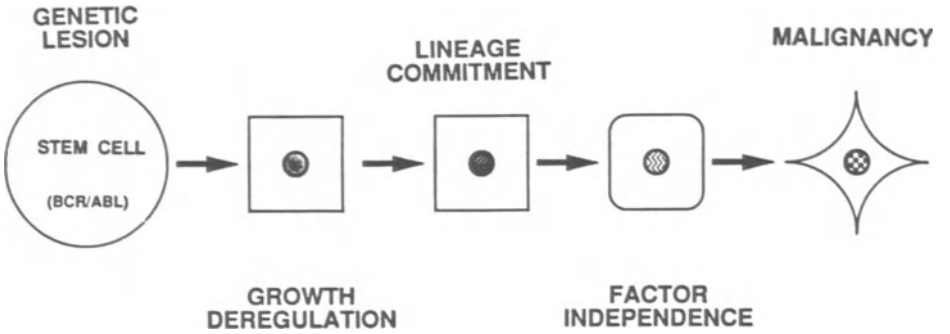


Figure 4: Schematic representation of discrete stages in the progression of BCR/ABL induced multi-potent hematopoietic progenitor cell transformation.

A characteristic of stem cells is their ability to remain quiescent for long periods of time and when appropriately stimulated, to divide and repopulate cells of the different hematopoietic lineages. Regulating the transition between the quiescent state and cellular division requires an intricate balance between external influences, such as growth factors, cell contact and autonomous cellular mechanisms. As our data demonstrate, the effect of BCR/ABL is very subtle and could serve to either heighten the cellular response to positive stimuli or attenuate the response to negative effectors of growth. Previous work from this laboratory exploited the growth stimulatory effect of the BCR/ABL oncogene in establishing a B-lymphoid progenitor culture system (**Scherle 1990**). Our data demonstrating that BCR/ABL can enhance growth of MPPC without abrogating their differentiation potential suggests that BCR/ABL may be a useful reagent to establish analogous systems for growing MPPC's in vitro.

REFERENCES

Daley, G.A., R.A. Van Etten, D. Baltimore, Science **247**:824-830 (1990)

Gishizky, M.L. and O. N. Witte, Science **256**:836 (1992)

Hariharan, I.K. and J.M. Adams, Oncogene Res. **3**:387 (1988)

Kelliher, M.A., J. McLaughlin, O.N. Witte, N. Rosenberg, Proc. Natl. Acad. Sci. U.S.A. **87**:6649-6653 (1990)

Kurzrock, R., J.U. Gutterman, and M. Talpaz, New Engl. J. Med. **319**:990, (1989).

Scherle, P.A., K. Dorshkind, O.N. Witte, Proc. Natl. Acad. Sci. U.S.A. **87**:1908-1912, (1990).

Young, J.C., M.L. Gishizky and O.N. Witte, Mol. Cell. Biol. **11**:854 (1991).

ACKNOWLEDGMENTS

We are grateful to D. Saffran, and C. Sawyers for critical review of the manuscript; and to N. Parada for photography. Murine GM-CSF, rat SLF, human G-CSF, human IL-6 and human erythropoietin were kindly provided by Ian McNeise and Kris Zsebo, Amgen Corp. Murine IL-2, murine IL-3 were purchased from Biosource International, Westlake Village, CA. M.L.G. is a recipient of a Leukemia Society of America Fellowship. O.N.W. is an Investigator of the Howard Hughes Medical Institute. This work was supported by grants to O.N.W. from the National Institutes of Health.

Repopulation of SCID Mice with Fetal-Derived B-Lineage Cells

R. R. HARDY, S. A. SHINTON and K. HAYAKAWA

Institute for Cancer Research, Fox Chase Cancer Center
7701 Burholme Ave., Philadelphia, PA 19111

Introduction

B lineage cells in the bone marrow can be recognized by the expression of the high molecular weight form of the common leukocyte antigen CD45, known as B220. We showed recently [1, 2] that a small portion of these cells in bone marrow co-express leukosialin, CD43 [3, 4], and that many of these cells possessed D-J, but not V-D-J rearrangements. Further analysis with other markers revealed that this D-J rearranging population was contained in the fraction of $B220^{+}CD43^{+}$ cells that also expressed intermediate levels of the heat stable antigen, HSA. We showed that in short term culture, many of these cells would progress to the Pre-B and B cell stages. We have termed cells with this phenotype "Pro-B". These Pro B cells can be detected both in bone marrow and fetal, known sources of B lymphopoiesis (Figure 1).

More recently [5] we compared the capacity of these cells to differentiate *in vivo* by transferring limited numbers into immunodeficient SCID mice [6] that lack their own lymphoid populations. We used these animals as recipients because good engraftment can be achieved with low dose irradiation (350R), which allows survival of animals receiving only lymphoid-restricted precursors. We found that B cells could be readily detected in the spleen and peritoneal cavities of such mice receiving as few as 10^4 such cells when analyzed 3 weeks following transfer. More typically we used 10^5 cells per mouse. At this dose B cells constituted approximately 5% of spleen and T cells were infrequent or absent. Thus transfer of this Pro B population provided repopulation limited to the B lineage. Furthermore, such repopulation proceeded in a "wave" of differentiation, since Pre-B cells were not detected in the bone marrow of these recipients three weeks after transfer.

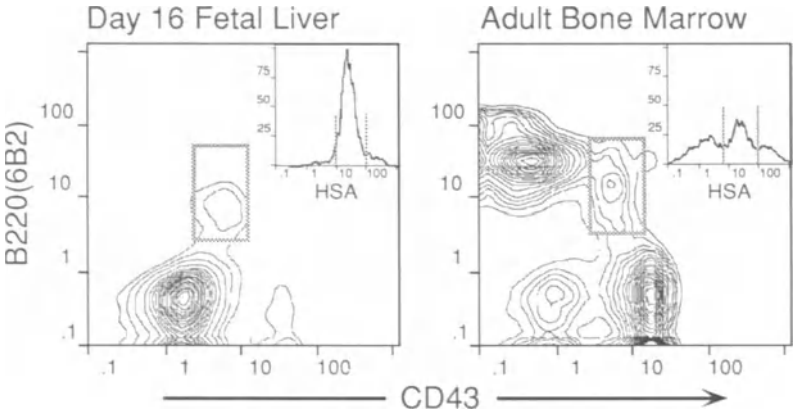


Fig. 1. Analysis of bone marrow and fetal liver for expression of B220, CD43 and HSA reveals a fraction of cells expressing all three determinants in these tissues. Note that while there are many $B220^{+}CD43^{-}$ cells in bone marrow, such cells are lacking from fetal liver. Gates used in sorting are indicated by dotted boxes and lines

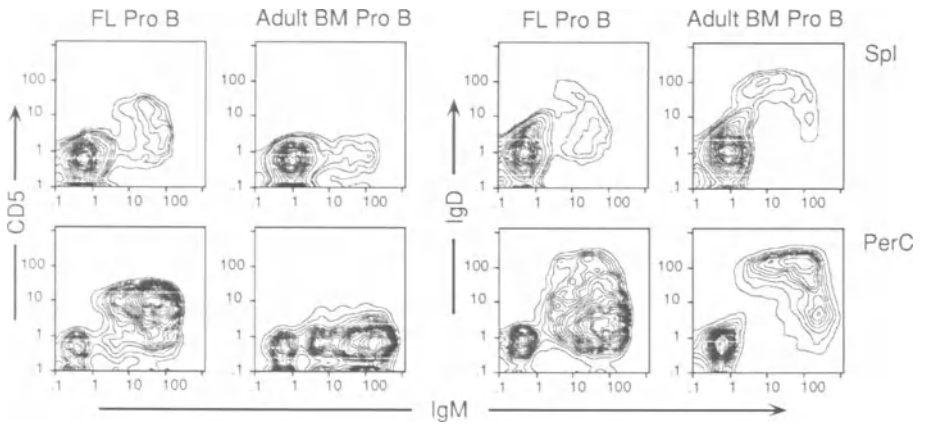


Fig. 2. B cells with distinct phenotypes are repopulated in SCID mice 3 weeks after transfer of fetal liver or adult bone marrow derived Pro B cells. 1 x 10^5 Pro B cells (defined as $B220^+CD43^+HSA^+$) were isolated by cell sorting from either source and then injected i.v. into SCID recipients irradiated the previous day (350R, Cs). Three weeks later recipients were sacrificed and spleen cell suspensions stained with indicated reagents. Cells were analyzed on a dual laser flow cytometer operating in four-color mode ($FACStar^{PLUS}$)

We then compared the phenotype of B cells generated by Pro B cells from two distinct developmental timings, adult bone marrow and fetal liver. Previously [7] we had suggested that B cells bearing a novel surface phenotype, $IgM^{high}IgD^{low}CD5^+$ (hereafter referred to simply as $CD5^+$ B cells), were largely generated early in development and only poorly repopulated using bone marrow stem cells, largely giving rise to B cells with a typical phenotype, $IgD^{high}IgM^{low}CD5^-$ (hereafter referred to as IgD^{++} B cells). In contrast, fetal liver stem cells efficiently reconstituted all lymphoid subpopulations. However, since transfer of stem cells repopulates the bone marrow, generating Pre-B and B cells there, it was unclear whether both $CD5^+$ and IgD^{++} B cells were simultaneously generated in fetal liver and then only the latter in bone marrow or alternatively whether $CD5^+$ B cells were produced in fetal liver and IgD^{++} B cells in adult bone marrow.

The results of these transfer experiments (Figure 2) supported the latter alternative. Thus, transfer of Pro B cells from fetal liver efficiently generated B cells, but few expressed high levels of IgD and many were $CD5^+$ (both in spleen and peritoneal cavity). In contrast, Pro B cells from bone marrow generated B cells in similar numbers, but most were IgD^{++} and few expressed CD5. We interpreted this as supporting a fetal origin for most $CD5^+$ B cells found in the adult animal and further, that most IgD^{++} cells do not arise from this fetal source. This distinction in precursors is important, since $CD5^+$ and IgD^{++} cells exhibit numerous functional distinctions. Further, many B cell lymphomas are $CD5^+$, both in mouse and human [8, 9]. Thus it is critical to establish whether $CD5^+$ B cells arise independently of IgD^{++} cells and if so to characterize differences in both the progenitors and their progeny.

Time course of B cell repopulation

We have carried out a time course of the repopulation of B cells using fetal-derived Pro B cells. We find detectable B cell engraftment at 2 weeks, peaking by 3-4 weeks and thence remaining relatively stable for months. As can be seen in Figure 3, many of the B cells in spleen express CD5 at all time points tested. Further, analysis for IgD expression (Figure 4) shows that most bear distinctive low levels of IgD together with high levels of IgM as a stable phenotype (out to 10 months).

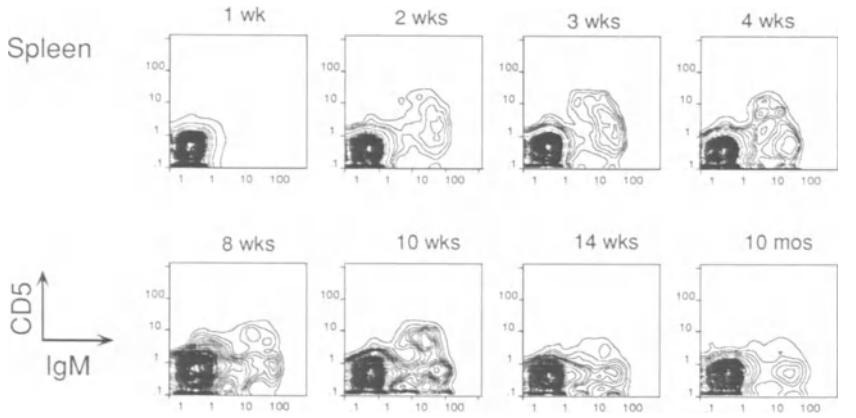


Fig. 3. Flow cytometry of CD5/IgM expression in SCID spleen as a function of time after transfer of fetal Pro B. The IgM^+ cell frequencies (where visible) vary from 2-7%. Data shown is gated for "lymphoid" forward/right angle scatter values. Representative data from numerous analyses (at least two at each time point). Note that CD5 expression, normally rare on B cells in spleen, is readily detectable in these analyses

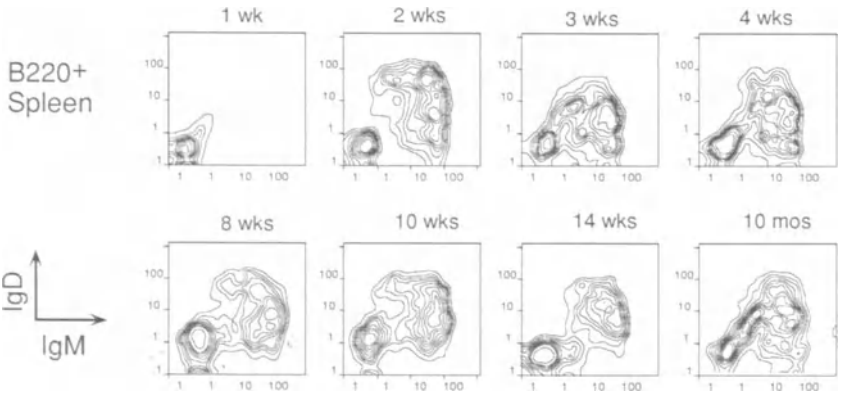


Fig. 4. Flow cytometry of IgM/IgD expression in SCID spleen as a function of time after transfer of fetal Pro B. Analyses comparable to those in Figure 3. Note that in contrast with the bulk of B cells in normal spleen, most B cells show a distinctive $IgM^{high}IgD^{low}$ phenotype

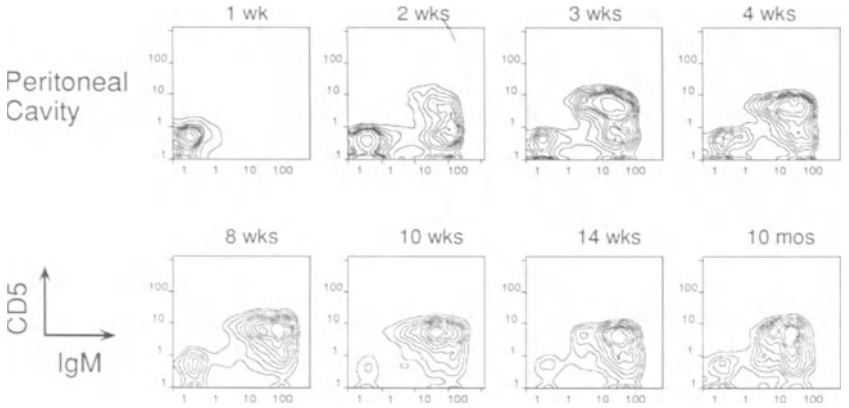


Fig. 5. Flow cytometry of CD5/IgM expression in SCID peritoneal cavity as a function of time after transfer of fetal Pro B. The IgM^+ cell frequencies (where visible) vary from 10-30%. Representative data gated as in Figure 3. Note that the bulk of B cells are $CD5^+$ at all but the first detectable time point, week two

A similar time course analysis of cells in peritoneal cavity also reveals first detectable B cells 2 weeks after injection (Figure 5). Interestingly, there is a reproducible increase in the expression of CD5 between 2 and 3 weeks; thereafter the percentage is relatively stable. This may reflect the induction of CD5 on an initially $CD5^-$ population during a process of antigen selection (see below).

Careful analysis of the bone marrow for engraftment of Pre B cells (as distinguished from Pro B cells that are always present in SCID bone marrow) shows that a transient population of $B220^+CD43^-$ cells is detected one week after injection (Figure 6). At this point all $B220^+$ cells are IgM^- (not shown). At later time points (2, 3 wks) this fraction is undetectable. Furthermore this population is never seen in control SCID bone marrow, whether irradiated or unmanipulated. A transient expansion of the Pro B fraction ($B220^+CD43^+$) is seen shortly after irradiation of SCID mice (see control, 1wk, Figure 6).

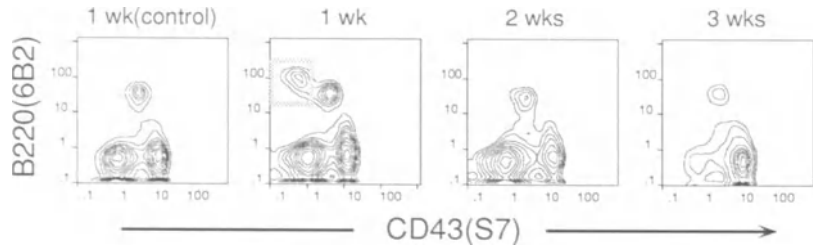


Fig. 6. Flow cytometry analysis of bone marrow from SCID recipients at different times after transfer of fetal Pro B reveals a transient population of Pre B cells ($B220^+CD43^-$, dotted box) one week after transfer. For these data, unlike all other experiments presented, 3-5 x 10^5 fetal Pro B cells were injected per animal to enhance the ability to detect early engraftment. Note that early after irradiation, control SCID bone marrow contains an elevated percentage of $B220^+CD43^+$ cells. In all plots, it is likely that most cells with this phenotype are SCID-derived and not from the transferred population

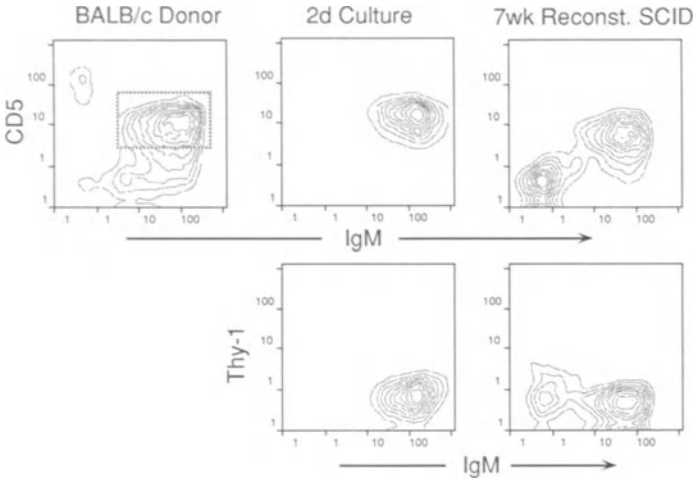


Fig. 7. Sorted CD5$^+$ B cells retain CD5 expression after transfer to and expansion in SCID mice. CD5$^+$ B cells were sorted from BALB/c peritoneal cavity, cultured for two days, then a fraction restained and the majority injected into SCID mice (irradiated the previous day, 350R, Cs). After 7 weeks, recipients were sacrificed and peritoneal cavity cells analyzed for the presence of CD5$^+$ B cells by flow cytometry. Thy-1 serves as a control for CD5 staining on B cells in these analyses

CD5 expression on B cells as a stable phenotype

If CD5$^+$ B cells are not constantly replenished from unrearranged sources in the adult, then how is the population maintained? We had previously shown that transfers of small numbers of IgM$^+$ B cells from peritoneal cavity could reconstitute and maintain the CD5$^+$ B cell population in irradiated recipients [10]. Thus CD5$^+$ B cells are a long-lived self-maintaining population, with little or no need for input from a newly rearranging precursor pool, in contrast with bone marrow derived IgD^{++} B cells that presumably turn over with a shorter half-life. Further, we have found that CD5 expression is a relatively stable feature of the CD5$^+$ B cell population since such cells when carefully purified from the peritoneal cavity will stably repopulate only CD5$^+$ B cells in SCID mice (Figure 7).

It appears that the Ig repertoire of CD5$^+$ B cells is more restricted in the adult compared to the newborn. This is particularly evident when one focuses on genes encoding novel self-reactivities enriched in this B cell subset such as antibody to a cryptic determinant on mouse red blood cells exposed by treatment with the proteolytic enzyme bromelain [11-13]. Thus we suggest that CD5$^+$ B cells have undergone selection from the original pool of Ig$^+$ cells with only those encountering their antigen at an appropriate (as yet undetermined) affinity being recruited into the long-lived adult population [14]. As such, they constitute a type of "memory" encoded in the germline for self determinants. The functional significance of such a self-memory population remains a topic of speculation for the moment and further investigation. Our repopulation system provides a powerful new tool for solving this intriguing puzzle.

Conclusions

Since their initial identification as a subset of B cells in normal mice [15] Ly-1/CD5$^+$ B cells have been shown to possess a set of properties that distinguish them from normal (IgD^{++}) B cells. Among these are production of certain self-reactive antibodies [16] and a propensity to clonal expansion and even neoplasia [17]; for a review see [18]. In particular, recent immunoglobulin V region gene sequence analysis of hybridomas derived from the normal CD5$^+$ B cell population provides some of the best evidence to date connecting them with neoplastic CD5$^+$ B cells [12, 19, 20]. Further, the definition of a homologous population of CD5$^+$ B cells in human that shares at least some properties with murine CD5$^+$ B cells provides further impetus to understand their significance ([21, 22]).

A recurrent issue in the study of this population has been it's relationship with the bulk of IgD^{++} B cells. Early work demonstrating differences in the efficiency of repopulating irradiated recipients using fetal liver versus adult bone marrow led us to suggest that CD5$^+$ B cells arise relatively independently from the bulk of B cells, primarily during fetal and neonatal development [7]. An alternative hypothesis, based on the observation that CD5 could be induced on human B cells by phorbol ester treatment [23], was that such cells were simply an activated stage of a single population of B cells. More recently, this latter view has received support from a demonstration that CD5 expression could be induced on murine CD5$^-$ B cells by treatment with anti-IgM antibody [24].

However a number of observations argue against the interpretation that the majority of CD5$^+$ B cells seen in normal mice arise by activation of typical IgD^{++} splenic B cells. First, it is difficult to explain the transfer results based on an activation model. Our recent Pro B cell transfer data (including that presented here) argue that most committed B cell progenitors present during fetal development contribute to the adult CD5 population and not to the IgD^{++} population whereas the converse is true for Pro B cells in bone marrow of adult mice. A counter to this interpretation of our data is that CD5 expression is only induced by certain antigens (possibly those known as "type II") and that such specificities are uniquely restricted to the fetal-derived B cell population, possibly due to low N region addition. While it is clear that N region addition is low in the progeny of fetal Pro B cells [25] it remains unclear how the lack of N regions would give such a biased reactivity (or alternately how the presence of N addition would preclude it). Furthermore, not all Ly-1 B cells show an absolute lack on N-addition [26].

Another suggestion is that preferential V_H/V_L expression restricted to fetal B cells might give the type of reactivity that induces CD5 expression. But again, there is no clear data suggesting that preferential V gene usage would produce this type of reactivity and further, such preferential usage has been reported in early B lineage cells of both fetal liver and adult bone marrow [27, 28]. Even if if it turns out that fetal and adult B cells do express such distinct repertoires, then the argument that CD5 is simply an activation antigen on B cells becomes in essence semantic, since it would then serve to mark the progeny of B cells that can only be generated during fetal development. The data in human is less clear-cut, due to the inability to perform transfer experiments, but the large population of CD5$^+$ B cells in cord blood [21] argues against it's expression simply being the result of recurrent infections. Further, immunoglobulin gene sequence analysis suggests that at least some CD5$^+$ human neoplasias arise preferentially from a selected population of B cells expression novel V_H/V_L combinations [22], analogous to findings in the mouse.

In summary, even though CD5 expression can be induced on bone marrow derived B cells by a type of "activation", it appears likely that CD5 expression *in vivo* normally serves to mark the progeny of a distinctive wave of B cell development separate from adult bone marrow derived B cells. Thus, as suggested long ago, CD5$^+$ B cells constitute a separate developmental pathway or

pathway or lineage with novel molecular and functional properties. The associations with autoreactivity and B cell neoplasia make this an important population to understand more fully.

Acknowledgments

This work was supported by grants from the National Institutes of Health (CA-06927, RR-05539, AI-26782, and CA-37252), the American Cancer Society (IM-529), the Pew Charitable Trust (86-5043HE) and by an appropriation from the Commonwealth of Pennsylvania

References

1. Hardy RR, Kemp JD, Hayakawa K (1989) Analysis of lymphoid population in scid mice; detection of a potential B lymphocyte progenitor population present at normal levels in scid mice by three color flow cytometry with B220 and S7. Curr Top Microbiol Immunol 152:19-25
2. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K (1991) Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med 173:1213-1225
3. Baecher CM, Infante AJ, Semcheski KL, Frelinger JG (1988) Identification and characterization of a mouse cell surface antigen with alternative molecular forms. Immunogenetics 28:295-302
4. Gulley ML, Ogata LC, Thorson JA, Dailey MO, Kemp JD (1988) Identification of a murine pan-T cell antigen which is also expressed during the terminal phases of B cell differentiation. J Immunol 140:3751-3757
5. Hardy RR, Hayakawa K (1991) A developmental switch in B lymphopoiesis. Proc. Natl. Acad. Sci. USA 88:11550-11554
6. Bosma MJ (1989) The scid mutation: occurrence and effect. Curr Top Microbiol Immunol 152:3-9
7. Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA (1985) Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J Exp Med 161:1554-1568
8. Boumsell L, Bernard A, Lepage V, Degos L, Lemerle J, Dausset J (1978) Some chronic lymphocytic leukemia cells bearing surface immunoglobulins share determinants with T cells. Eur J Immunol 8:900-904
9. Haughton G, Arnold LW, Bishop GA, Mercolino TJ (1986) The CH series of murine B cell lymphomas: neoplastic analogues of Ly-1$^+$ normal B cells. Immunol Rev 93:35-51
10. Hayakawa K, Hardy RR, Stall AM, Herzenberg LA, Herzenberg LA (1986) Immunoglobulin-bearing B cells reconstitute and maintain the murine Ly-1 B cell lineage. Eur J Immunol 16:1313-1316
11. Mercolino TJ, Arnold LW, Hawkins LA, Haughton G (1988) Normal mouse peritoneum contains a large population of Ly-1$^+$ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. J Exp Med 168:687-698
12. Hardy RR, Carmack CE, Shinton SA, Riblet RJ, Hayakawa K (1989) A single V_H gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the V_H11 family. J Immunol 142:3643-3651
13. Carmack CE, Shinton SA, Hayakawa K, Hardy RR (1990) Rearrangement and selection of V_H11 in the Ly-1 B cell lineage. J Exp Med 172:371-374
14. Hayakawa K (1989) Autoreactivity and CD5$^+$ B cells. Curr Opin Immunol 2:582-587

15. Hayakawa K, Hardy RR, Parks DR, Herzenberg LA (1983) The "Ly-1 B" cell subpopulation in normal immunodefective, and autoimmune mice. J Exp Med 157:202-218
16. Hayakawa K, Hardy RR, Honda M, Herzenberg LA, Steinberg AD, Herzenberg LA (1984) Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. Proc Natl Acad Sci U S A 81:2494-2498
17. Stall AM, Farinas MC, Tarlinton DM, Lalor PA, Herzenberg LA, Strober S, Herzenberg LA (1988) Ly-1 B-cell clones similar to human chronic lymphocytic leukemias routinely develop in older normal mice and young autoimmune (New Zealand Black-related) animals. Proc Natl Acad Sci U S A 85:7312-7316
18. Hayakawa K, Hardy RR (1988) Normal, autoimmune, and malignant $CD5^+$ B cells: the Ly-1 B lineage? . Annu Rev Immunol 6:197-218
19. Pennell CA, Mercolino TJ, Grdina TA, Arnold LW, Haughton G, Clarke SH (1989) Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. Eur J Immunol 19:1289-1295
20. Pennell CA, Arnold LW, Haughton G, Clarke SH (1988) Restricted Ig variable region gene expression among $Ly\text{-}1^+$ B cell lymphomas. J Immunol 141:2788-2796
21. Hardy RR, Hayakawa K, Shimizu M, Yamasaki K, Kishimoto T (1987) Rheumatoid factor secretion from human Leu-1+ B cells. Science 236:81-83
22. Kipps TJ (1989) The CD5 B Cell. Adv. Immunol. 47:117-185
23. Miller RA, Gralow J (1984) The induction of Leu-1 antigen expression in human malignant and normal B cells by phorbol myristic acetate (PMA). J Immunol 133:3408-3414
24. Cong YZ, Rabin E, Wortis HH (1991) Treatment of murine CD5- B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. Int Immunol 3:467-476
25. Feeney AJ (1990) Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J Exp Med 172:1377-1390
26. Gu H, Forster I, Rajewsky K (1990) Sequence homologies, N sequence insertion and JH gene utilization in V_HDJ_H joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. Embo J 9:2133-2140
27. Yancopoulos GD, Malynn BA, Alt FW (1988) Developmentally regulated and strain-specific expression of murine V_H gene families. J Exp Med 168:417-435
28. Perlmutter RM, Kearney JF, Chang SP, Hood LE (1985) Developmentally controlled expression of immunoglobulin V_H genes. Science 227:1597-1601

Early B-Cell Repertoires

J. F. KEARNEY

Division of Developmental and Clinical Immunology, Department
of Microbiology and the Comprehensive Cancer Center, University of Alabama
at Birmingham, Birmingham, Alabama 35294

Introduction

It is clear now from many studies, that the early B cell repertoire differs significantly from that expressed in the adult. These differences result from not only the variety of V genes that are expressed but also the mechanisms involved in V gene rearrangements [1-5]. Additionally, the phenotypes of early B cells are typical of the B-1 phenotype while the majority of adult cells are of the B-2 type [6]. Previous studies have also established that the ability of B cells to respond to different antigens develops in a hierarchy with a particularly late development of the ability to respond to polysaccharide antigens [1,7,8]. In contrast to the late appearance of B cells capable of responding to specific antigens, the early B cell repertoire is marked by the properties of interconnectivity, autoreactivity and multispecificity which result from the character of the receptors expressed by early B cells.

Interconnectivity

Previous studies have shown that early B cell derived antibodies are highly connected when analyzed for binding to each in vitro [9,10]. Several fundamental questions relate to the origin of this connectivity. Are these interactions between IgM antibodies the result of selection of B cells expressing particular genetically related V genes within a given strain or are these V region connections "learned" and develop as the result of mutual selection during B cell development? We have attempted to study the two alternatives by comparing interconnectivity that develops within perinatal antibodies derived from two distinct parental strains, BALB/c and C57BL/6, compared to the connectivities developing between such parental antibodies when these are derived from F1 mice of the same parental strains. When we analyzed the connectivity between parentally-derived BALB/c and C57BL/6 hybridomas it was clear that while there were differences in interconnectivity apparent within the BALB/c compared to C57BL/6 sets of IgM antibodies, interconnectivity across strains was much less. However, when the allotypically distinct hybridomas were isolated from F1 hybrid mice and tested in the same way, such differences were not obvious and there appeared to be equivalent connectance between antibodies even though they were derived from the different parental chromosomes. These results suggest that the connectance between immunoglobulins observed during early life results from an interplay of the V regions expressed by both alleles and do not appear to be

Current Topics in Microbiology and Immunology, Vol. 182

genetically restricted. These results favor the second hypothesis that mutual selection by interaction of B cell receptor immunoglobulins are involved in the connectivity observed that develops within the individual [9,10].

Self-reactivity and Multispecificity

Although autoantibody production is characteristic of many autoimmune diseases, it is also a hallmark of developing B cell repertoires in mouse and man [9-11]. Self-reactivity can be manifested as anti-idiotypic activity as described in the previous section or as reactivity to other cell surfaces structures.

During development in the mouse the most 3' D proximal genes are rearranged and expressed first in early development at a frequency out of proportion to the size of the V_H gene families. In most strains of mice studied a particular member of the V_H7183 family, V_H81X, is functionally rearranged at a high frequency in fetal liver and adult bone marrow yet never appears to be expressed as IgM expressed by B cells [4,5,12]. In our collection of perinatal hybridomas we have isolated several secretory hybridomas expressing V_H81X in conjunction with a light chain. The secreted IgM antibody from these hybridomas is highly self-reactive in that it binds to many IgM's derived from the perinatal period and in this sense is one of the most highly connected of perinatal antibodies. In addition, this antibody reacts with the membrane of a variety of self-cell types and cell lines as detected by FACS analysis. A clue to its highly reactive nature was obtained by analysis of the derived amino acid sequence which revealed that within the VDJ region of these germline rearranged genes here were five pairs of positively charged amino acids. Analysis of other Ig V regions by search of GenBank revealed that this is a very unique characteristic and it is rare to find even one pair of such charged amino acids in a given V_H region. This unusual characteristic suggests a reason why the rare V_H81X containing antibodies isolated have such high self-reactivity towards self-Igs and other cell surface membranes.

This high degree of self reactivity may be responsible for the lack of expression of B cells expressing the V_H81X gene since this gene is rearranged at a high frequency in B cell generative sites. B cells expressing functional rearrangements appear to be deleted [12]. It is not clear as to the function of such B cells or pre-B cells that would express such a receptor early in development. To try to understand why this occurs and to determine a possible function of such cells expressing a rearranged V_H gene we have constructed a transgenic mouse expressing a functionally rearranged V_H81X gene. Preliminary results from mice in which the V_H81X Cμ (IgH6a) is expressed on a IgH6b background have shown that these mice appear to contain normal numbers of B cells expressing the IgM transgene but no serum transgene IgM could be detected. A small amount of serum IgM could be detected from the expression of endogenous genes. These results suggest that the V_H81X gene can be expressed in periphral B cells but that its presence renders the B cells anergic in some way and IgM is not secreted into the serum.

Apart from the apparent self-reactivity that occurs between Ig V regions, other autoreactivities that are often observed in human and mouse autoimmune diseases were also detected in perinatal hybridomas. Approximately 11% of hybridomas from the perinatal period reacted with thymocytes and/or subpopulations of peripheral T cells and sometimes B cells. A majority of these antibodies appeared to bind to epitopes which were sensitive PI-PLC treatment [13] and several antibodies which appeared to react with a similar epitope, precipitated a 100 Kd molecule from the surface of thymocytes which appeared to be associated with Thy-1 and Th-B molecules [2]. Nucleotide sequence analysis of this set of antibodies showed a high degree of homology with almost identical V_H genes from the SM7 family and with identical V-D-J_H joins [14]. Since these hybridomas were isolated from separate fusions it is likely that B cells expressing this particular specificity are generated regularly during the perinatal period. What particular role, if any, do B cells expressing such specificities play in early lymphocyte repertoire development? We are currently investigating the role of such anti-T cell specific B cells in secondary lymphoid organs since B cells are the first lymphoid cells to enter spleen and lymph nodes and locate in areas which rapidly become T cell populated compartments.

Neonatal B Cells

It is clear from the above, that perinatal B cells differ in many ways from their adult equivalents. Apart from the fetal liver as a site for B cell development we have recently found that mesodermal derived tissues including omentum and mesentery also appear to be sites for B cell development in mouse and man [15,16]. In mice, fetal omentum transferred into subkidney capsular sites of scid mice gave rise to $CD5^+$ B cells but not conventional B cells or T cells within the peritoneal cavity of the recipient. Grafting of this tissue also produce approximately normal levels of serum IgM of donor origin as well as IgG (mainly IgG3) in the serum. In addition, donor-derived IgA plasma cells were found in the lamina propria [15,17]. The nature of the precursors is not yet clear, but these experiments clearly showed that the mouse omentum contained precursors for $CD5^+$ B cells. Similarly in humans we could show that fetal omentum is a site for B cell generation since cells with all the criteria of precursor B cells including CD24 positive cells and TdT activity could be found in early fetal life [16]. More recently a preliminary study of other mesodermally derived tissues in human fetuses including kidney and lung also showed evidence for pre-B cell development [18]. All of these studies suggest that fetal liver is not the only site where B cells develop in the embryo and that there may be multifocal sites in which the early B cells, most notably CD5 B cells which predominate early in development, are generated in sites other than the fetal liver.

These results not only define new sites for B lymphopoiesis but also may relate to the tendency for plasmacytomas to arise in these tissues as described extensively by Potter (in this volume).

References

1. Perlmutter RM (1986) Programmed development of the antibody repertoire. Curr Top Microbiol Immunol 135:95.
2. Feeney AJ (1990) Lack of N regions in fetal and neonatal mouse immunoglobulins V-D-J functional sequences. J Exp Med 172:1377.
3. Gu H, Tarlenton D, Müller W, Rajewsky K, Förster I (1991) Most peripheral B cells in mice are ligand selected. J Exp Med 173:1357.
4. Yancopoulos GD, Desiderio SV, Paskind M, Kearney JF, Baltimore, D Alt FN (1984) Preferential utilization of the most J_H-proximal gene segments in pre-B cell lines. Nature 311:727.
5. Perlmutter RM, Kearney JF, Chang SP, Hood LE (1985) Developmentally controlled expression of immunoglobulin V_H genes. Science 227:1597.
6. Herzenberg LA, Stall AM, Lalor PA, Sidman C, Moore WA, Parks DR, Herzenberg LA (1986) The Ly-1 B cell lineage. Immunol Rev 93:81.
7. Klinman NR, Press JL (1975) The B cell specificity repertoire: its relationship to definable subpopulations. Transplant Rev 24:41.
8. Stohrer R, Kearney JF (1984) Ontogeny of B cell precursors responding to $\alpha 1 \rightarrow 3$ dextran in BALB/c mice. J Immunol 133:2323.
9. Holmberg D, Forsgren S, Forni L, Ivars F, Coutinho A (1984). Reactions among IgM antibodies derived from normal neonatal mice. Eur J Immunol 14:435.
10. Vakil M, Sauter H, Paige C, Kearney JF (1986) *In vivo* suppression of perinatal multispecific B cells results in a distortion of the adult B cell repertoire. Eur J Immunol 16:1159.
11. Vakil M, Kearney JF (1988) Expression of idiotypes and anti-idiotypes during development: Frequencies and functional significance in the acquisition of the adult B cell repertoire. In: *Elicitation and Use of Anti-Idiotype Antibodies and their Biological Properties.* Boca Raton, CRC Press. p. 75.
12. Decker DJ, Boyle NE, Klinman NR (1991) Predominance of non-productive rearrangements of V_H81X gene segments evidences a dependence of B cell clonal maturation on the structure of nascent H chains. J Immunol 147:1406.
13. Lehuen A, Monteiro R, Bartels J, Kearney JF (1992) Characterization, specificity and Ig V gene usage of anti-lymphocyte monoclonal antibodies from perinatal mice. Inter Immunol (in press).
14. Lehuen A, Kearney JF (1992) Identification of a 100 kD molecule associated with two PI-linked molecules: Thy-1 and the ThB. Eur J Immunol (in press).
15. Solvason N, Lehuen A, Kearney JF (1991) An embryonic source of Ly1 but not conventional B cells. Inter Immunol 3:543.
16. Solvason N, Kearney JF (1992) The human fetal omentum: A site of B cell generation. J Exp Med 175:397.
17. Kroese FGM, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg L (1988) Many of the IgA plasma cells for the murine gut are derived from self replenishing precursors in the peritoneal cavity. Inter Immunol 1:75.
18. Nunez C, Kubagawa H, Stankovic A, Cooper MD (1992) Widespread tissue distribution of fetal pre-B cells in humans. FASEB J 6(5):4444.

In Vitro Growth and Maturation of Human B-Cell Precursors

S. Saeland, I. Moreau, V. Duvert, D. Pandrau and J. Bancherau

Schering-Plough Laboratory for Immunological Research, Dardilly, France

Purified B cell precursors (BCP) (CD10+ CD19+ surface-membrane (s)Ig- cells) isolated from human fetal bone marrow (BM) were cultured with various cytokines, in the presence or absence of a fibroblastic stromal cell layer derived from adult human BM. We demonstrated that IL-7, IL-3, and stem-cell factor (SCF) participate in inducing low magnitude BCP proliferation in the absence of stroma. Addition of either IL-4, IFN (α and γ), or TGFß, resulted in significant inhibition of proliferation.
Strikingly, BCP proliferated at remarkably higher levels when cultured on BM stromal cells, and this effect was further enhanced by exogenously supplied IL-7. Proliferating cells were mostly CD20+, and included both cμ- and cμ+ cells.

Furthermore, BCP proliferated in response to anti CD40 antibody presented by FcγRII-transfected murine fibroblastic Ltk- cells (CD40 system) (Banchereau et al. 1991), demonstrating a functional role for CD40 in B cell ontogeny. However, this effect was shown to require a second signal, which could be specifically provided by IL-3 among a panel of cytokines examined.
Finally, although suggestive of BCP maturation, the culture systems examined did not permit the transition to mature B cells (sIgM+ sIgD+).

Introduction

A number of groups have devoted major efforts to understand the regulatory mechanisms controlling BCP proliferation and maturation. Progress has been most notable in the murine system (reviewed by Rolink and Melchers 1991), largely following the establishment of long-term stroma-dependent BCP cultures (Whitlock and Witte 1982). Such cultures permitted the identification and molecular cloning of stromal-cell derived interleukin (IL)-7 (Namen et al. 1988), which represents a central growth-factor in murine B cell ontogeny. In addition, a number of other cytokines have been described as positive (stem cell factor (SCF), IL-4, IL-3), or negative (IL-1, IL-4, TGFß), regulators of murine BCP proliferation at various stages of development. Although cytokines (IL-1, IL-4) have also been implicated in BCP maturation, this process is thought to be largely driven by expression of the Ig genes (Reth et al. 1987; Era et al. 1991).
In humans, lack of appropriate culture systems has long constituted a major obstacle for the study of B lymphopoiesis (reviewed by LeBien 1989). Recently, however, we and others (Wolf et al. 1991) succeeded in expanding human BCP for several weeks in vitro. Here, we summarize our studies on the proliferation and maturation of human BCP.

Current Topics in Microbiology and Immunology, Vol. 182

Materials and Methods

Isolation of human BCP

Human BCP were isolated from femurs of spontaneously aborted fetuses between 18-24 weeks of gestation. BM was extracted, and centrifuged on Ficoll-Hypaque (d=1.077). Light-density cells were subjected to overnight adherence at 37°C, and non-adherent cells were subsequently fractionated by panning with anti-CD10 MAb (ALB-1, Immunotech, Marseille, France). CD10+ cells were recovered and depleted of sIg+ cells by a cocktail of MAbs (anti μ, α, δ, κ, λ) and immunomagnetic beads. This procedure led to the isolation of a highly purified CD10+ sIg- BCP population, which uniformly expressed CD19 antigen.

Cultures of BCP in the presence of cytokines

Freshly isolated CD10+ sIg- BCP were cultured in OptiMEM liquid medium (Gibco, Grand Island, NY), containing 10%v/v fetal bovine serum (FBS), 5 x 10E-5 M 2-ß-mercaptoethanol (2-me), and antibiotics. For 3H-thymidine (3H-Thd) incorporation assays, BCP were seeded in 96-well culture plates at 10E4 cells/well. In parallel, cultures were initiated at 3 x 10E5 cells/ml in 24-well plates, for cell-counts and phenotype analysis. Human cytokines were used as purified recombinant preparations, at concentrations indicated in Figs. and Tables.

Cultures of BCP on stromal cells

Freshly-isolated CD10+ sIg- BCP were cultured on confluent monolayers of human BM stromal cells prepared as follows. Briefly, adult BM from femur fragments obtained during orthopedic surgery was seeded in α-MEM medium supplemented with 12.5%v/v FBS, 12.5%v/v horse serum, 10E-6 M hydrocortisone, and 10E-4 M 2-me. Non-adherent cells were removed following 18 hours of incubation. Adherent cells were cultured for 7-10 days, detached with trypsin, and depleted of cells of hematopoietic origin by using a cocktail of MAbs (anti CD45, CD14, CD20, CD33, CD19, CD2, CD3) and immunomagnetic beads. Cells recovered were replated and grown to confluence, yielding uniform fibroblastic-like cultures. After washing off spent medium, BCP were added (in OptiMEM medium) to the stromal cells, at the same cell densities as described above.

Cultures of BCP in CD40 system

BCP were recovered after expansion of CD10+ sIg- cells on BM stroma and IL-7 for 10 days (see above), washed, and seeded onto irradiated (5000rad) murine fibroblastic Ltk- cells transfected with CDw32, the human FcγII-receptor (CDw32 L cells) (ratio BCP/L cells=2). Anti CD40 MAb (MAb 89) (Vallé et al. 1989) was added at 500ng/ml, in the presence or absence of cytokines. As control, a MAb of same isotype (IgG1) but unrelated specificity was supplemented (500ng/ml) to the cultures. BCP were seeded at the same densities as above. The CD40 system has been previously described in detail for cultures of mature human B cells (Banchereau et al. 1991).

Results and Discussion

Phenotype of fetal CD10+ sIg- BCP

We first characterized the phenotype of freshly-isolated fetal BCP by flow cytometry.
The CD10+ sIg- cells were found to have the following phenotype: CD1a-, 2-, 3-, 5-,8-, 9+, 10+, 11a+ (LFA-1), 13+ subset, 19+, 20+ subset, 21-, 22±, 23-, 24+, 25-, 28-, 33-, 34+ subset, 37+, 38+, 40+, 43+, 44+, 45R0-, 45RA+, 47+, 48+, 49d+ (VLA-4), 49e+ (VLA-5), 54+ (ICAM-1), 55+, 58+ (LFA-3), 59+, w78+, c-kit-, sIg-, cμ+ subset, cκ-, cλ-.
In addition, Hoechst 33342 fluorescence analysis indicated that a proportion of freshly-isolated CD10+ sIg- cells were in cycle (10.4% cells in S+G2/M) (data not shown).

Effect of cytokines on human BCP

As illustrated in Table 1, among a panel of factors tested, only IL-7 and IL-3 induced significant levels of 3H-Thd uptake by fetal CD10+ sIg- BCP. Human BCP have previously been reported to proliferate in response to IL-7 (Saeland et al. 1991; Uckun et al. 1991), and IL-3 (Wörmann et al. 1989). However, the effect of IL-7 is of much lower magnitude than that described on murine BCP (Lee et al. 1989). In this context, although early BCP respond to IL-7 (Saeland et al. 1991), we did not detect high-proliferative B lineage cells within the CD34+ compartment, indicating that our present BCP selection does not exclude such a putative CD10- population.

The association of IL-7 and IL-3 provides a stronger proliferative signal for CD10+ sIg- BCP than either cytokine used alone (Saeland et al. 1991; and Table 1). However, these effects remain of low magnitude, and such cultures did not permit an increase of cell numbers (not shown). Furthermore, IL-7 and IL-3 dependent cultures could only be maintained for approximately two weeks.

IL-7 dependent proliferation of CD10+ sIg- cells was potentiated by soluble SCF (Table 1), which, unlike IL-3, lacked proliferative activity per se (Table 1). In this respect, SCF has been reported to synergize with IL-7 for the proliferation of early murine BCP (McNiece et al. 1991). However, murine BCP have been reported to express high levels of the c-kit encoded SCF-ligand (Rolink et al. 1991), whereas human BCP seem to lack detectable c-kit expression (personal observations; and Ashman et al. 1991).

Table 1. Proliferation of CD10+ sIg- BCP in response to cytokines

Culture conditions	cpm Exp 1	Exp2	Exp 3
Medium	525 ± 86	205 ± 15	317 ± 82
IL-7	1137 ± 163	2694 ± 72	1548 ± 18
IL-3	1337 ± 230	790 ± 56	
IL-6	500 ± 114		
IL-10	313 ± 102		
IFN-γ	585 ± 149		
TNF-α	441 ± 53		
TGFß	136 ± 15		
SCF			447 ± 126
IL-7 + IL-3		5911 ± 405	
IL-7 + SCF			2424 ± 267

^{3}H-Thd incorporation ± SD in triplicate wells, after 6 days of culture of CD10+ sIg- cells (10E4/well). Cytokine concentrations as follows: IL-7: 10 ng/ml, IL-3: 10 ng/ml, IL-6: 20 U/ml, IL-10: 100 ng/ml, IFN-γ : 500 U/ml, TNFα: 25 ng/ml, TGFß: 2 ng/ml, SCF: 100 ng/ml

As shown in Table 2, several cytokines, such as IFN-γ, IFN-α, IL-4, and TGF-ß, were able to inhibit IL-7 and IL-3 dependent BCP proliferation. In this context, TGF-β and IL-4 have been described as growth-inhibitors in murine B cell ontogeny (Lee et al. 1989; Rennick et al. 1987). Finally, we did not detect an inhibitory effect of IL-1α on BCP proliferation in response to IL-7 and IL-3 (Table 2). This contrasts with the IL-1α induced inhibition of IL-7 dependent murine BCP growth (Suda et al. 1989).

Table 2. Inhibitory effect of cytokines on IL-7 + IL-3 dependent proliferation of CD10+ sIg- BCP

Culture conditions	cpm Exp 1	Exp2
Medium	723 ± 71	1050 ± 515
IL-3 + IL-7	2808 ± 130	5721 ± 709
IL3 + IL-7 + IFNγ	1531 ± 283	
IL-3 + IL-7 + IFNα	1729 ± 186	
IL-3 + IL-7 + TGFß	729 ± 77	
IL-3 + IL-7 + IL-1α	3067 ± 522	
IL-4		916 ± 78
IL-3 + IL-7 + IL-4		1541 ± 517

Experimental conditions as in Table 1, except IFNγ: 1000 U/ml, IFNα: 1000 U/ml, IL-1α: 1000 U/ml, IL-4: 100 U/ml.

Effect of bone marrow stromal cells on human BCP

High-level BCP proliferation in the presence of stromal cells

In the mouse, stromal cells from the BM are well known to play a key role in B lymphopoiesis, serving as accessory cells producing cytokines and providing membrane contact.

Consequently, we generated stromal cell cultures from human BM and evaluated their effect on the proliferation and maturation of fetal CD10+ sIg- BCP. The stromal cultures were depleted of cells bearing hematopoietic markers, thus abrogating endogenous hematopoiesis, and eliminating lymphoid and myeloid cells (data not shown). This appeared particularly important in order to establish BCP cocultures without irradiation and subsequent physiological alterations of the stromal cells.

As illustrated in Fig. 1, CD10+ sIg- cells cultured in the presence of confluent stromal cells incorporated high levels of 3H-Thd as compared to BCP alone. This reflected BCP proliferation, as stromal cells alone incorporated only low levels of 3H-Thd (Fig. 1). The supportive effect of stromal cells on BCP proliferation was considerably enhanced by the addition of exogenous IL-7 upon initiation of the cocultures (Fig. 1). These results confirm recently reported experiments (Wolf et al. 1991), and illustrate the importance of the association of stromal cells and IL-7 in human B-lymphopoiesis, as widely documented in the mouse.

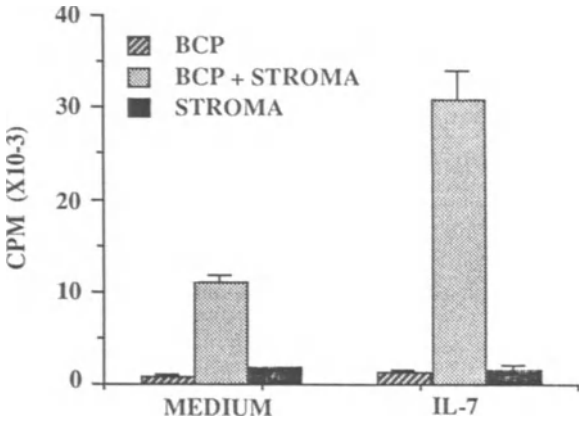


Fig. 1. High-level proliferation of CD10+ sIg- BCP cultured on bone marrow stromal cells.

3H-Thd incorporation ±SD in triplicate wells seeded with freshly-isolated CD10+ sIg- BCP (10E4/well), in the absence or presence of stromal cells, or with stromal cells alone. Cultures performed with or without exogenous IL-7 (10ng/ml)

Lymphoid cells were recovered from BCP cocultures as non-adherent and stroma-adherent fractions. In line with the data presented in Fig. 1, lymphoid cell numbers were found to increase in cultures supplemented with IL-7, leading to a several fold expansion as compared to input BCP (Table 3). Such cultures could be maintained up to one month, without significant loss of cell viability. In contrast, cocultures performed in the absence of IL-7 contained fewer cells (Table 3), and were associated with a lower cell viability.
Finally, we also observed high-level proliferation of adult CD10+ sIg- BCP on stromal cells, indicating that this effect is not restricted to fetal B cell ontogeny (not shown).

Table 3. Expansion of lymphoid cells in cultures of CD10+ sIg- BCP on bone marrow stroma

Days of culture	cell numbers (x10E-5) + Medium	+ IL-7
0	3.0	3.0
5	3.8	7.2
10	6.4	16.2
15	7.9	21.7
20	9.2	23.6
25	2.8	22.7
30	0.8	21.8

Data represent total recovery of viable lymphoid cells (stroma adherent + non adherent) in cultures performed in the presence or absence of IL-7 (10 ng/ml).

Effect of stromal cell culture on BCP phenotype

We next studied the phenotype of lymphoid cells recovered from cocultures of fetal CD10+ sIg- cells with BM stromal cells, in the presence or absence of exogenous IL-7.
Thus, virtually all cells were CD19+ CD10+ (Fig. 2), demonstrating their B lineage affiliation. Only a minor proportion of cells expressed sμ (maximally in the order of 10%) (Fig. 2), in association with sκ or sλ (not shown), independently of the presence of IL-7 (Fig. 2).These data are in accordance with other authors (Wolf et al. 1991).
However, we failed to detect cells bearing other isotypes (not shown), indicating that the great majority of the cells in our cultures are BCP, and that the present system does not support the emergence of mature B cells. In contrast, stromal cells have been described to provide sufficient signals for the maturation of human BCP to Ig plaque-forming cells in agar cultures (McGinnes et al. 1991). The reasons for these differences remain to be investigated. In this context, we neither detected cκ+ or cλ+ cells in our cultures, nor the presence of Ig in the supernatants, indicating the absence of plasmocytes.
As shown in Fig. 2, BCP cultured on stromal cells expressed high levels of CD20 antigen in the presence of exogenous IL-7. We further observed the appearance of CD21+ cells, and occasional CD23+ cells, under the same culture conditions (not shown). In contrast, we noted a reduction of the CD34+ BCP subset, and an importance decrease in the expression of CD9 antigen (not shown). Taken together, these results suggest that our BM stromal cell cultures support a partial BCP maturation sequence, in which IL-7 plays an active role.
With the exception of an increase in LFA-1 levels, which has previously been described during human B cell ontogeny (Kansas and Dailey 1989), our stromal culture system did not modulate the expression of a number of adhesion receptors (VLA-4, VLA-5, ICAM-1, LFA-3) (not shown). Binding of BCP to stromal cells involves VLA-4 (Ryan et al. 1991), but our data suggest that expression levels of this molecule cannot explain the reported maturation-associated decrease in the capacity of BCP to bind to stroma (Ryan et al. 1990).
We neither detected expression of c-kit, nor of the transferrin receptor, following culture of BCP on stromal cells. In contrast, however, and unlike freshly-isolated BCP, a subset of the cells recovered from the cultures was found to express CD25, the 55kD chain of the IL-2 receptor (data not shown).

Finally, CD40 was expressed on most BCP following culture on stroma, with a pattern similar to that of freshly-isolated CD10+ sIg- cells (not shown).

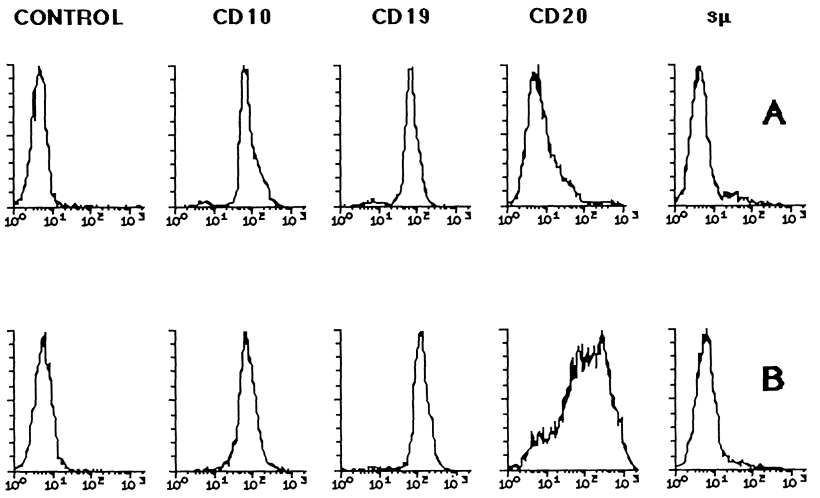


Fig. 2. Phenotype of lymphoid cells following culture of CD10+ sIg- BCP on bone marrow stromal cells.

Freshly-isolated CD10+ sIg- BCP were cultured for 11 days on stroma (see Materials and Methods), in the absence (A) or presence (B) of exogenous IL-7 (10ng/ml). Histograms represent log of fluorescence (horizontal axis) versus relative cell numbers (vertical axis) for non-adherent lymphoid cells (FACScan analysis). Similar results were obtained on stroma adherent lymphoid cells (not shown)

Characterization of proliferating BCP in stromal cultures

Next, we characterized proliferating cells in cultures of CD10+ sIg- BCP and BM stroma. Thus, we performed cell-cycle analysis (Hoechst 33342 fluorescence) on stroma adherent and non-adherent lymphoid fractions.

As shown in Fig. 3, a comparable proportion of cells were in S+G2M phase in the two fractions (adherent: 23.7%; non-adherent: 26.5%) following 4 days of culture. Similar results were obtained on day 10 cultures (not shown). These data indicate that the capacity to adhere to stroma does not permit a segregation of proliferating and non-proliferating BCP. We further observed that the majority of cycling cells, both within stroma adherent and non-adherent fractions, expressed CD20 in the presence of IL-7 (Fig. 3). However, we recently detected high-proliferative capacity cells both within CD20- and CD20+ BCP fractions (not shown). These data would suggest that IL-7 is involved in the induction of CD20, leading to a rapid predominance of proliferating CD20+ cells. Accordingly, as discussed above, a much lower proportion of CD20+ cells was found in cultures not supplemented with IL-7 (Fig. 2). Finally, similar proportions of cμ+ and cμ- cells were identified in stroma-adherent and non adherent populations (Fig. 3). Furthermore, proliferating cells were found within the cμ+ and cμ- subsets of both populations (Fig. 3).

Our observation that cμ- sIg- cells are not restricted to the stroma-adherent fractions indicate that some of the cells preceding the cμ+ stage have lost their adhesion capacity. Our findings suggest that the progressive loss of BCP capacity for stroma adherence (Ryan et al. 1990) is not an obligatory pathway.

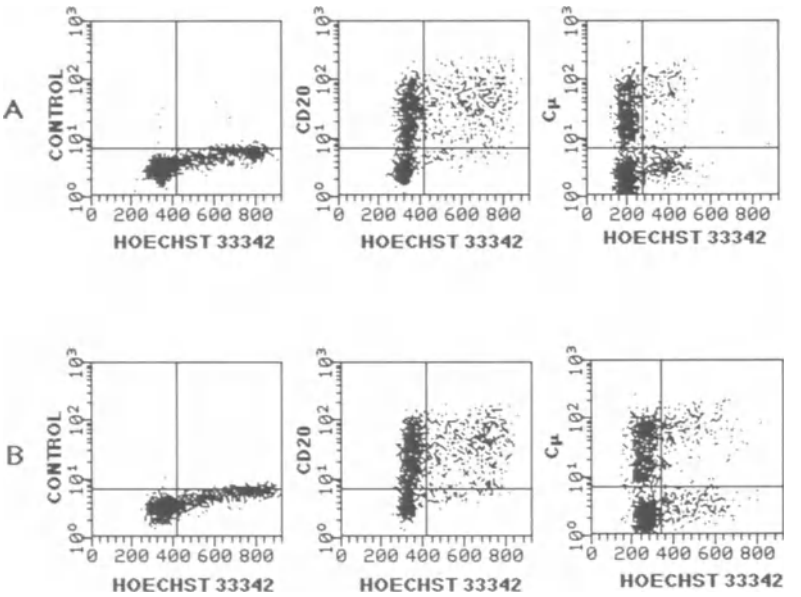


Fig. 3. Cell cycle analysis of CD10+ sIg- BCP cultured on bone marrow stromal cells.

Freshly-isolated CD10+ sIg- cells were cultured 4 days on stroma (see Materials and Methods), in the absence (A) or presence (B) of exogenous IL-7 (10ng/ml). Dot plots represent DNA content (Hoechst 33342 fluorescence) (horizontal axis) versus phenotype (vertical axis) (FACStar+ analysis)

Role of surface membrane CD40 in BCP development

The 45kD CD40 glycophosphoprotein has been demonstrated to participate in the activation and proliferation of mature human B cells. In particular, triggering through anti CD40 antibodies presented by murine CDw32 L cells results in long-lasting proliferation of normal human B cells (CD40 system), which is further enhanced by IL-4 (Banchereau et al. 1991). Since CD40 is also expressed on human BCP (Law et al. 1990; Saeland et al. 1992), we investigated the role of this molecule in human B cell ontogeny.

As illustrated in Fig. 4, significant levels of 3H-Thd incorporation were observed when fetal BCP expanded on stroma (see Materials and Methods) were cultured in the presence of anti CD40 MAb, CDw32 L cells, and human IL-3. Similar results were obtained using freshly-isolated CD10+ sIg- cells (not shown), indicating that the effect is not associated with preculture on stromal cells. The observed proliferation was specific for CD40, as it was not observed with an isotype-related control MAb (Fig. 4). However, anti CD40 signaling in the absence of IL-3 was largely ineffective in inducing proliferation, either through soluble MAb, or upon presentation in the context of CDw32 L cells (Fig. 4). These data are in accordance with the description that soluble or cross-linked anti CD40 MAbs do not induce BCP proliferation (Law et al. 1990).

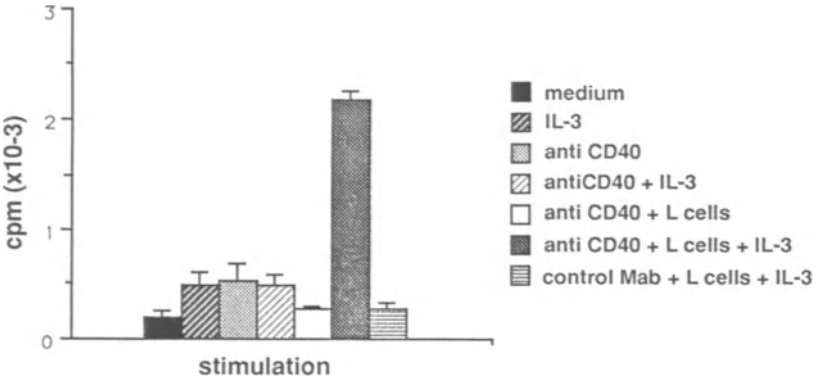


Fig. 4. CD40 dependent proliferation in the presence of IL-3.

BCP recovered from culture of CD10+ sIg- cells on stroma were seeded either in CD40 system (anti CD40 + L cells) (see Materials and Methods), or in control conditions, in the presence or absence of IL-3 (10ng/ml). Control MAb is of same isotype as anti CD40 (IgG1). 3H-thymidine incorporation ±SD in triplicate wells (10E4 cells), at day 8 of culture

Among a panel of cytokines tested, only the addition of IL-3 resulted in proliferation in the CD40 system (Table 4), indicating that IL-3 plays a particular role in CD40 dependent BCP activation.
IL-4 was ineffective as a cofactor for BCP proliferation (Table 4), in contrast with the co-stimulatory role of this cytokine on mature B cells (Gordon et al. 1988).These data confirm that our present description of CD40 dependent proliferation represents an effect on BCP and not on mature cells.

Table 4. IL-3 acts as a specific cofactor for CD40 dependent BCP proliferation

Culture conditions	cpm
Medium	417 ± 68
IL-3	3143 ± 759
IL-1α	362 ± 39
IL-2	500 ± 127
IL-4	344 ± 53
IL-6	358 ± 103
IL-7	873 ± 73
IL-10	652 ± 137
IFNγ	480 ± 35
TNFα	259 ± 56
TGFß	385 ± 82

BCP recovered from culture of CD10+ sIg- cells on stroma were seeded in CD40 system, as described in Materials and Methods. Data represent 3H-Thd incorporation ± SD in triplicate wells (10E4 cells), after 8 days of culture. Cytokine concentrations as in Table 1, except IL-1α: 20 U/ml, IL-2: 20U/ml, IFNγ: 500 U/ml.

Finally, we observed that BCP cultured following CD40 stimulation only included a low proportion of sIgM+ cells, and we did not detect cells bearing other isotypes (not shown).
Taken together, our results identify a role for CD40 in B cell ontogeny. Further studies will be required to characterize the target BCP for CD40 dependent proliferation, and to address the mechanisms underlying this effect.

Concluding remarks

We have described different culture systems for investigating the regulatory mechanisms in human B lymphopoiesis.
Of particular interest, BCP were maintained several weeks in culture in the presence of stromal cells, providing a model to address the role of stromal cell-derived molecules in BCP proliferation. In addition, a practical consequence of our results is the possibility to generate relatively large numbers of BCP for characterization studies.
Our present data confirm and extend recent observations of Wolf et al (Wolf et al. 1991). In particular, it is clear that, as in the mouse, IL-7 plays a critical role in the proliferation of human BCP. Another similarity with the murine system is the requirement for additional signal(s) for optimal proliferation. Further work will be required to dissect the factors acting at the different stages in human B lymphopoiesis.
Our observations that the CD40 molecule signals BCP proliferation in the presence of IL-3 raises the question of the physiological relevance of this effect. In this context, both IL-3 and a recently identified CD40 ligand (Armitage et al. 1992) are produced by activated T cells, suggesting the possibility that CD40 could be involved in T cell-dependent regulation of B lymphopoiesis.
Finally, the culture systems presented herein did not permit the generation of sIgM+ sIgD+ B cells from BCP. Consequently, investigation of the factors required for the transition step to mature B cells represents an important priority.

Acknowledgments

We would like to thank I. Durand and V. Bouchut for expert flow cytometry analysis. We are grateful to the groups of Pr. J.L. Touraine, Dr. R. Bouvier, and Dr. Cordier-Alex, for samples of fetal bone marrow. Finally, we would like to acknowledge the assistance of M. Vatan in preparing this manuscript.

References

Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, Anderson DM, Gimpel SD, Davis-Smith T, Maliszewski CR, Clark EA, Smith CA, Grabstein KH, Cosman D, Spriggs MK (1992) Molecular and biological characterization of a murine ligand for CD40. Nature 357: 80-82

Ashman LK, Cambareri AC, Bik To L, Levinsky RJ, Juttner CA (1991) Expression of the YB5.B8 antigen (c-kit protooncogene product) in normal human bone marrow. Blood 78: 30-37

Banchereau J, de Paoli P, Vallé A, Garcia E, Rousset F (1991) Long term human B cell lines dependent on interleukin 4 and anti-CD40. Science 251: 70-72

Era T, Ogawa M, Nishikawa SI, Okamoto M, Honjo T, Akagi K, Miyazaki JI, Yamamura KI (1991) Differentiation of growth signal requirement of B lymphocyte precursor is directed by expression of immunoglobulin. EMBO J. 10: 337-342

Gordon J, Millsum MJ, Guy GR, Ledbetter JA (1988) Resting B lymphocytes can be triggered directly through the CDw40 (Bp50) antigen. A comparison with IL-4 mediated signaling. J. Immunol. 140: 1425-1430

Kansas GS, Dailey MO (1989) Expression of adhesion structures during B cell development in man. J. Immunol. 142: 3058-3062

Law CL, Wörmann B, LeBien TW (1990) Analysis of expression and function of CD40 on normal and leukemic human B cell precursors. Leukemia 4: 732-738

LeBien TW (1989) Growing human B-cell precursors in vitro: the continuing challenge. Immunol. Today 10: 296-298

Lee G, Namen AE, Gillis S, Ellingsworth LR, Kincade PW (1989) Normal B cell precursors responsive to recombinant murine IL-7 and inhibition of IL-7 activity by transforming growth factor-ß. J. Immunol. 142: 3875-3883

McGinnes K, Quesniaux V, Hitzler J, Paige C (1991) Human B-lymphopoiesis is supported by bone marrow-derived stromal cells. Exp. Hematol. 19: 294-303

McNiece IK, Langley KE, Zsebo KM (1991) The role of recombinant stem cell factor in early B cell development. Synergistic interaction with IL-7. J. Immunol. 146: 3785-3790

Namen AE, Lupton S, Hjerrild K, Wignall J, Mochizuki DY, Schmierer A, Mosley B, March CJ, Urdal D, Gillis S, Cosman D, Goodwin RG (1988) Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 333: 571-573

Rennick D, Yang G, Muller-Sieburg C, Smith C, Arai N, Takabe Y, Gemmell L (1987) Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. Proc. Natl. Acad. Sci. USA 84: 6889-6893

Reth M, Petrac E, Wiese P, Lobel L, Alt FW (1987) Activation of Vk gene rearrangement in pre-B cells follows the expression of membrane-bound immunoglobulin heavy chains. EMBO J. 6: 3299-3305

Rolink A, Melchers F (1991) Molecular and cellular origins of B lymphocyte diversity. Cell 66: 1081-1094

Rolink A, Streb M, Nishikawa SI, Melchers F (1991) The c-kit-encoded tyrosine kinase regulates the proliferation of early pre-B cells. Eur. J. Immunol. 21: 2609-2612

Ryan DH, Nuccie BL, Abboud CN, Liesveld JL (1990) Maturation-dependent adhesion of human B cell precursors to the bone marrow microenvironment. J. Immunol. 145: 477-484

Ryan DH, Nuccie BL, Abboud CN, Winslow JM (1991) Vascular cell adhesion molecule-1 and the integrin VLA-4 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells. J. Clin. Invest. 88: 995-1004

Saeland S, Duvert V, Caux C, Pandrau D, Favre C, Vallé A, Durand I, Charbord P, de Vries JE, Banchereau J (1992) Distribution of surface-membrane molecules on bone marrow and cord blood CD34+ hematopoietic cells. Exp. Hematol. 20: 24-33

Saeland S, Duvert V, Pandrau D, Caux C, Durand I, Wrighton N, Wideman J, Lee F, Banchereau J (1991) Interleukin-7 induces the proliferation of normal human B-cell precursors. Blood 78: 2229-2238

Suda T, Okada S, Suda J, Miura Y, Ito M, Sudo T, Hayashi SI, Nishikawa SI, Nakauchi H (1989) A stimulatory effect of recombinant murine interleukin-7 (IL-7) on B-cell colony formation and an inhibitory effect of IL-1α. Blood 74: 1936-1941

Uckun FM, Dibirdik I, Smith R, Tuel-Ahlgren L, Chandan-Langlie M, Schieven GL, Waddick KG, Hanson M, Ledbetter JA (1991) Interleukin-7 receptor ligation stimulates tyrosine phosphorylation, inositol phospholipid turnover, and clonal proliferation of human B-cell precursors. Proc. Natl. Acad. Sci. USA 88: 3589-3593

Vallé A, Zuber CE, Defrance T, Djossou O, De Rie M, Banchereau J (1989) Activation of human B lymphocytes through CD40 and interleukin 4. Eur. J. Immunol. 19: 1463-1467

Whitlock CA, Witte ON (1982) Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci. USA 79: 3608-3612

Wolf ML, Buckley JA, Goldfarb A, Law CL, LeBien TW (1991) Development of a bone marrow culture for maintenance and growth of normal human B cell precursors. J. Immunol. 147: 3324-3330

Wörmann B, Gesner TG, Mufson RA, LeBien TW (1989) Proliferative effect of interleukin-3 on normal and leukemic human B cell precursors. Leukemia 3: 399-404

Modulation of Signal Transduction in Phosphocholine-Specific B Cells from μκ Transgenic Mice

J. J. KENNY[1], D. G. SIECKMANN[2], C. FRETER[3], R. HODES[4], K. HATHCOCK[4] and D. L. LONGO[5]

[1]PRI/DynCorp, Frederick, MD 21702;
[2]Naval Medical Research Inst., Bethesda, MD 20889;
[3]Lombardi Cancer Center, Georgetown University, Washington, D.C. 20057,
[4]Experimental Immunology Branch, NCI-NIH, Bethesda, MD 20205;
[5]Biological Response Modifiers Program, NCI-FCRDC, Frederick, MD 21702.

INTRODUCTION

The bone marrow of an adult mouse produces approximately 16 million new B cells each day (1). The vast majority of these cells appear to be rapidly turned over since 2/3 of the peripheral B cell pool is comprised of long-lived $\mu^+\delta^+$ B cells (2). Recent studies indicate that newly generated B cells are selected into this long-lived pool via an Ig V region-mediated process that appears to involve internal autoantigens or external environmental antigens (3-7); and that this receptor-driven selection process is influenced by both the anatomical and physiological environment of the B cell (5,7) as well as the genetic make up of the host (8). Thus, we have recently demonstrated that phosphocholine-(PC)-specific B cells are positively selected into the peripheral lymphoid tissues of M167 μ-H-chain transgenic mice that express a normal X-chromosome but they are clonally deleted in both M167 μ and μκ transgenic mice which coexpress these M167 transgenes with the X-linked immune deficiency gene, xid (6,8). Idiotype and antigen binding analysis of antibodies generated via transfection of variant V_H1 genes in conjunction with the κ8, κ22, and κ24 light chain genes suggest that both the positive and negative selection of idiotype-positive, PC-binding B cells are antigen-mediated and not idiotype-mediated processes (6)(Kenny,J.J. et al., submitted). In as much as >97% of the peripheral B cells in M167 μκ transgenic mice express the transgene-encoded IgM product as an antigen-specific receptor on their surface (9), it was of interest to determine whether or not these antigen-selected B cells had been altered during their positive selection process. The data presented in this paper suggest that the initial encounter with antigen has resulted in a selective form of tolerance in that extensive cross-linking of the IgM receptors with soluble anti-Ig leads to the death of these B cells.

RESULTS AND DISCUSSION

Thymus Dependent Immune Responses Appear to be Normal in M167 Transgenic Mice

To analyze the in vivo and in vitro in immune responses of B cells from M167 μκ transgenic mice to PC, mice were either immunized i.p. with PC-KLH in CFA and their spleen cells assayed 5 days later for PC-specific PFC, or unprimed splenic B cells were set up in vitro with PC-KLH and a KLH-specific T helper cell line. The data in table 1 demonstrate that large numbers of M167-id$^+$ PFC were generated in the spleens of transgene positive (TG$^+$) mice while the TG$^-$ littermates produced the expected T15-id dominant response. In as much as TG$^+$ mice have more than 10 x 10^6 PC-specific B cells in their spleens prior to immunization (6,9), it is somewhat surprising that the TG$^+$ mice produce less than 10^6 PC-specific PFC/spleen following immunization with

Current Topics in Microbiology and Immunology, Vol. 182

PC-KLH. This could indicate that: 1) very few of these B cells are capable of developing into antibody secreting cells; or 2) that T cell help and/or other physiological or anatomical requirements may be limiting in situ. Pinkert et al. (10) have shown that only 1 in every 10^3 B cells from these M167 $\mu\kappa$ transgenic mice is capable of responding to PC in the splenic fragment assay, where presumably every B cell should be provided with maximum T cell help. However, as shown in Fig. 1 and in reference (11), the B cells from these mice make excellent antibody responses when placed in culture with PC-KLH and KLH-specific T helper cells. PC-specific antibody responses of 2.5 μg/ml were produced with as few as 3 x 10^3 B cells per well. Due to the low number of B cells used, no anti-PC antibody was produced from TG^- B cells in assays performed in 96 well plates (data not shown).

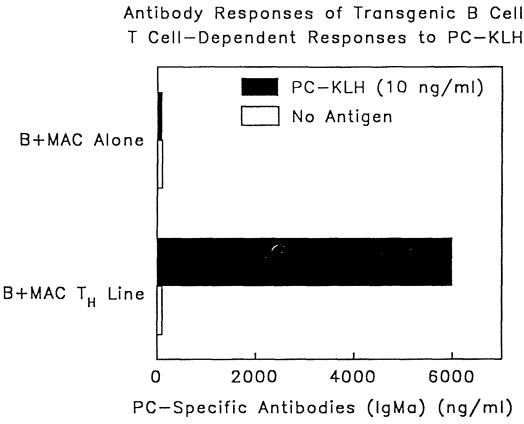
The data in Table 1 and Fig. 1 indicate that the B cells from M167 $\mu\kappa$ TG^+ mice are capable of responding normally in vivo and in vitro when provided with cognate T cell help. However, when spleen cells from TG^+ and TG^- mice were cultured with optimally stimulatory concentrations of soluble anti-μ, or Sepharose-conjugated anti-μ, anti-μ^a, anti-μ^b, anti-id antibodies, or PC and analyzed for proliferation by ^{3}H-TdR uptake, spleen cells from TG^+ mice were unresponsive to soluble goat anti-μ while the normal TG^- cultures were stimulated ~30-fold over the medium control. In contrast, the TG^+ spleen cells gave a 15-fold higher response than the medium control after stimulation with the same preparation of goat anti-μ conjugated onto Sepharose beads. TG^+ cultures also responded to Sepharose conjugated anti-id, anti-μ^a-allotype, and PC and to LPS (Table 2). Overall, these results suggest that the transgene-encoded sIgM receptor is capable of transducing mitogenic signals when stimulated by Sepharose conjugated anti-Ig, but not soluble anti-Ig.

The unresponsiveness of T^+ B cells to soluble anti-Ig could be due to: 1) FcR-mediated inhibition; 2) T-cell suppression; 3) developmental arrest of these B cells; 4) anti-μ induced receptor modulation; or 5) receptor-induced cell death. It has been demonstrated that anti-μ-induced activation can be inhibited by the Fc portion of the antibody molecule acting through the B cell FcR (12). To investigate FcR-mediated inhibition as a possible cause of the unresponsiveness in TG^+ B cells, the spleen cells from TG^+ and TG^- mice were: 1) cultured with soluble goat anti-μ in the presence of purified

Table 1. Primary in vivo immune response of M167 $\mu\kappa$ transgenic mice to phosphocholine[a]

Mouse Phenotype	Plaque Forming Cells Per Spleen			
	Total IgM	% V_H1-id	% T15-id	% M167-id
Transgene Positive	260,008	99	0	100
Transgene Negative	92,537	100	95	0

a) Mice were immunized I.P. with 100 ug of PC-KLH in CFA. Direct PFC were assayed on PC-SRBC 5 days after immunization.



Microtiter wells were set up in triplicate with 10^4 anti-Thy1 + C' treated M167 $\mu\kappa$ spleen cells (B + MAC) with or without 10^4 B10 KLH-specific T_H-cells. Media was changed at day 4 and supernatants assayed on PC-BSA-coated microtiter plates (9) at day 10.

Table 2. In vitro activation of spleen cells from M167 $\mu\kappa$ transgenic mice

	Transgene +	Transgene -
	CPM/Culture	
Media	4,372	5,281
Anti-μ	4,453	155,331
Anti-μ-Seph.	62,078	153,550
Anti-V_H-id-Seph.	221,022	6,882
Anti-IgM^a-Seph.	36,120	5,868
Anti-IgM^b-Seph.	2,970	79,641
PC-Seph.	184,871	4,987
MOPC-21-Seph.	3,081	5,406
Rat-IgG-Seph.	8,055	5,441
LPS	51,409	73,242

a) Spleen cells (3×10^5) from individual T^+ and T^- 207-4 mice were cultured in the presence of soluble goat anti-μ (100 μg/ml), goat anti-μ-Sepharose (1:150), anti-μ^a-Sepharose (DS1-Sepharose, 1:100), anti-μ^b-Sepharose (AF6-78.25-Sepharose, (1:100) for 48 hr. prior to pulsing with ^{3}H-thymidine for 16 hrs.
b) Data represent the geometric mean of triplicate cultures. The standard error for all groups exhibiting proliferation above that of the medium control was less that 5 % of the mean.

monoclonal 2.4G2 anti-FcR antibody (13); and, 2) stimulated with goat $F(ab')_2$ anti-μ. The data in Table 3 demonstrate that anti-FcR antibody had no effect on the anti-μ response of the TG^+ spleen cells. Treatment of TG^+ and TG^- spleen cells with $F(ab')_2$ anti-μ resulted in a 3-fold increase in proliferation of the TG^+ spleen cells, while the proliferation of C57BL/6 and TG^- spleen cells was enhanced an average of 1.6-fold over the responses obtained with an equal molar concentration of intact anti-μ-antibody (Table 4). The low response obtained with the $F(ab')_2$ anti-μ was still 3 times lower than the response of the same cells to anti-μ-conjugated Sepharose and 10 times lower than the response to anti-V_H1-id-conjugated beads. From these experiments, it is evident that FcR mediated inhibition is not the primary reason for the lack of anti-μ induced responses in TG^+ B cells.

The removal of T cells by treatment with anti-Thy 1.2 + C′ also had no effect on the ability of the TG^+ B cells to respond to anti-μ, and the coculture of TG^+ and TG^- spleen cells in the presence of anti-μ did not suppress the TG^- spleen cell response (data not shown). Since the anti-Thy + C′ treatment completely eliminated the proliferative response to Con A, the presence of an active anti-μ-specific or non-specific suppressor T cell or suppressor factors in the TG^+ spleen can be dismissed.

Anti-μ Induced Killing of B Cells from 207-4 Transgenic Mice.

We have previously shown that the B cells in M167 $\mu\kappa$ transgenic mice express high levels of sIgM and do not express sIgD even though ~20% of these B cells coexpress endogenous sIgM (9). In as much as this cell surface phenotype is similar to that of immature B cells that have recently emerged from the bone marrow (14), the restricted anti-μ stimulation defect observed in the B cells from 207-4 transgenic mice could be: 1) due to arrest of these B cells at a stage of development which is easily tolerizable, i.e. $sIgM^+IgD^-$; 2) the

Table 3. Anti-μ stimulation of Transgene Positive and Transgene Negative Spleen Cells in the Presence of Anti-Fc Receptor Antibody[a]

Mitogens	Anti-FcR	C57BL/6	Transgene Positive	Transgene Negative
			CPM/Culture	
Medium	-	4,456	424	1,352
	+	7,750	327	3,659
Goat anti-μ	-	86,203	861	20,066
	+	86,464	795	67,444
Goat anti-μ-Seph.	-	129,239	15,567	63,649
LPS	-	76,393	24,320	61,309

a) Spleen cells (3×10^5) of individual T^+ and T^- mice were cultured in triplicate wells in the presence of soluble goat anti-μ (100 μg/ml) and 2.4G2 monoclonal anti-FcR antibody, or Sepharose conjugated goat anti-μ (1:200) or LPS (50 μg/ml) for 48 hr. prior to pulsing with ^{3}H-thymidine for 16 hrs. Result are represented as a geometric mean of the CPM/culture, and standard errors of the mean were less than 5 % in activated cultures.

Table 4. $F(ab')_2$ Anti-μ Stimulation of Transgene Positive and Transgene Negative Spleen Cells[a]

Stimulating Agent	Transgene Positive	Transgene Negative
	CPM/Culture[b]	
Media	3,599	5,356
LPS	43,750	88,404
Goat anti-μ-Seph.	39,475	77,671
Goat anti-μ 100 μg/ml	6,100	69,157
$F(ab')_2$ anti-μ 61 μg/ml	12,354	207,095

a,b) Spleen cells were cultured and data analyzed as described in table 2.

consequence of a previous encounter with autologous or environmental PC which has resulted in a selective inactivation of certain biochemical activation pathways; or, 3) an activation defect common to all μκ transgenic mice, which results from transgene-induced alteration in B cell development. This latter possibility was addressed by anti-μ stimulation of spleen cells from the μκ anti-TNP Sp6 transgenic mouse line (15). The data in Table 5 show that both soluble anti-μ and anti-μ Sepharose beads induced significant proliferation in both TG^+ and TG^- B cell populations; thus, the defect in B cells from M167 μκ transgenic mice is not simply the result of μκ transgene expression but may be related to the changes induced in these B cells following antigen-induced selection or to an arrest in their development. It is known that the TG^+ TNP-specific B cells that coexpress endogenous sIgM also express sIgD (16). This suggests that the B cells in the TNP-transgenic mice have fully matured, whereas, those in the PC-transgenic mice may be immature.

It has been shown that anti-μ treatment of neonatal B cells, which express predominantly sIgM-only, results in either down modulation and failure to reexpress sIgM receptors (17) or the induction of apoptosis (Chang, T.L., submitted). On the other hand, mature sIgM:sIgD positive B cells will reexpress their receptors within 18 hr of anti-Ig stripping (17) and will then proliferate. It was therefore possible that soluble anti-μ caused either receptor down modulation on the IgM-only TG^+ B cells and thus, no response would be seen because multiple rounds of anti-μ signaling are required to get effective induction of proliferation (18). Alternatively, the anti-μ stimulation could result in the death of these cells. To test both of these posibilities, TG^+ and TG^- spleen cells were incubated with soluble goat anti-μ antibody or control goat IgG for one hr at 37°C to allow binding and capping of the sIgM on the B cells. The cell suspensions were then washed and a portion of the cells stained with biotin conjugated anti-B220 antibody plus either FITC conjugated goat anti-μ, anti-μ^a, or rabbit anti-goat-IgG. Only low levels of goat antibody remained after the stripping and wash procedure (not shown). The remainder of the cells were incubated overnight in RPMI-1640 + 10% FCS to allow regeneration of the membrane IgM and were then stained for B220, IgM and IgM^a-allotype. The results of one representative experiment are shown in Table 6. The TG^+ and TG^- spleen cell populations initially had 13.6 and 36.5% B cells respectively. After 1 hr incubation with soluble anti-μ, staining of sIgM was reduced to less than 1%; thus, sIgM was efficiently capped and removed from both cell types. In both the TG^+ and TG^- populations, the loss of $B220^+IgM^+$ cells was balanced by an increase in the number of $B220^+IgM^-$cells. Twenty four hours after treatment with goat anti-μ, 48.3% of the TG^- spleen cells reexpressed sIgM as compared with 52.5% in the control. In marked contrast, only 6.4% of the TG^+ cells reexpressed their surface IgM and very few $B220^+IgM^-$ cells remain in these cultures. These results indicate that approximately 2/3 of the TG^+ B cells actually die within the first 24 hr following anti-μ stimulation. The B

Table 5. Anti-μ Induced Activation of Spleen Cells from Sp6 μκ Anti-TNP Transgenic Mice[a]

Stimulating Agent	Transgene Positive Mouse 1	Transgene Positive Mouse 2	Transgene Negative Mouse1	Transgene Negative Mouse 2
	CPM/Culture			
Media	2,490	1,783	3,680	5,321
Anti-μ	42,922	51,591	63,561	81,565
Anti-μ-Seph.	81,536	82,656	139,872	119,206
LPS	61,555	54,780	94,014	97,602

a) Spleen cells from two TG^+ and TG^- mice were cultured and data calculated as described in table 2.

cells that remain do not appear to down modulate their receptors. These findings suggest that the failure of TG^+ B cells to proliferate following anti-μ stimulation is due to preferential killing of these sIgM-only B cells.

Early Signal Transduction Events in Transgenic B Cells Appear to be Normal

The B cells from these M167 $\mu\kappa$ anti-PC transgenic mice present a unique opportunity for elucidating the difference(s) in the biochemical pathway(s) that lead either to cell proliferation or cell death following signal transduction through the same Ig-receptor. High concentrations of soluble anti-μ have been shown to induce both intracellular and extracellular calcium transport and increased phosphotidylinositol (PI) turnover within minutes of sIgM receptor cross-linking (19). Thus, we analyzed PI turnover in the B cells from M167 $\mu\kappa$ transgenic mice to determine whether or not this early activation event was altered in these cells. The data in Table 7 show that there was no difference in PI turnover in TG^+ vs TG^- B cells following activation with anti-μ, LPS or aluminum fluoride. Preliminary analysis of Ca-flux in these cells also indicates that this activation step is unaltered, although initial unstimulated Ca levels may be higher in the B cells from TG^+ mice (J. Mond, unpublished data). However, Hornbeck et al. (manuscript in preparation) have demonstrated that phosphomyristin C levels in unstimulated TG^+ B cells are elevated 5 fold over those of TG^- B cells. Since phosphomyristin C is a primary substrate for protein kinase C and it

Table 6. Goat anti-μ treatment of spleen cells from transgene positive mice induces B-cell death

		Percent of Total Cells[b]				
	Treatment[a]	Transgene Positive			Transgene Negative	
		μ^- $B220^+$	μ^+ $B220^+$	μ^{a+} $B220^+$	μ^- $B220^+$	μ^+ $B220^+$
Day 1	Goat IgG	0.7	13.6	15.1	0.3	36.5
	Goat anti-μ	13.8	0.3	0.2	32.5	0.4
Day 2	Goat IgG	0.4	18.3	16.5	1.8	52.5
	Goat anti-μ	1.3	6.4	6.3	3.1	48.3

a) Spleen cells (1×10^7/ml) from T^+ and T^- 207-4 mice were cultured in the presence of soluble goat anti-μ (100 μg/ml) for 1 hr. at 37°C, washed 3 times and cultured overnight at 37°C.
b) Spleen cells (1×10^6) were stained before and after anti-μ treatment with FITC-conjugated anti-μ and biotin-conjugated anti-B220 plus PE-Streptavidin and analyzed as described in the methods section.

Table 7. Anti-μ induced activation of the phosphatidylinositol cycle in B cells from M167 $\mu\kappa$ transgenic mice[a]

	Ratio: PI/Total Myoinositol $\times 10^{-2}$	
Inducing Agent	Transgene Positive	Transgene Negative
None	5.0	5.7
Anti-μ	8.0	8.4
LPS	6.0	6.3
AlF	10.8	11.1

a) Anti-Thy + C' treated spleen cells from M167 $\mu\kappa$ TG^+ and TG^- littermates were cultured for 4 hours in inositol-free RPMI supplemented with 200-400 μCi of ^{3}H-myo-inositol (58.5 Ci/mMole). Cells were washed, stimulated with soluble anti-μ for 30 min. and PI turnover measured as previously described (20).

is induced following cross-linking of sIgM, this observation may indicate that a biochemical change has indeed been induced in these cells following their initial in vivo selective encounter with antigen.

The data presented above indicate that the initial biochemical pathways that ultimately lead to either the induction of proliferation or cell death following high dose anti-μ cross-linking of sIgM receptors appear to be shared. However, it is not yet clear whether the cell death pathway has been programmed into the PC-specific B cells as a result of their positive selection by antigen or that it results from a developmental arrest of these cells as $sIgM^+$:$sIgD^-$ cells which are extremely susceptible to tolerance induction following excessive cross-linking of their IgM receptors (17). It will be necessary to analyze several strains of sIgM-only transgenic mice before we can rule out developmental arrest as a possible explanation.

When B cells are stimulated with soluble anti-μ, most sIgM receptors are engaged and modulated from the surface membrane, whereas, anti-μ on a Sepharose bead may engage only a limited number of receptors. It is therefore possible that the extent of receptor cross-linking is responsible for the difference in signaling. Thus, limited receptor cross-linking leads to proliferation of these cells while extensive cross-linking leads to cell death purhaps by apoptosis. Studies are in progress using different conjugation ratios of PC-dextran to elucidate the cross-linking parameters that differentiate between induction of death and proliferation. Brunswick et al. (21) have shown that very low concentrations of dextran-conjugated anti-μ or anti-δ can lead to B cell proliferation in the absence of Ca-flux or PI turnover, while higher doses of this polyclonal activator induce Ca-flux, PI turnover and proliferation. Thus, there appears to be at least 3 separate biochemical pathways that can be activated following cross-linking of the sIgM receptor. Elucidating these pathways and distinguishing what determines which pathway will be utilized should lead to new insights on B cell development and regulation.

Acknowledgements

The authors are grateful to Drs. Ursula Storb, David Lo and Ralph Brinster for providing the original M167 transgenic mice used to establish our breeding colony, and to Dr. Matthew Scharff for providing us with the anti-T15 and anti-V_H1 hybridomas. The excellent technical assistance of Ms. Gretchen Guelde and Ms. Erin Martin also gratefully acknowledged. We appreciate the able assistance of Ms. Laura Martinez in the preparation of the manuscript. Research sponsored, at least in part, by the National Cancer Institute, DHHS, under contract NO1-CO-74102 with Program Resources, Inc. This work was also supported in part by the Naval Medical Research and Development Command Research and Technology Work Unit # 3M161102BS12.AA.112. Sponsored in part by the Office of Naval Research, contract N00014-89-C-0305. The contents of this publication are the private views of the authors and do not necessarily reflect the views or policies of the DHHS or the Department of Defense, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, National Research Council, DHHS Publication (NIH) 86-23, (1985).

References

1. Osmond, D.G. 1986. Population dynamics of bone marrow B lymphocytes. Immunol. Rev. 93:103.
2. Forster, I. and K. Rajewsky. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. Proc. Natl. Acad. Sci. USA 87:4781.
3. Freitas, A., M-P. Lembezat, and A. Coutinho. 1989. Expression of antibody V-regions is genetically and developmentally controlled and modulated by the B lymphocyte environment. Int. Immunol 1:342.
4. Gu, H., D. Tarlinton, W. Miller, K. Rajewsky, and I. Forster. 1991. Most Peripheral cells in mice are ligand selected. J. Exp. Med. 173:1357.
5. Freitas, A.A., A-C. Viale, A. Sundblad, C. Huesser, and A. Coutinho. 1991. Normal serum immunoglobulins participate in the selection of peripheral B cell repertoires. Proc. Natl. Acad. Sci. USA. 88:5640.
6. Kenny, J.J., C. O'Connell, D. G. Sieckmann, R. T. Fischer, and D. L. Longo. 1991. Selection of antigen-specific, idiotype positive B cells in transgenic mice expressing a rearranged M167-μ heavy chain gene. J. Exp. Med. 174:1189.
7. Andrade, L., F. Huetz, P. Poncet, V. Thomas-Vaslin, M. Goodhardt, and A. Coutinho. 1991. Biased V_H gene expression in murine CD5 B cells results from age-dependent cellular selection. Eur. J. Immunol. 21:2017.
8. Kenny, J.J., A. M. Stall, D. G. Sieckmann, M. C. Lamers, F. Finkleman, L. Finch, and D. L. Longo. 1991. Receptor-mediated elimination of phosphocholine-specific B cells in X-linked immune deficient mice. J. Immunol. 146:2568.
9. Kenny, J.J., F. Finkleman, F. Macchiarini, W. C. Kopp, U. Storb, and D. L. Longo. 1989. Alteration of the B cell surface phenotype, immune response to phosphocoline and the B cell repertoire in M167 μ plus κ transgenic mice. J. Immunol. 138:1363.
10. Pinkert, C.A., J. Manz, P-J. Linton, N. R. Klinman, and U. Storb. 1989. Elevated PC responsive B cells and anti-PC antibody production in transgenic mice harboring anti-PC immunoglobulin genes. Veter. Immunol. Immunopath. 23:321.
11. O'Brien, R.L., P. Marrack, U. Storb, and J. W. Kappler. 1988. B cells expressing Ig transgenes respond to a T-dependent antigen only in the presence of Ia-compatible T cells. J. Immunol 141:3335.
12. Scribner, D. J., H. L. Weiner, and J. W. Morehead. 1978. Anti-immunoglobulin stimulation of murine lymphocytes: V. Age-related decline in Fc receptor-mediated immunoregulation. J. Immunol. 121:377.
13. Unkeless, J.C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc-receptors. J. Exp. Med. 150:580.
14. McNagy, K.M., P-A. Cazenave, and M. D. Cooper. 1988. BP-3 alloantigen: A cell surface glycoprotein that marks early B lineage cells and mature myeloid lineage cells in mice. J. Immunol. 141:2551.
15. Rusconi, S. and G. Kohler. 1985. Transmission and expression of a specific pair of rearranged immunoglobulin μ and κ genes in a transgenic mouse line. Nature 314:330.
16. Lamers, M.C., M. Vakil, J. F. Kearney, J. Langhorne, C. J. Paige, M. H. Julius, H. Mossmann, R. Carsetti, and G. Kohler. 1989. Immune status of a μ,κ transgenic mouse line. Deficient response to bacterially related antigens. Eur. J. Immunol. 19:459.
17. Sidman, C.L. and E. R. Unanue. 1975. Receptor-mediated inactivation of early B lymphocytes. Nature 257:149.
18. Sieckmann, D.G. 1980. The use of anti-immunoglobulin to induce a signal for cell division in B lymphocytes via their membrane IgM and IgD. Immunol. Rev. 52:181.

19. Cambier, J.C., L. B. Justement, M. K Newell, Z. Z. Chen, L. K. Haris, M. Sandoval, M. J. Klemsz, and J. T. Ramsom. 1987. Transmembrane signals and intracellular "second messengers" in the regulation of quiescent B-lymphocyte activation. Immunol. Rev. 95:37.
20. Freter, C.E., M.E. Lippman, A. Cheville, S. Ainn, and E.P. Gelmann. 1988. Alterations in phosphoinositide metabolism associated with 17β-estradiol and growth factor treatment of MCF-7 breast cancer cells. Mol. Endocrinol. 2:159.
21. Brunswick, M., C.H. June, F.D. Finkelman, H.M. Dentzis, J.K. Inman, and J.J. Mond. 1989. Surface immunoglobulin-mediated B-cell activation in the absence of detectable elevations in intracellular ionized calcium: a model for T-cell-independent B-cell activation. Proc. Natl. Acad. Sci. USA 86:6724.

Immunoglobulin Gene Rearrangement

Activities Involved in V(D)J Recombination

G. E. Taccioli, G. Rathbun, Y. Shinkai, E. M. Oltz, H. Cheng, G. Whitmore[1], T. Stamato[2], P. Jeggo[3] and F. W. Alt

The Howard Hughes Medical Institute, The Children's Hospital and
The Department of Genetics, Harvard University Medical School,
300 Longwood Ave. Boston, Massachusetts.
[1] Ontario Cancer Institute, Experimental Therapeutics, 500 Sherbourne St.,
Toronto, Ontario, Canada.
[2] The Lankenau Medical Res. Center, 100 Lancaster Ave., Wynnewood,
Pennsylvania.
[3] MRC Cell Mutation Unit, University of Sussex, Falmer, Brighton, UK.

Introduction.

The assembly of immunoglobulin (Ig) and T cell receptor (TCR) variable region gene segments (V,D,J) is a complex reaction that likely involves numerous components. In a simplified view, the recombination mechanism involves recognition of conserved heptamer-spacer-nonamer recombination sequences (RS) that flank each germline V, D, or J segment, introduction of double stranded breaks between the elements to be joined and the flanking RS elements, potential loss and/or addition of nucleotides at the coding junctions, polymerization and ligation activities to complete the joining process (Fig.1; Alt and Baltimore 1982; for review see Blackwell and Alt 1989; Lieber 1991). The RS sequences that flank V, D, and J segments are sufficient to target the site-specific activities of the V(D)J recombination system to the adjacent "coding" sequences (Akira et al. 1987; Hesse et al. 1989). A notable aspect of this recombination reaction is the asymmetric processing of the coding and RS joins; the latter rarely involve nucleotide deletion and/or addition (Lieber 1991). The relative orientation of the sequences in the chromosome determines the fate of the reaction products. If the two coding gene sequences are in "opposite" transcriptional orientation the reaction will lead to inversion of the segment of DNA between the coding and RS joins with retention of all products in the chromosome. If the two sequences are in the same transcriptional orientation, the coding joins will be retained in the chromosome while the RS joins will be deleted as a circle (Okasaki and Sakano 1988; Toda et al. 1988; Fig.1). However, linear deletion products have also been observed to accumulate in thymus providing more direct evidence for the occurrence of double stranded breaks during this recombination process (Roth et al. 1992).

Two Recombination Activating Genes (RAG-1 and RAG-2) have been isolated that are completely sufficient to generate VDJ recombinase activity when expressed simultaneously in all examined cell types (Schatz et al. 1989; Oettinger et al. 1990). The RAG gene products have not been identified as actual components of the V(D)J recombinase, hence the somewhat ambiguous nomenclature. It is possible that one or both RAG gene products serve to regulate expression of the actual recombinase gene(s); alternatively, these products in some way may activate the actual V(D)J recombinase. Consistent with the distribution of V(D)J recombinase activity, the RAG-1 and RAG-2 genes were found to be co-expressed at substantial levels only in primary lymphoid tissues

Current Topics in Microbiology and Immunology, Vol. 182

and in cell lines that represent precursor lymphocytes (Shatz et al. 1989; Oettinger et al. 1990). However, expression of one or the other of these genes has been found in additional tissues, leading to speculation that one or both of the gene products might have activities in more mature lymphoid cells or in non-lymphoid cells (Chun et al. 1991; Carlson et al. 1991). The RAG genes are conserved in vertebrates but, to date, no close homologs have been found in lower organisms. The RAG-2 gene product has no clear homology to any other gene product (Oettinger et al. 1990). However, the RAG-1 protein does have limited homology to the yeast protein, HPR-1 which is apparently involved in recombination (Aguilera and Klein 1990).

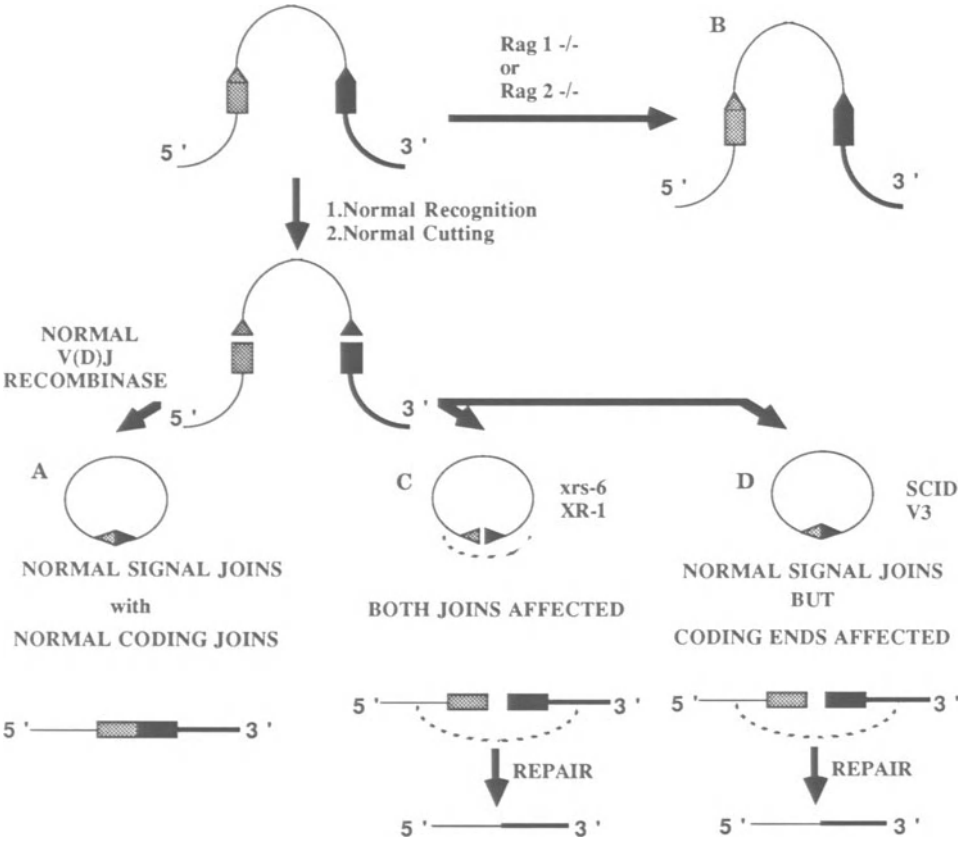


Fig.1. Model for V(D)J recombinase-mediated rearrangements.
RS elements are indicated by black and white triangles and coding sequences by boxes. (A) During normal V(D)J recombination double strans breaks are introduced between the RS and adjacent coding sequences, folowing by proccessing and ligation. (B) in RAG-deficient mice the recombination process is not initiated and coding sequences remain unrearranged (C) In xrs-6 and XR-1 double stranded breaks appear to bc properly introduced but normal joining of both RS and coding sequences does not occur. (D) In scid and in the V3 dsbr mutant only the joining of the coding sequences is significantly impaired.

Based on expression pattern, the RAG gene products (or activities they regulate) are likely the tissue-specific components of the V(D)J recombinase. In addition, this system employs at least one non-essential lymphoid specific activity, TdT, that qualitatively modifies V(D)J junctions via N region addition (Alt and Baltimore 1982). Finally, it seems likely that the specific components of V(D)J recombinase can recruit ubiquitously expressed cellular activities to perform certain aspects of the rearrangement process. One such activity may be encoded by the gene affected by the murine scid mutation. This defect was initially defined as an autosomal recessive mutation that resulted in a general (but often not complete) absence of mature B and T lymphocytes due to an impairment in one of the terminal steps of the V(D)J recombination reaction (Bosma et al. 1983; Shuler et al. 1986). The homozygous scid mutation also has additional manifestations in both lymphoid and non-lymphoid cells, most notably an increased sensitivity to ionizing radiation (e.g.. an impairment in the ability to repair double-stranded DNA breaks Fulop and Phillip 1990; Biedermann et al. 1991; Hendrickson et al. 1991). Thus, the scid mutation may affect a more generalized activity involved in double-strand break repair that is recruited by the V(D)J recombination system to perform one of its terminal steps.

Results and Discussion.

RAG Gene Expression is Required for Initiation of V(D)J Recombination.

To unequivocally evaluate the function of the RAG-2 gene product, we eliminated a major portion of the RAG-2 coding region in mice by ES cell gene-targeting technologies (Shinkai et al. 1992); others have targeted the RAG-1 gene (Mombaerts et al. 1992). Mice lacking either RAG-1 or RAG-2 gene function have identical and very specific phenotypes: both are viable but have a complete severe combined immune deficiency. These RAG deficient mice have no mature B or T cells in either primary or peripheral lymphoid tissues However, primary tissues from these animals accumulate lymphoid cells that appear to represent very early T and B cell progenitors. These accumulated pre-lymphocytes have no detectable rearrangements of either endogenous Ig or TCR loci.

In accord with the findings on primary lymphocyte differentiation organs, A-MuLV transformed pre-B cell lines generated from either fetal liver or adult bone marrow of RAG-1 or RAG-2 mutant mice have absolutely no rearrangements of any endogenous antigen receptor loci. This is in contrast to A-MuLV transformants derived from normal mice which invariably have at least DJ_H rearrangements (Shinkai et al. 1992; Mombaerts et al. 1992). The specificity of the RAG-2 defect has been demonstrated by transfection of RAG-2 expression vectors into RAG-2 deficient (RAG-2 -/-) A-MuLV transformants; such cells acquire ability to efficiently undergo rearrangements of both transfected V(D)J recombination substrates (Shinkai et al. 1992) and endogenous J_H loci (Fig.2) (Rathbun et al. in preparation). Thus, the endogenous IgH loci are fully competent for rearrangement in RAG-2 -/- pre-B cells but cannot undergo the process in the absence of the RAG-2 product (see below). Together, these findings indicate that in the absence of the RAG-1 or RAG-2 gene products, B and T cell development is blocked at a very early stage due to an inability to initiate V(D)J rearrangement.

Analyses of RAG-1 or RAG-2 deficient mice have, thus far, revealed no abnormalities in any tissue or developmental system outside of the immune system. The NK and myeloid compartments appear completely intact in these animals and there are no obvious neurological abnormalities. The lack of a detectable neurological phenotype in RAG-1 (or RAG-2) deficient mice would appear to eliminate any obvious function for these gene products in development of the central nervous system (Chun et al. 1992;

Matsuoka et al. 1991). However, the possibility of a more subtle function for RAG-1 (or RAG-2) in maintenance of neurological function (Chun et al. 1992) including a role in DNA repair (see below) requires more detailed and long term analyses of the mutant animals. Likewise, a potential role for RAG gene products in more mature stages of lymphocyte development remains possible and can be tested by complementation of the RAG defect with assembled Ig (or TCR) transgenes.

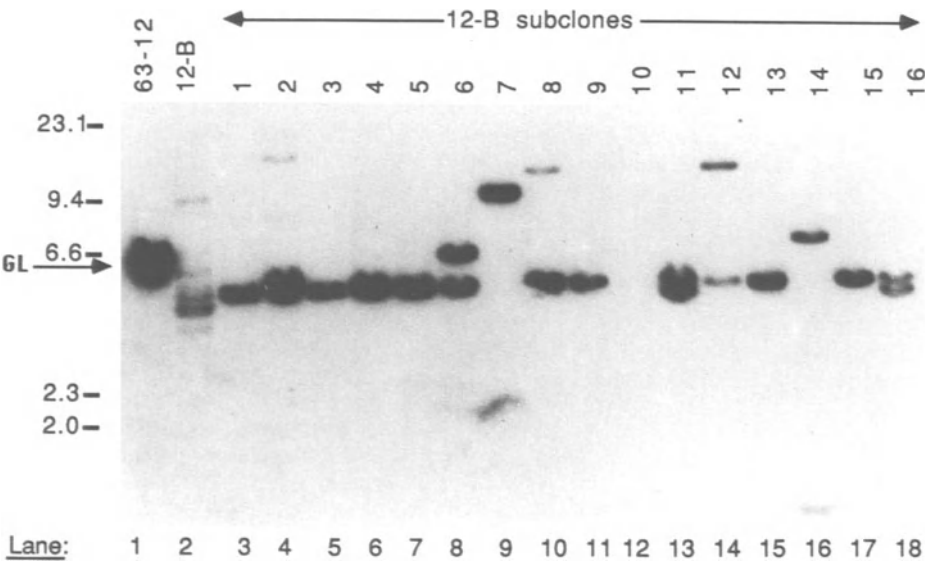


Fig.2. Onset of DJ_H rearrangements in reconstituted RAG-2 -/- A-MuLV transformed pre-B cells.
DNA from the parent cell line or from transfectants with RAG-2 expression vector was digested with EcoRI and assayed by Southern blot procedure for hybridization to a J_H 4 probe. Lane1: parent RAG-2-/- A-MuLV transformed pre-B cell line. Note germline (GL) configuration of the J_H loci. Lane 2: J_H rearrangement status (showing virtually no GL J_H loci) of p12-B, a 63-12 transfectant containing a stably integrated RAG-2 expression construct.. Lanes 3-18: various DJ_H rearrangements in subclones of p12-B.

Unlike the RAG mutant pre-B cells, scid pre-B cells undergo the initial steps of VDJ recombination relatively efficiently, including RS recognition and introduction of precise double stranded breaks (Malynn et al. 1988; Lieber et al. 1988; Blackwell et al. 1989: Hendrickson et al. 1990). The scid defect is manifested as an inability to form coding joins, however, RS joins are generated with relatively normal fidelity and efficiency (Fig.1). In contrast to the RAG-deficient mice, the severe combined immune deficiency of scid mice is "leaky". In other words, scid mice develop populations of peripheral mature lymphocytes in a time dependent fashion (Bosma and Carroll 1991). The leakiness of the scid mutation may, in some cases, be explained by somatic reversion events (Petrini et al. 1990). It is more likely, however, that much of this leakiness results from the rescue of the liberated coding joins in scid pre-lymphocytes by an illegitimate recombination mechanism that occasionally restores what appear to be normal coding joins (Ferrier et al. 1990; Hendrickson et al. 1990; Bosma et al. 1988). Because RAG-

deficient animals cannot even initiate the V(D)J recombination process, the Ig and TCR variable region loci are as recombinationally inert as any other genetic loci in these animals. Such a defect cannot be leaky without reversion of the mutation, a situation which can not occur in gene-targeted deletions.

Somatic Cell Genetic Approach To Define Additional Activities Involved in VDJ Recombination.

Mutations in yeast that have impact in recombination frequently affect the DNA repair process, particularly the double-strand break repair (dsbr) pathway (for review see Friedberg 1988). In this regard, V(D)J recombination and dsbr, in addition to involving double strand breaks, probably also utilize common enzymatic activities such as exonuclease, polymerase, and ligase. Thus, some of the ubiquitous factors recruited by V(D)J recombinase may also participate in dsbr. To test this notion, a large battery of existing mutant CHO cell lines with known defects in either excision repair (UV sensitivity) (for review see Hoeijmakers and Bootsma 1990) or dsbr (X-ray sensitivity) (review in Jeggo 1990) was assayed for ability to rearrange transiently introduced V(D)J recombination substrates when co-transfected with of constitutively expressing RAG

TABLE 1. Signal and Coding Joins formation in CHO cell lines

CELL LINE	SENS		RepAmpRCamR/AmpR x100		%correct joints
	UV	X-ray	pJH200(Sig)	pJH290(Cod)	
V3	-	S	**2.4**	***0.07***	**45**
AA8(wt)	-	-	4.0	*6.9*	99
Mock	-	-	0.05	*0.07*	NA
xrs-6	-	S	**0.10**	***0.11***	**14**
K1(wt)	-	-	2.3	*1.8*	99
Mock	-	-	<0.01	*ND*	NA
xrs-6/Ch2(D5)	-	P	1.4	*ND*	99
XR-1	-	S	**<0.1**	***<0.2***	**10**
4362A(wt)	-	-	1.0	*0.92*	99
Mock	-	-	<0.04	*ND*	NA
XR-1:Ch5	-	P	3.0	*ND*	99
BLM-1	-	S	5.0	*>4.0*	99
BLM-2	S	S	>3.0	*>3.0*	99
ADR-3	-	S	5.0	*10.0*	99
K1(wt)	-	-	5.4	*9.3*	99
Mock	-	-	<0.01	*ND*	NA
Scid (SCGR11)	-	S	5.6	*0.30*	80

wt (wild type), ND (not determined), NA (not applicable), S (sensitive), P (partial), (-) indistinguisable from wt. The last column represents the % of correct RS joints screened by digestion of the recombinant substrates with HgiAI, a site that is generated only after precise fusion of heptamers (Hesse et al. 1987). Mock transfections were performed in the corresponding wt cell line transfected only with the recombination substrate. The UV sensitive cell lines (UV-, EM9) were provided by Dr. L. Thompson. SCGR11 is a scid fibroblast cell line that was kindly provided by Dr. D. Weaver. The bleomycin (BLM-) and adryamicin (ADR-3) resistant CHO cell lines were provided by Dr. I. Hickson and Dr. C. Robson.

expression vectors (providing the specific V(D)J recombination functions to the non-lymphoid cells) (Alt et al. 1992) . For these analyses, we used the pJH290 and pJH200 transient recombination substrates (Hesse et al. 1987) that, respectively, allow rough quantitation of coding and RS join formation by recovery from mammalian cells followed by assays of conferred drug resistance in bacteria (Table 1).
None of a variety of different excision repair mutants showed any impairment in ability to undergo V(D)J recombination (data not shown). However, 2 of 5 tested dsbr mutants (Xrs-6 and XR-1) demonstrated a striking decrease in ability to form both coding and RS joins . A third dsbr mutant (V3) showed a preferential impairment in coding join formation with a relatively normal level of RS join formation (Table 1). The latter phenotype is reminiscent of the V(D)J recombination defect found in scid pre-B cells (Lieber et al. 1988) and in scid fibroblasts (Table 1). Each of the CHO mutants belong to a different complementation group indicating that the defect(s) may be encoded by different genes. Revertants of the Xrs-6 and XR-1 mutants were obtained by introduction of human chromosomes 2 and 5, respectively, (Jeggo et al. 1992; Giaccia et al. 1990); notably, these revertants, D5 and XR-1:Ch5, also showed completely normal ability to undergo V(D)J recombination when assayed as described above (Table 1). The xrs-6 and XR-1 mutants are likely to affect different activities than the murine scid defect (evidenced both by the manifestation of the defect and by complementation studies) (Taccioli et al. submitted). The V3 mutation, on the other hand, generates a V(D)J recombinational defect quite similar to that of the scid mutation. Fusion between scid fibroblasts and V3 cells are in progress to determine whether the defect involves the same gene.

The above studies strongly suggest that DNA repair system and V(D)J recombination share a number of different components. In other words, the free ends generated by the V(D)J recombinase complex might be a substrate for the general non-lymphoid specific end-joining activities of the DNA repair system. One could envision that mutated genes that affect DNA repair may exert their effects in a number of direct or indirect fashions, including mutation of an essential component of the reaction (e.g.. a ligase, Barnes et al. 1992), mutation of a factor necessary for expression of a reaction component, or mutation of a factor that although not directly involved in the repair process may yield an activity that interferes with proper expression or activity of a required component (Jentsch et al. 1987). Therefore, it should be possible to design strategies that will allow selective cloning of genes that encode the products that complement these mutations. Further definition of activities involved in V(D)J recombination may also yield insight into defects that underlie a number of human diseases that affect the immune system. In this regard, it seems possible that various human diseases that affect both DNA repair processes as well as aspects of lymphocyte development may involve a shared factor.

Acknowledgements.

This work was supported by the HHMI and NIH grant A.I. 20047. G.E.T. is an Irvington Institute Fellow, G.R and Y.S. are fellows of the HHMI, and E.M.O is a Freddy Cunha IV fellow of the Cancer Research Institute.

References.

Aguilera A, Klein, HL (1990) HPR1, a Novel Yeast Gene That Prevents Intrachromosomal Excision Recombination, Shows Carboxy-Terminal Homology to the Saccharomyces cerevisiae TOP1 Gene. Mol Cell Biol 10:1439-1451

Akira S, Okazaki K, Sakano H (1987) Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 238:1134-1138

Alt FW, Rathbun G, Oltz E, Taccioli GE, Shinkai Y (1992) Function and Control of Recombination-Activating Gene Activity. Ann N Y Acad Sci 651:277, 294

Alt FW, Blackwell TK, Yancopoulos GD (1987) Development of the Primary Antibody Repertoire. Science 238:1079-1087

Alt FW, Baltimore D (1982) Joining of immunoglobulin heavy chain gene segments: Implication from a chromosome with evidence of three D-J_H fusions. Proc Natl Acad Sci USA 79:4118-4122

Barnes DE, Tomkinson AE, Lehmann AR, Webster DB, Lindahl T (1992) Mutations in the DNA Ligase I Gene of an Individual with Immunodeficiency and Cellular Hypersensitivity to DNA-Damaging Agents. Cell 69:495-503

Biedermann KA, Sun J, Giaccia AJ, Tosto LM, Brown M (1991) scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc Natl Acad Sci USA 88:1394-1397

Blackwell TK, Malynn BA, Pollock RR, Ferrier P, Covey LR, Fulop GM, Phillips RA, Yancopoulos GD, Alt FW (1989) Isolation of scid pre-B cells that rearrange kappa light chain genes: formation of normal signal and abnormal coding joints. EMBO J 8(3):735-742

Blackwell TK, Alt FW (1989) Mechanism and developmental program of immunoglobulin gene rearrangements in mammals. Annu Rev Genet 23:605-636

Bosma, MJ, and Carroll, AM (1991) The scid mouse mutant: definition, characterization, and potential uses. Annu Rev Immunol 9:323-350

Bosma GC, Fired M, Custer RP, Carroll A, Gibson DM, Bosma, MJ (1988) Evidence of functional Lymphocytes in some (LEAKY) scid Mice. J Exp Med 167:1016-1033

Bosma GC, Custer RP Bosma, MJ (1983) A severe combined immunodeficiency mutation in mouse. Nature 298:184-186

Carlson LM, Oettinger MA, Schatz DG, Masteller EL, Hurley EA, McCormack WT, Baltimore D, Thompson CB (1991) Selective expression of RAG-2 in chicken B cells undergoing conversion gene conversion. Cell 64: 201-208

Chun JJ, Schatz DG, Oettinger MA, Jaenisch R, Baltimore D (1991) The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. Cell 64:189-200

Ferrier P, Covey LR, Li SC, Suh H, Malynn BA, Blackwell K, Moureen AM, Alt FW (1990) Normal recombination substrate V_H to DJ_H rearrangements in pre-B cell lines from scid mice. J Exp Med 171:1909-1918

Fulop GM, Phillips RA (1990) The scid mutation in mice causes a general defect in DNA repair. Nature 346:479-482

Friedberg EC (1988) Deoxyribonucleic Acid Repair in the Yeast Saccharomyces cerevisiae. Microbiol Rev 52(1):70-102

Giaccia AJ, Denko N, MacLaren R, Mirman D, Waldren C, Hart I, Stamato TD (1990) Human Chromosome 5 Complements the DNA Double-Strand Break-Repair Deficiency and Gamma-Ray Sensitivity of the XR-I Hamster Variant. Am J Hum Genet 747:459-469

Hendrickson EA, Schlissel MS, Weaver DT (1990) Wild-type V(D)J recombination in scid pre-B cells. Mol Cell Biol 10:5397-5407

Hendrickson EA, Qin X-Q, Bump EA, Schatz DG, Oettinger M, Weaver DT (1991) A link between double-strand break-related repair and V(D)J recombination: The scid mutation. Proc Natl Acad Sci USA 88:4061-4065

Hesse JE, Lieber MR, Gellert M, Mizuuchi K (1987) Extra chromosomal DNA substrates in pre-B cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. Cell 49:775-783

Hesse JE, Lieber MR, Mizuuchi K, Gellert M (1989) V(D)J recombination: a functional definition of the joining signals. Gene Dev 3:1053-1061

Hoeijmakers JHJ, Bootsma D (1990) Molecular Genetics of Eukaryotic DNA Excision Repair. Cancer Cells 2(10):311-320

Jeggo PA (1990) Studies on mammalian mutants defective in rejoining double-strand break in DNA. Mutat Res 239:1-16

Jeggo PA, Hafezparast M, Thompson AF,Broughton BC, Kaur GP, Zdzienicka M, Athwal RS (1992) Localization of a DNA repair gene (XRCC5) involved in double strand repair break rejoining to human chromosome 2 (in press)

Jentsch S, McGrath JP, Varshavsky A (1987) The yeast DNA repair RAD6 encodes a ubiquitin-conjugating enzyme. Nature 329:131-134

Lieber MA (1991) Site-specific recombination in immune system. FASEB J 5:2934-2944

Lieber MR, Hesse JE, Mizuuchi K, Gellert M (1987) Developmental stage specificity of the lymphoid V(D)J recombination activity. Genes Dev 1:751-761

Lieber MR, Hesse JE, Lewis S, Bosma GC, Rosenberg N, Mizuuchi K, Bosma MJ, Gellert M (1988) The defect in murine severe combined immune deficiency: joining of signal sequences but not coding segments in V(D)J recombination. Cell 55:7-16

Matsuoka M, Nagawa F, Okazaki K, Kingsbury L, Yoshida K, Muller U, Larue D., Winer JA, Sakano H (1991) Detection of somatic DNA recombination in the transgenic mouse brain. Science 254:81-86

Malynn BA, Blackwell TK, Fulop GM, Rathbun GA, Furley AJW, Ferrier P, Heinke LB, Phillips RA, Yancopoulos GD, Alt FW (1988) The scid defect affects the final step of the immunoglobulin VDJ recombinase mechanism. Cell 54:453-460

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonewaga S, Papaionnou VE (1992) RAG-1 Deficient Mice have no Mature B and T Lymphocytes. Cell 68:869-867

Oettinger MA, Schatz DG, Gorka C, Baltimore D (1990) RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science 248:1517-1523

Okasaki K, Sakano H (1988) Thymocyte circular DNA excised from T cell receptor a-d gene complex. EMBO J 7(6):1669-1674

Petrini JHP, Carroll AM, Bosma MJ (1990) T-cell receptor gene rearrangements in functional T-cell clones from severe combined immune deficient (scid) mice. reversion of the scid phenotype in individual lymphocyte progenitors. Proc Natl Acad Sci USA 87:3450-3453

Roth DB, Nakajima PB, Menetski JP, Bosma MJ, Gellert M (1992) V(D)J Recombination in Mouse Thymocytes: Double-Strand Breaks Near T Cell Receptor d Rearrangements Signals. Cell 69:41-53

Schatz DG, Oettinger MA, Baltimore D (1989) The V(D)J recombination activating gene, RAG-1. Cell 59:1035-1048

Schuler W, Weiler IJ, Schuler A, Phillips RA, Rosenberg N, Mak TW, Kearney JF, Perry RP, Bosma JJ (1986) Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. Cell 46:963-972

Shinkai Y, Rathbun G, Lam K, Oltz EM, Stewart V, Mendelsohn M, Charon J, Datta M, Young F, Stall AM, Alt FW (1992) RAG-2 Deficient Mice Lack Mature Lymphocytes due to Inability to Initiate VDJ Rearrangement. Cell 68:855-867

Toda M, Fujimoto S, Iwasato T, Takeshita S, Tezuka K, Ohbayashi T, Yamagishi H (1988) Structure of Extrachromosomal Circular DNAs Excised from T-cell Antigen Receptor Alpha and Delta-chain Loci. J Mol Biol 202:219-231

Double-Strand Breaks Associated with V(D)J Recombination at the TCRδ Locus in Murine Thymocytes

D. B. Roth[1], P. B. Nakajima[2], J. P. Menetski[1], M. J. Bosma[2], and M. Gellert[1]

[1]Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health Bethesda, MD 20892, (301) 496-5888
[2]Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

Introduction

Somatic recombination events are responsible for assembling the variable regions of immunoglobulin and T cell receptor genes from germline-encoded DNA segments (Tonegawa, 1983; Lewis and Gellert, 1989). These rearrangements are mediated by a recombination activity that recognizes signal sequences (consisting of conserved heptamer and nonamer elements separated by nonconserved spacer regions of 12 or 23 nucleotides) located adjacent to the V, D, and J coding segments. Although the mechanism of the reaction remains obscure, recombination is thought to involve either single-stranded or double-stranded cleavage at the border between a signal heptamer and a coding segment, followed by rejoining of the DNA ends in a new configuration (Alt and Baltimore, 1982; Lewis and Gellert, 1989).

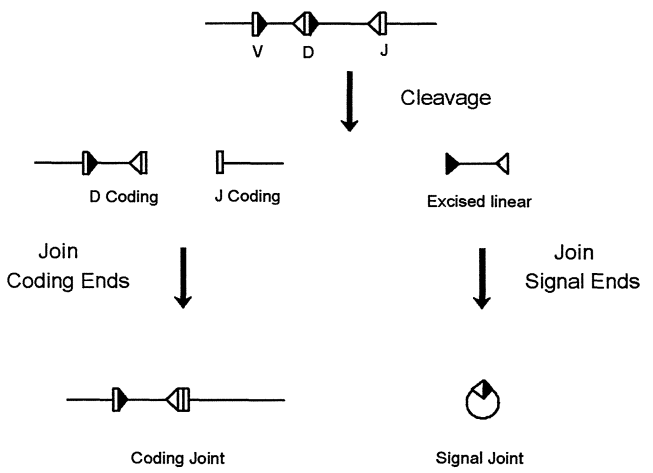


Fig.1. A breakage-reunion model for V(D)J recombination. Recombination signal sequences are represented by triangles, with heptamers adjacent to coding segments, which are shown as rectangles. Signals with 23 and 12 nucleotide spacers are represented by solid and open triangles, respectively.

One possible mechanism is diagrammed in Fig. 1. According to this scheme, double-strand cleavage creates two sets of termini, coding ends and signal ends, which are subsequently joined to generate coding joints (the junction between coding segments) and signal joints (the reciprocal junction with the recombination signals fused heptamer-to-heptamer). One prediction of this model is that molecules containing double-strand breaks adjacent to recombination signals might accumulate in tissues containing a large pool of recombinationally active cells, such as the thymus. We have used the T cell receptor δ locus as a model system to search for such broken molecules in murine thymocytes (Roth et al., 1992). This locus provides a relatively simple system for the study of cleavage events, as it contains only two D and two J elements, and most recombination events in newborn mice involve D2 and J1. We therefore assayed

genomic DNA preparations from BALB/c thymocytes for the presence of molecules containing double-strand breaks near D2 and J1.

Results

The arrangement of the recombination signal sequences adjacent to D2 and J1 allows these elements to participate in a variety of rearrangement events, including V-D2, D2-J1, V-D2-J1, and V-D1-D2-J1 joining. Possible products of cleavage at these signals are illustrated in Fig. 2. To assay for these species, thymus DNA was digested with EcoRI and analyzed by Southern blotting using the TCR δ probes shown in Fig. 2. Fig. 3 shows a blot of EcoRI digested DNA hybridized to the 5'D2 probe. In addition to the germline fragment (7400 bp) several

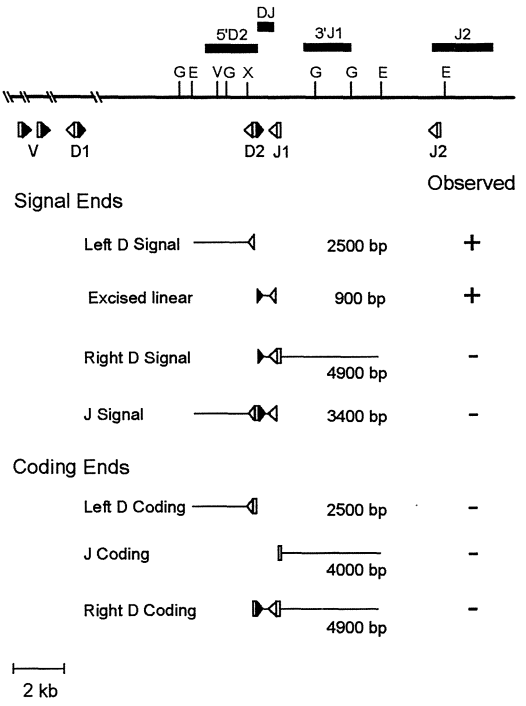


Fig. 2. Possible products of double-strand breaks near the TCRδ D2 and J1 elements. Cleaved molecules observed in this study are indicated in the right column. Restriction sites: E, EcoRI; G, BglII; V, PvuII; X, XmnI.

fragments are present in thymus DNA from newborn, 2-3 day old, and 5 week old BALB/c mice (lanes 3-5), including a 6600 bp fragment resulting from D2-J1 rearrangement (Carroll and Bosma, 1991), and species of approximately 3300, 3000, and 2500 bp. The 3300 and 3000 bp fragments represent reciprocal products of D1-D2 and V-D2 rearrangements, as they contain signal joints (Roth et al., 1992 and our unpublished observations). The nongermline fragments are not present in DNA preparations from adult liver (lane 1), day 15 fetal liver (lane 2), adult spleen, or adult testis (not shown), indicating that these species are specifically associated with tissues undergoing TCR gene rearrangement. The 2500 bp fragment could result from double-strand cleavage near the D2 element, since an EcoRI site is located approximately 2500 bp to the left of D2 (Fig. 2).

To confirm that the 2500 bp fragment results from double-strand cleavage, and to test whether any of the other nongermline fragments might be due to cleavage events, we developed an exonuclease assay.

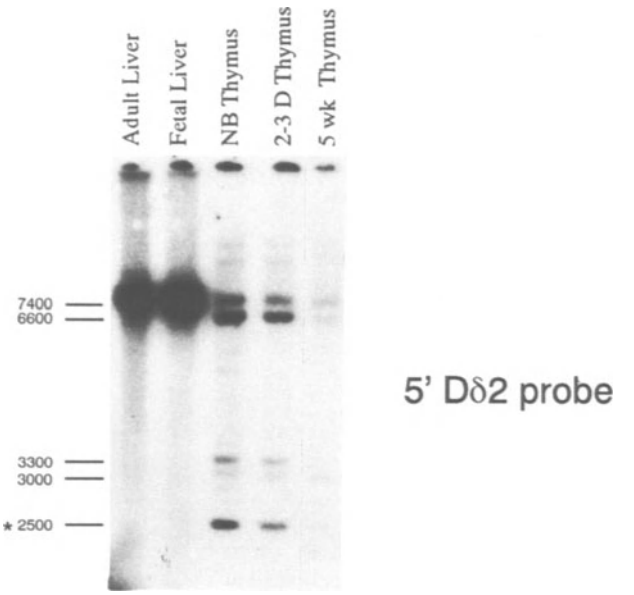


Fig. 3. EcoRI mapping of double-strand breaks. Approximately 8 μg of each sample of genomic DNA was digested with EcoRI and electrophoresed through a 1% agarose gel. The membrane was probed with the 5'D2 probe.

Exonuclease treatment of genomic DNA prior to digestion with a restriction enzyme should specifically destroy molecules with broken ends. Newborn thymus DNA was treated with *Micrococcus luteus* ATP-dependent exonuclease, and then digested with EcoRI. The resulting fragments were analyzed by Southern blotting using the 5'D2 probe (Fig. 4). Exonuclease treatment prior to digestion with EcoRI destroys only the 2500 bp fragment (lane 4), demonstrating that this fragment results from a double-strand break. The remaining nongermline fragments (6600, 3300, and 3000 bp) are exonuclease-resistant, indicating that they do not contain exonuclease-sensitive ends. As a control for exonuclease activity, thymus DNA was digested with EcoRI prior to exonuclease treatment, resulting in loss of greater than 95% of the hybridizable material (lane 2). This assay provides a general method for the identification of fragments that contain free broken ends, and is particularly useful for characterizing samples containing numerous nongermline restriction fragments.

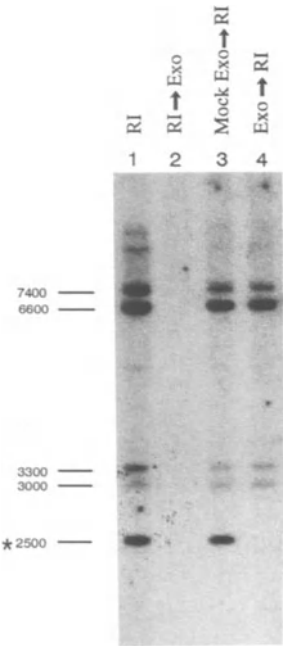


Fig. 4. An exonuclease assay for molecules containing double-strand breaks.

The location of the double-strand break was mapped more precisely using additional restriction enzymes. Digestion with PvuII, BglII, and BamHI produced fragments of sizes consistent with the presence of a double-strand break very near the D2 element (Roth et al., 1992). Fine mapping of the broken end was performed using XmnI, which cleaves 298 bp to the left of D2. In principle, cleavage could occur at the recombination signals on either side of D2, generating species terminating in either D signal or D coding ends (Fig. 2). These molecules would differ by only 16 nucleotides, the length of the D2 element. Digestion with XmnI mapped the cleaved terminus to within five nucleotides of the left end of the D2 element, consistent with cleavage at the recombination signal to the left of D2, corresponding to a signal end. Cleavage at this signal might be related to rearrangement with elements leftward of D2, such as V-D2 or D1-D2 joining. No coding ends could be detected (Roth et al., 1992).

To quantitate the species resulting from double-strand breaks near D2, the amount of radioactivity hybridizing to the fragments shown in Fig. 3 was measured using a phosphorimager. To normalize for the amount of DNA in each lane, the blot was rehybridized with a probe specific for a nonrearranging sequence (RAG1 probe). This analysis revealed that molecules with double strand breaks near D2 comprise about 2-3% of total genomic DNA in newborn or 2-3 day old thymus. Although the abundance of all fragments hybridizing to the 5'D2 probe is severely diminished in 5-6 week old Balb/c thymus DNA (lane 5), longer exposures of the autoradiogram reveal that the 2.5 kb species is still present in this sample (not shown), and its abundance relative to the germline fragment is similar to that in newborn DNA. Thus, double strand breaks near D2 are relatively abundant, and are present well past the newborn period.

In the autoradiogram shown in Fig. 3, the abundance of the 2500 bp fragment appears to be much greater than 2-3% of total hybridizing signal in newborn thymus DNA. This is because a significant fraction of total thymus DNA does not hybridize to the 5'D2 probe.

The loss of hybridization is significantly greater in the DNA preparation from 5-6 week old thymus (Fig. 3, lane 5). This does not simply reflect the amount of DNA in this sample, as reprobing the blot with the nonrearranging RAG1 probe reveals abundant hybridization to germline fragments (not shown). Hybridization to the 5'D2 probe could be lost because of TCR α rearrangements, as the δ locus is embedded in the TCR α locus in such a way that Vα - Jα rearrangements excise the entire δ locus. To test this hypothesis, the blot shown in Fig. 3 was rehybridized to with a probe specific for Jδ2. Sequences hybridizing to this probe should be deleted along with the rest of the δ locus by Vα- Jα rearrangements. As shown in Fig. 5, some loss of hybridization to this probe occurs with increasing age, but not much; 2-3 day thymus and 5 week thymus have lost 20 and 30 percent, respectively, of hybridization to this probe. Therefore, deletion of the entire TCRδ locus by Vα-Jα recombination does not play a major role in the loss of sequences able to hybridize to the 5'D2 probe. Rearrangements involving sequences to the left of D2, which would not affect hybridization to the J2 probe (such as V to D2 or D1 to D2 joining) are more likely to be responsible for this phenomenon. The presence of molecules cleaved to the left of D2 (possible intermediates in V to D2 or D1 to D2 rearrangement) is consistent with this interpretation.

Discussion

Here we describe the identification of DNA molecules containing a double-strand break near the TCRδ D2 element. Observation of these broken molecules in thymus, but not in liver or spleen, provides the first direct evidence for an association between specific cleavage of chromosomal DNA and recombination in mammalian cells, and supports a breakage-reunion model of V(D)J recombination. Cleavage occurs within several nucleotides of the recombination signal on the left side of D2, generating a signal end (Roth et al., 1992).

Fig. 5. Use of the J2 probe to measure extent of TCRα rearrangement. The blot shown in Fig. 3 was stripped and rehybridized to the J2 probe. Quantitation was performed using a Molecular Dynamics model 400E phosphorimager, which was also used to generate this autoradiographic image.

These broken molecules could be intermediates in V-D2 or D1-D2 rearrangement, and might correspond to excised linear fragments containing the sequences between D1 and D2 or a V segment and D2. This is supported by the recent observation of an approximately 10kb fragment in undigested thymus DNA, which could represent a D1-D2 excised linear molecule (unpublished). Another excised linear fragment, resulting from double-strand cleavage at the recombination signal to the right of D2 and at the signal to the left of J1, has been identified (Roth et al., 1992); these molecules also terminate in signal ends. This excised linear fragment might arise as a result of D2-J1 recombination; corresponding circular forms containing perfect signal junction have been detected (Roth et al., 1992). Although molecules with signal ends are relatively abundant, species with coding ends could not be detected. The overall results of these investigations are summarized in the right column of Fig. 2.

The relatively high abundance of cleaved molecules with signal ends suggests that they are quite stable. Long-lived intermediates resulting from double-strand cleavage have been observed in both Tn7 and Tn10

transposition in *E. coli* (Bainton et al., 1991; Haniford et al., 1991), as well as in yeast mating type switching (Raveh et al., 1989; White and Haber, 1990). The double-stranded transposon ends produced during Tn10 and Tn7 transposition are present in extremely stable protein-DNA complexes and are protected until they are joined to target DNA (Bainton et al., 1991; Haniford et al., 1991). Perhaps signal ends are similarly protected, providing an explanation for the remarkable preservation of signal ends seen in signal junctions, as well as the apparent lack of heterogeneity present in the molecules cleaved at D2.

The observation that molecules with signal ends are much more abundant than the molecules terminating in coding ends suggests that these two types of ends are treated differently. This is supported by two additional lines of evidence. First, nucleotides are normally lost only from coding ends (Lieber et al., 1988). Second, the murine severe combined immunodeficiency (*scid*) mutation affects coding joint formation much more severely than signal joint formation (Lieber et al., 1988). Perhaps signal ends are sequestered in a protein-DNA complex, and joined by a specific pathway. In contrast, coding ends might be handled by general, nonspecific cellular end-joining activities (Roth and Wilson, 1988). This is consistent with the observation that the *scid* mutation affects general double-strand break repair functions. The frequent presence of short sequence homologies at coding joints (Gu et al., 1990; Feeney, 1992) as well as their apparent use in random end joining of transfected DNA molecules (Roth and Wilson, 1988) lends support to this notion. Perhaps the mechanism of V(D)J recombination is analogous to certain transposition events (Engels et al., 1990) in which excision of the transposable element and integration into the target DNA are performed by the transposase, while repair of the broken donor site is mediated by general repair pathways .

This work was supported in part by National Institutes of Health grants AI-13323, CA-04946, and RR-05539 and an appropriation from the Commonwealth of Pennsylvania. J.P.M. was supported by a National Research Council-NIH research associateship. D.R. is a Howard Hughes Medical Institute Physician Research Fellow.

References

Alt, F. W. and D. Baltimore (1982). Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-J_H fusions. Proc. Natl. Acad. Sci. USA **79**: 4118.

Bainton, R., P. Gamas and N. L. Craig (1991). Tn7 transposition in vitro proceeds through an excised transposon intermediate generated by staggered breaks in DNA. Cell **65**: 805.

Carroll, A. M. and M. J. Bosma (1991). T-lymphocyte development in scid mice is arrested shortly after the initiation of T-cell receptor δ gene recombination. Genes Devel. **5**: 1357.

Engels, W. R., D. M. Johnson-Schlitz, W. B. Eggleston and J. Sved (1990). High frequency P element loss in Drosophila is homolog dependent. Cell **62**: 515.

Feeney, A. J. (1992). Comparison of junctional diversity in the neonatal and adult immunoglobulin repertoires. Intern. Rev. Immunol. **8**: 113.

Gu, H., I. Forster and K. Rajewsky (1990). Sequence homologies, N sequence insertion and J_H gene utilization in V_HDJ_H joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J. **9**: 2133.

Haniford, D. B., H. W. Benjamin and N. Kleckner (1991). Kinetic and structural analysis of a cleaved donor intermediate in Tn10 transposition. Cell **64**: 171.

Lewis, S. and M. Gellert (1989). The mechanism of antigen receptor gene assembly. Cell **59**: 585.

Lieber, M. R., J. E. Hesse, S. Lewis, G. C. Bosma, N. Rosenberg, K. Mizuuchi, M. J. Bosma and M. Gellert (1988). The defect in murine severe combined immunodeficiency: joining of signal sequences but not coding segments in V(D)J recombination. Cell **55**: 7.

Lieber, M. R., J. E. Hesse, K. Mizuuchi and M. Gellert (1988). Lymphoid V(D)J recombination: nucleotide insertion at signal joints as well as coding joints. Proc. Natl. Acad. Sci. USA **85**: 8588.

Raveh, D., S. H. Hughes, B. K. Shafer and J. N. Strathern (1989). Analysis of the HO-cleaved MAT DNA intermediate generated during the mating type switch in the yeast Saccharomyces cerevisiae. Mol. Gen. Genet. **220**: 33.

Roth, D. B., P. B. Nakajima, J. P. Menetski, M. J. Bosma and M. Gellert (1992). V(D)J recombination in mouse thymocytes: double-strand breaks near T cell receptor δ rearrangement signals. Cell **69**: 41.

Roth, D. B. and J. H. Wilson (1988). Illegitimate recombination in mammalian cells. Genetic Recombination. Washington, DC, ASM Press. 621.

Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature **302**: 575.

White, S. I. and J. E. Haber (1990). Intermediates of recombination during mating type switching in Saccharomyces cerevisiae. EMBO J. **9**: 663.

Replication, Transcription, CpG Methylation and DNA Topology in V(D)J Recombination

C.-L. HSIEH, G. GAUSS and M. R. LIEBER

Laboratory of Experimental Pathology, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305-5324

Introduction

The seven antigen receptor loci are targeted for V(D)J recombination at different times, different developmental stages, and with B or T lineage specificity by unknown mechanisms. The physical basis for the differential accessibility in this site-specific recombination reaction has been a matter of much speculation. Possibilities have included the processes of transcription (Ferrier et al., 1989; Martin et al., 1991; Schlissel et al., 1991) and DNA replication, and the structural features of DNA methylation and chromatin structure (Mather and Perry, 1983; Persiani and Selsing, 1989; Storb and Arp, 1983; Yancopoulos et al., 1986). The relevant order and hierarchy of these parameters in controlling accessibility of the V(D)J recombination activity are unknown. Although some studies have raised the possibility that transcription is a requirement for recombination, the temporal resolution of such studies has been limiting. They have not permitted determination of whether transcription and recombination are consequences of a common chromatin change or whether transcription precedes and, thereby, activates recombination.

Recent findings have intensified an interest in locus targeting of V(D)J recombination (Lieber, 1991). First, the length of the essential portions of the recombination signal sequences may not be long enough to stringently specify these sites in the genome. The heptamer can serve as a signal without the nonamer at a reduced, but significant, frequency, potentially allowing cutting at many adventitious sites. Within the heptamer, only 4 base pairs appear to be critical to

initiating and completing the reaction (Hesse et al., 1989). Second, it appears that the V(D)J recombination activity may be able to initiate nucleolytic cuts at a single signal (Lewis et al., 1988; Hendrickson et al., 1991). These cuts are then reclosed, with some base loss and addition. Hence, the sequence requirements for initiation of the reaction may lie in one rather than two signals. These findings indicate that without some form of locus targeting, V(D)J recombination may catalyze cuts and rearrangements much more frequently than one would expect to be compatible with cell viability. Here we summarize our recent investigations of the role that DNA replication, transcription, CpG methylation and DNA topology have in targeting the V(D)J recombination reaction.

V(D)J Recombination and DNA Replication

We have examined a series of extrachromosomal DNA substrates for V(D)J recombination under replicating and non-replicating conditions (Hsieh et al., 1991). Complete and partial replication were examined by monitoring the loss of prokaryotic-specific adenine methylation at 14 to 22 MboI/DpnI restriction sites (GATC) on the substrates. Some of these sites are within 2 bases of the signal sequence ends. We find that neither coding joint nor signal joint formation requires substrate replication.

After ruling out replication as a substrate requirement, we determined if replication had any effect on the efficiency of V(D)J recombination. Quantitation of V(D)J recombination efficiency on non-replicating substrates requires some method of monitoring the entry of substrate molecules into the cells. We devised such a method by monitoring DNA repair of substrates into which we had substituted deoxyuridine for 10 to 20% of the thymidine nucleotides in the DNA. The substrates which enter the lymphoid cells are repaired efficiently in vivo by the eukaryotic uracil DNA repair system. Upon plasmid harvest, we distinguish repaired (entered) from unrepaired (not entered) plasmids by cleaving unrepaired molecules with uracil DNA glycoylase and E. coli endonuclease IV in vitro. This method of monitoring DNA entry does not

appear to underestimate or overestimate the amount of DNA entry. Using this method, we find no significant quantitative effect of DNA replication on V(D)J recombination efficiency.

V(D)J Recombination and Transcription

It has been shown previously by others that transcription is temporally correlated with the onset of V(D)J recombination at the endogenous antigen receptor loci (Alt et al., 1987). We have been interested in determining whether this temporal correlation indicates a causal connection between these two processes. We have compared V(D)J recombination minichromosome substrates that have transcripts running through the recombination zone with substrates that do not in a transient transfection assay (Hsieh, McCloskey and Lieber, 1992). In this system, the substrates acquire a minichromosome conformation within the first several hours after transfection. We find that the substrates recombine equally well over a 100-fold range in transcriptional variation. In additional studies, we have taken substrates that have low levels of transcription and inhibited it further by methylating the substrate DNA or by treating the cells with a general transcription inhibitor (a-amanitan). Although these treatments decrease the level of expression an additional 10- to 100-fold, there is still no observable effect on V(D)J recombination.

Based on these results, we conclude that transcription is not necessary for the V(D)J reaction mechanism and does not alter substrate structure at the DNA level or at the simplest levels of chromatin structure in a way that affects the reaction.

CpG Methylation and V(D)J Recombination

We have used minichromosome substrates to study the role that CpG methylation might play in controlling V(D)J recombination site accessibility (Hsieh and Lieber, 1992). We

find that CpG methylation decreases the V(D)J recombination of these substrates more than 100-fold. The decrease correlates with a considerable increase in resistance to endonuclease digestion of the methylated minichromosome DNA. The minichromosomes acquire resistance to both the intracellular V(D)J recombinase and exogenous endonuclease only after DNA replication. Therefore, CpG methylation specifies a chromatin structure that, upon DNA replication, is resistant to eukaryotic site-specific recombination. The implications of these findings to V(D)J recombination as well as to the chromatin assembly of methylated DNA during replication are discussed below.

CpG Methylation, V(D)J Recombination and Accessible Domain Size

CpG methylation on V(D)J recombination substrates results in a striking inhibition of V(D)J recombination. This is clearly not a direct interference of the interaction of the recombination activity with the methylated DNA because no inhibition of recombination is observed in the case of non-replicating substrates. The inhibition due to methylation is only apparent for transfected plasmids that form replicating minichromosomes in the lymphoid cells. This inhibition of site-specific recombination correlates with a degree of restriction endonuclease resistance that is only apparent after substrates have replicated. We infer that replication of methylated DNA results in a chromatin structure that is resistant to lymphoid V(D)J recombination.

Using a V(D)J recombination substrate that is capable of DNA replication in human cells, we have found that V(D)J recombination is inhibited more than 200-fold by CpG methylation in a human lymphoid pre-B cell line (unpublished observations). As in the case of the polyoma replicon, CpG methylation did not inhibit origin firing. Therefore, our conclusions regarding the inhibition of V(D)J recombination by CpG methylation are not restricted by species or the choice of replicon. These extents of inhibition are likely to be 10-fold underestimates because what little recombination that does

occur happens on the small sub-population of demethylated molecules.

Inaccessibility occurs regardless of the exact placement of the CpG sites relative to the signals, indicating that a regional structure is important rather than a localized interference with the heptamer or nonamer recognition. We find that a CpG gap of 150 bp around one signal and a 91 bp gap around the other signal in one of our substrates (pJH229) still resulted in a 100-fold inhibition of V(D)J recombination. The inhibition does not increase for a substrate with CpG sites located within 4 bp to 8 bp of each signal. It is not clear in any system what length of DNA is critical for the formation of an inaccessible domain. Because the domain of inaccessibility does not appear to be at the length of hundreds of base pairs, one suspects that it is considerably longer.

CpG Methylation and V(D)J Recombination at the Endogenous Antigen Receptor Loci

Analysis of over 50,000 bp of sequence at the endogenous Ig and TCR V, D, and J segments in 8 vertebrate species indicates they are CpG-rich relative to the bulk genome. In addition, where long stretches of sequence are available at the immunoglobulin loci, the CpG density also appears to be higher than the bulk genome. In a continuous 14,928 bp stretch of the human D locus that includes twelve DH segments, the CpG density is one in 55 b (Ichihara et al., 1988). With respect to the T-cell receptor loci, analysis of TCR α, β and γ sequences in human and mouse indicate that these sequences are also richer in CpG than the bulk genome (Kabat et al., 1987). Of the Ig and TCR V, D, and J segments in these 8 vertebrate species, only the V_H and V_λ genes of mouse are as CpG-deficient as bulk vertebrate DNA, and these recombine with segments that are CpG-rich.

The CpG densities of our substrates are very similar to the observed CpG richness in the endogenous loci. For the substrates, the local CpG densities (within 50 bp) are between one in 15 and one in 48 bp. The regional densities (within 1 to

3 kb) are between one in 19 and one in 37 bp. We see no diminution in the effect of CpG methylation over this range. In fact, we see the highest inhibition on the substrates with the lowest regional and local CpG densities over these ranges. We conclude that the CpG densities studied here are comparable and relevant to the densities at endogenous loci.

Model of the Order of Events Required for Locus Activation for V(D)J Recombination

Based on these functional and structural observations and the structural analyses of others on the endogenous loci, we suggest a model in which targeted demethylation leads to an endonuclease sensitive chromatin structure which then allows for recombination to occur (Fig. 1). How might the targeted demethylation occur? This is not yet clear. It is possible that specific sites are recognized by demethylation activities at each locus. Alternatively, binding of transcription factors may target a region for demethylation.

Fig. 1. Proposed Order of Events in the Activation of Antigen Receptor Loci for V(D)J Recombination

CpG Methylation and Genome Stability

Our observations that CpG methylated DNA that has undergone replication is resistant to site-specific endonucleolytic action by the V(D)J recombinase is of intrinsic significance from a chromatin structure standpoint. If one

considers the V(D)J recombinase as a nuclease, then these studies demonstrate that the nuclease resistant chromatin configuration induced by methylation is present prior to cell fractionation. The relevance of this is two-fold. First, if methylated DNA is as resistant to recognition by other nucleases as by the V(D)J recombinase, then CpG methylation may stabilize the genome against the inadvertent action of the enzymes of DNA metabolism. Second, because 75% of the CpG sites in the genome are methylated, much of the genome in lymphoid cells is likely to be protected from rearrangements at adventitious sites that might otherwise be catalyzed by the V(D)J recombination activity. Given the minimal requirements for cutting by the V(D)J recombination activity, there may be as many as 5000 adventitious cut sites for every one signal sequence at a V, D, or J segment. The chromosomal translocations that occur in childhood lymphoid leukemia and lymphoma are likely to represent only a fraction of what would occur if a major portion of the genome were left unprotected. The studies here suggest that at least a significant portion of the protection against V(D)J recombination outside of the targeted loci is mediated by CpG methylation.

From an evolutionary standpoint it is interesting to note that, within the animal kingdom CpG methylation is used on a genome-wide basis only in the vertebrates. It is possible that the combination of a longer lifespan and a more complex genome made the stability against inadvertent recombination a selective advantage for retaining some minimal density of methyl-CpG. It is also interesting, in light of the observations here, that the only physiologic, site-directed recombination reaction that has been described in animal cells appears to be restricted to vertebrates, suggesting that the protection provided by methylation might have been a prerequisite for the evolutionary introduction of a site-directed endonuclease.

Topological Bias in V(D)J Recombination

One of many questions about V(D)J recombination concerns how the two signals and the recombination activity assemble. One hypothesis has been that the recombination

activity binds to one signal, and collisional interactions of this protein-DNA complex with the other signal generates the ternary complex. A second hypothesis has been that the protein-DNA complex formed at one signal tracks processively along the DNA until it reaches a second signal (Yancopoulos et al., 1988). This hypothesis predicts that a signal will recombine with the nearest compatible signal with some preference to distant ones. Though the second hypothesis seems at odds with the notion that V(D)J recombination is a random exon assembly system, work from several laboratories has been cited in favor of some form of tracking. First, the joining at the murine lambda locus markedly favors proximal over distal segments (Storb et al., 1989). Second, at the TCR g locus, the temporal onset of Vγ2, Vγ3, and Vγ4 rearrangement correlates with distance. Third, the utilization of V_H gene segments that are more proximal to DJ_H is 3- to 30-fold greater than the utilization of more distal V_H gene segments during fetal development (Yancopoulos et al., 1988). Finally, the strongest support for the tracking hypothesis (Kurosawa and Tonegawa, 1982) lies in the data for which it was originally proposed to explain: the well-established fact that deletional V(D)J recombination occurs one- to two-orders of magnitude more frequently than inversional recombination in D_H to J_H joining at the heavy chain locus (Meek et al., 1989). The joining of D_H to J_H occurs predominantly by deletion even though inversion should be common based on the 12/23 joining rule (Hesse et al., 1987). Because the D_H element bears signal sequences on each side, inversion would be expected as often as deletion in D_HJ_H recombination, but in fact, the markedly favored outcome is deletion, entailing the utilization of the closer recombination site. The way a tracking model could explain this is that the 12-signal of the D_H element that gives rise to deletion is at least 10-bp closer to the 23-signal than the 12-signal that would give rise to inversion. Modified proposals invoking a looser type of tracking, such as jumping followed by local tracking, might account for the few inversions that do occur in D-J_H joining, and still allow for randomness of the exon assembly process.

We have studied how signals interact by looking for

biases in the outcome of the recombination reaction when multiple possible outcomes exist using extrachromosomal plasmid V(D)J recombination substrates (Gauss and Lieber, 1992). We find that there is no form of tracking between signals. Yet the large bias for deletional over inversional V(D)J recombination can be recapitulated on these substrates. Their analysis indicates that the large bias in favor of deletions is due to two factors: (1) sequence differences between the two sides of the D segment can favor usage of the proximal 12-signal which leads to deletion, and (2) reactions that require two joints to form (inversions) have a lower probability of going to completion than reactions that require only one joint to form.

REFERENCES

Alt, F. W., Blackwell, T. K., and Yancopoulos, G. D. (1987). Development of the primary antibody repertoire. Science 238, 1079-1087.

Ferrier, P., Covey, L. R., Suh, H., Winoto, A., Hood, L., and Alt, F. W. (1991). T cell receptor DJ but not VDJ rearrangement within a recombination substrate introduced into a pre-B cell line. Int. Immunol. 1, 66-74.

Gauss, G., and Lieber, M. R. (1992). The basis for the mechanistic bias for deletional over inversional V(D)J recombination. Genes Dev. 6(8), in press.

Hendrickson, E. A., Liu, V. F., and Weaver, D. T. (1991). Strand breaks without DNA rearrangement in V(D)J recombination. Mol. Cell. Biol. 11, 3155-3162.

Hesse, J. E., Lieber, M. R., Mizuuchi, K., and Gellert, M. (1987). Extrachromosomal DNA substrates in pre-B cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. Cell 49, 775-783.

Hesse, J. E., Lieber, M. R., Mizuuchi, K., and Gellert, M. (1989). V(D)J recombination: a functional definition of the joining signals. Genes Dev. 3, 1053-1061.

Hsieh, C.-L., McCloskey, R. P., Radany, E., and Lieber, M. R. (1991). V(D)J recombination: evidence that a replicative mechanism is not required. Mol. Cell. Biol. 11, 3972-3977.

Hsieh, C.-L. and Lieber, M.R. (1992). CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication. EMBO J. 11, 315-325.

Hsieh, C.-L., McCloskey, R. P., and Lieber, M. R. (1992). V(D)J recombination is not affected by transcription. J. Biol. Chem. 267 (in press).

Ichihara, Y., Matsuoka, H., and Kurosawa, Y. (1988). Organization of human immunoglobulin heavy chain diversity gene loci. EMBO J. 7, 4141-4150.

Kabat, E., Wu, T., Reid-Miller, M., Perry, H., and Gottesman, K. (1987) Sequences of Proteins of Immunological Interest, 4th Ed., U.S. Dept. HHS, Washington, D.C.

Kurosawa, Y. and Tonegawa, S. (1982) Organization, structure, and assembly of immunoglobulin heavy-chain diversity DNA segments. J. Exp. Med. 155, 201-218

Lewis, S. M., Hesse, J. E., Mizuuchi, K., and Gellert, M. (1988). Novel strand exchanges in V(D)J recombination. Cell 55, 1099-1107.

Lieber, M. R., Hesse, J. E. Mizuuchi, K., and Gellert, M. (1987). Developmental stage specificity of the lymphoid V(D)J recombination activity. Genes Dev. 1, 751-761.

Lieber, M.R. (1991) Site-specific recombination in the immune system. FASEB J. 5 (14): 2934-2944.

Martin, D., Huang, R., LeBien, T., and Van Ness R. (1991). Induced rearrangement of k genes in the BLIN-1 human pre-B cell line correlates with germline J-Ck and Vk transcription. J. Exp. Med. 173, 639-645.

Mather, E. L., and Perry, R. P. (1983). Methylation status and DNaseI sensitivity of immunoglobulin genes: changes associated with rearrangement. Proc. Natl. Acad. Sci. USA 80, 4689-4693.

Meek, K., Hasemann, C., and Capra, J.D. (1989) Novel rearrangements at the immunoglobulin D locus. J. Exp. Med. 170, 39-57

Persiani, D. M., and Selsing, E. (1989). DNaseI sensitivity of immunoglobulin light chain genes in Abelson murine leukemia virus transformed pre-B cell lines. Nucl. Acids Res. 17, 5339-5348.

Schlissel, M. S., Corcoran, L. M., and Baltimore, D. (1991) Virus-transformed pre-B cells show ordered activation

but not inactivation of immunoglobulin gene rearrangement and transcription. J. Exp. Med. 173, 711-720.

Storb, U. and Arp, B. (1983) Methylation patterns of immunoglobulin genes in lymphoid cells: correlation of expression and differentiation with undermethylation. Proc. Natl. Acad. Sci. USA 80, 6642-6646.

Storb, U., Haasch, D., Arp, B., Sanchez, P., Cazenave, P., and Miller, J. (1989) Physical linkage of mouse lambda genes by pulsed-field gel electrophoresis suggests that the rearrangement process favors proximate target sequences. Mol. Cell. Biol. 9, 711-718

Yancopoulos, G. D., Blackwell, T. K., Suh, H., Hood, L., and Alt, F. W. (1986). Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. Cell 44, 251-259.

Yancopoulos, G., Malynn, B., and Alt, F. (1988) Developmentally regulated and strain-specific expression of murine VH gene families. J. Exp. Med. 168, 417-435

Rearrangement and Expression of Immunoglobulin Genes in Transgenic Mice

U. STORB, P. ENGLER, E. KLOTZ, A. WENG, D. HAASCH, C. PINKERT[1], L. DOGLIO, M. GLYMOUR and R. BRINSTER[1]

Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637 and
[1]School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104

Transgenic mice are discussed which carry a rearrangment test transgene. The methylation status of the transgene varies, depending on the background mouse strain. When the transgene is bred into the C57BL/6 strain, it is completely methylated and not rearranged in lymphoid organs. After several generations of crossing into DBA/2 or SJL the transgene becomes unmethylated and rearranges at high frequency. A strain specific modifier of DNA methylation (*Ssm-1*) was mapped close to the Friend virus susceptibility locus (*Fv-1*) on mouse chromosome 4.

Rearranged transgenes from spleen, bone marrow and thymus of adult mice or fetal liver were cloned and sequenced. A great variety of joints was found, with about 1/3 being in the correct reading frame. Small deletions into the V- and J- coding ends as well as N region additions contributed to the variability. The fetal joints showed no N regions. Since no functional immunoglobulin (Ig) gene can be created from this artificial test gene, the data indicate that the rearrangement mechanism of the fetus differs from that of the adult.

Introduction

The rearrangement of Ig genes is controlled at several levels. Besides a requirement for the preB and preT cell spcific V(D)J recombinase, the target genes must be "accessible" (Blackwell and Alt 1989). We have constructed a rearrangement test gene, pHRD (Fig.1), which, when transfected into recombinase positive preB cells, is correctly rearranged in nearly every transfectant (Engler and Storb 1987; Engler et al. 1991a). In order to investigate competency for rearrangement during development, we produced transgenic mice with the pHRD test gene (Engler et al. 1991b; Engler et al. 1992). This report summarizes our findings with these mice.

Results and Discussion

The transgene is differentially methylated in different mouse strains

The pHRD transgenic mice were first propagated by crossing with C57BL/6 mice (Engler et al. 1991b). Unexpectedly, there was no rearrangment of the test gene in lymphoid organs. An analysis using methylation sensitive restriction enzymes showed that the transgenes were completely methylated in all tissues of the mice, suggesting that perhaps the transgenes were inaccessible to the V(D)J recombinase. The transgenic mice were then crossed with two other mouse strains, SJL and DBA/2. After two generations of such crosses, partial or complete undermethylation of the transgenes was seen (Fig.2) and additional crosses led to complete undermethylation in all cases. The undermethylation paralleled rearrangement of the test gene. This could be easily detected in spleen, lymph nodes and bone marrow by Southern blot analysis. Thus, hypermethylation prevented the rearrangement.

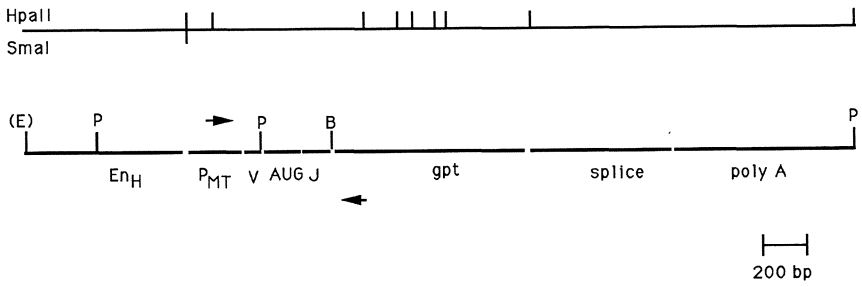


Fig.1. Map of the rearrangement test gene pHRD. The transgene pHRD contains the heavy chain intron enhancer (En_H), the metallothionein promoter (P_{MT}), a portion of Vκ including the RSS (V), a portion of the rat preproinsulin gene including the translation initiation region (AUG), part of Jκ1 including the RSS (J), the E.coli xanthine-guanine phosphoribosyl transferase gene (gpt), and the SV40 splicing (splice) and polyadenylation (poly A) signals. Primers for PCR are indicated by arrows. Restriction sites are indicated as E=EcoRI (eliminated in the transgene), B=BamHI, P=PstI (rearrangement deletes the middle PstI site). The methylation sensitive restriction sites HpaII (H) and SmaI (S) are shown.

Mapping the strain-specific modifier (*Ssm-1*)

When pHRD transgenic mice on a DBA/2 background in which the test genes are unmethylated are crossed with C57BL/6 mice, the transgenes become completely methylated in the first generation offspring (Engler et al. 1991b). We used this dominant C57BL/6 effect to map the modifier gene (*Ssm-1*) in BXD recombinant inbred mice. Males with unmethylated transgenes were crossed with BXD females and the methylation status of the transgenes was determined by Southern blots. The offspring gave one of two patterns: either complete methylation (as if bred to C57BL/6) or unmethylated (like the parent). We found an exact concordance of methylation with the *Fv-1* locus on chromosome 4.

Additional crosses with various inbred strains of mice which are either *Fv-1*^{b} or *Fv-1*^{n} showed no absolute correlation between pHRD methylation and *Fv-1*, indicating that *Ssm-1* and *Fv-1* are different genes.

Possible function of *Ssm-1*

The strain specific results described above were the same in 13 of 13 independent transgenic lines: on a C57BL/6 background the transgenes are hypermethylated. Many of the lines have been crossed with DBA/2, leading to undermethylation of the transgenes. Thus, the target for *Ssm-1* is the pHRD transgene independent of chromosomal position effects. We are in the process of defining the target sequences within pHRD and possible endogenous targets in C57BL/6 mice.

We are also trying to determine when in early development the *Ssm-1* effect is first seen. So far we know that in 7.5 day embryos the difference between undermethylation in DBA/2 and hypermethylation in C57BL/6 is clearly established.

What may be the action of *Ssm-1*? We assume that it is not itself a methylase, because the overall DNA methylation is the same in the different mouse strains and it appears that a single methyltransferase is responsible for the maintenance methylation in the mouse genome as well as presumably the *de novo* methylation early in embryogenesis (Bestor et al. 1988). It is perhaps more likely, that *Ssm-1* interacts with certain DNA sequences resulting in their priming for methylation by the classical methyltransferase (Fig.3). It would seem likely that mouse strains which are *Ssm-1* negative have other modifier genes which interact with different targets. Perhaps the function of this type of modifier is to promote an overall pattern of DNA methylation which aids in the regulated expression of specific genes.

Variability of rearranged pHRD joints

Rearrangement of the pHRD test gene occurs in mice in which the multiple transgene copies are partially or completely unmethylated (Engler et al. 1991b; P.E. and U.S., in

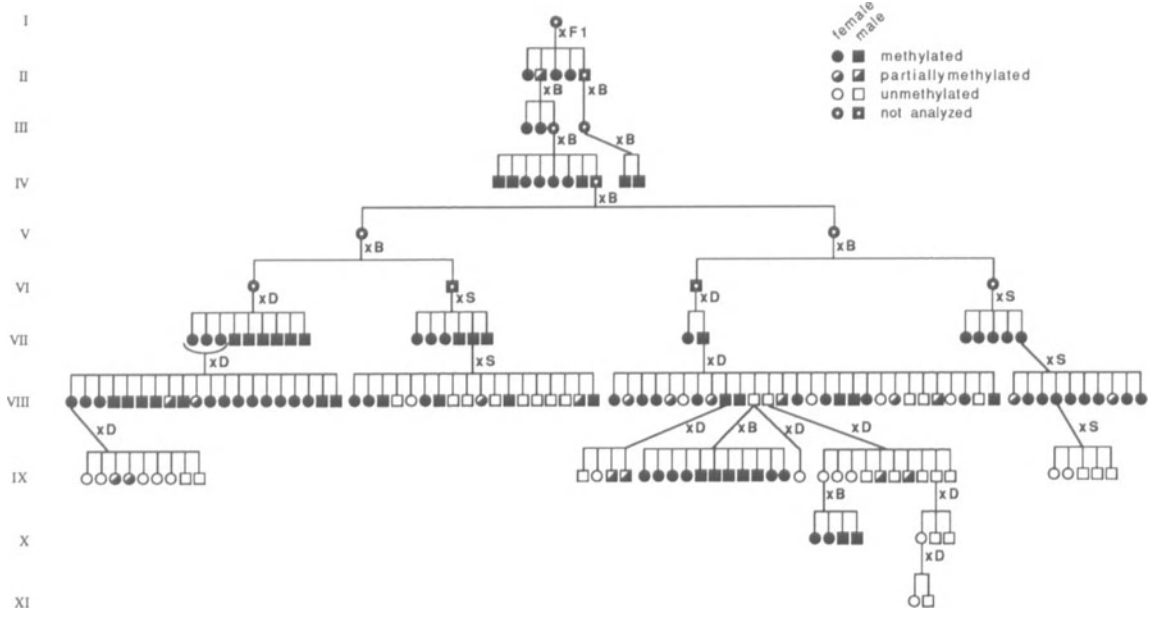


Fig.2. Pedigree of the pHRD transgenic line #342-2. Only offspring that inherited the transgene are shown; all mice were hemizygous for the transgene locus. The founder mice were derived from (B6xSJL)F1 eggs fertilized with F1 sperm. The initial breedings were to F1 males; thereafter the lines were crossed to B6 (B), DBA/2 (D), or SJL (S) strains as indicated. The methylation status was assessed by Southern blots of HpaII digested tail DNAs probed with gpt. All mice with a partially or completely unmethylated phenotype showed rearrangement of the test gene in lymphoid organs, but not in brain, liver, kidney or tail.

preparation). We have made a more detailed analysis in a transgenic line (#342-2) with 7 copies of pHRD. It appears that the partial methylation represents a mixed mosaicism, where in different cells varying numbers of the total CpG sites are methylated (Engler et al. 1991b). We do not know yet, how the different methylation patterns relate to the chromatin status of the transgenes (Fig.3). We also do not know, how the fine mapping of the methylated CpGs relates to accessibility to the V(D)J recombinase. Apparently, in a given cell, rearrangement can occur despite methylation of many of the potential methylation sites (Engler et al. 1991b; P.E. and U.S., in preparation).

Since the pHRD test gene does not encode functional Ig molecules it was interesting to analyze the VJ joints produced in these mice in order to obtain a sampling of unselected joints. DNA was prepared from lymphoid tissues and a stretch around the rearranged joint of pHRD was amplified by PCR, cloned and sequenced (P.E. et al. 1992). The joints were shown to resemble those found in endogenous Ig genes with no or moderate deletions (maximally ten nucleotides) into the V or J coding regions. Inverted repeats, often of considerable length, were seen. The study of joints arising from the pHRD transgene has allowed an appreciation of the enormous variability encoded by a single V-J gene pair. We sequenced 76 joints, which were all different, except for six which occurred two, three, or five times in the same or different organs. About one third of the joints are in frame with respect to the translational codons for the V and J region, as expected from random associations.

It had been found that endogenous Ig (and TCR) genes of fetal mice lack N nucleotide additions, whereas in adult joints such additions are frequent (Feeney 1990; 1991a and b; Meek 1990). This difference could be either mechanistic or due to selection of special repertoires at the different stages of mouse development. The pHRD transgene represents a neutral target to answer this question.

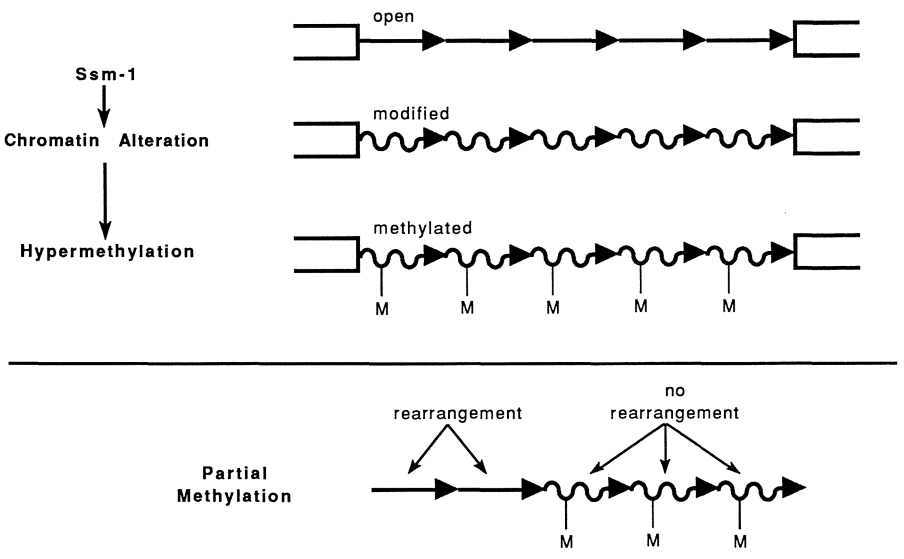


Fig.3. Model of the relationship of *Ssm-1* function to the rearrangement of the pHRD transgene. The five horizontal arrows represent tandem transgene copies. The straight arrows indicate unmodified DNA, the wavy arrows, DNA which has been modified by *Ssm-1*. The open boxes represent endogenous mouse DNA at the integration site; it is unknown if the *Ssm-1* effect spreads into flanking regions. M=methylation at all HpaII sites (see Fig.1). Only transgenes in which at least one HpaII site is unmethylated are rearranged. It is not yet known if, in the partial methylation state, the *Ssm-1* modified chromatin (wavy line) persists.

Normally, N regions are mainly seen in H genes which are rearranged in early preB cells. However, the pHRD transgene contains the heavy chain enhancer and its Vκ-Jκ joints are probably rearranged in early preB cells, because they are found to be rearranged in early fetal liver cells. No N regions were found in seven different joints from 15.5 day fetal liver, but 74% of joints from adult spleen, thymus or bone marrow showed N regions. This finding suggests that the rearrangment process is different in fetal and adult lymphoid tissues. This may be due to different levels of a terminal transferase (TdT) as has been suggested by others (Feeney 1990; Meek 1990). However, fibroblasts which are induced to rearrange Ig test genes by the expression of RAG-1 and RAG-2 genes show occasional N regions despite the apparent absence of TdT (Schatz et al. 1992). It will probably require a cell free system of V(D)J recombination to determine the molecular basis of the fetal/adult difference. Since normally N nucleotides are mainly seen in H genes whose rearrangment precedes that of L genes, perhaps the adult early preB cell has a different recombinase composition than that of the more mature preB cell, and the latter more closely resembles the fetal recombinase.

Acknowledgements

This work was supported by NIH grants HD23089 and AI24780. P.E. was supported by NIH training grant CA09594. E.K. and A.W are supported by NIH training grants GM07183 and GM07197 respectively. L.D. had a postdoctoral fellowship from the National Multiple Sclerosis Society.

References

Bestor T, Laudano A, Mattaliano R, Ingram V (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. J Mol Biol 203:971-983

Blackwell TK, Alt FW (1989) Mechanism and developmental program of immunoglobulin gene rearrangement in mammals. Annu Rev Genet 23:605-636

Engler P, Storb U (1987) High-frequency deletional rearrangement of immunoglobulin k gene segments introduced into a pre-B-cell line. Proc Natl Acad Sci USA 84:4949-4953

Engler P, Roth P, Kim JY, Storb U (1991a) Factors affecting the rearrangement efficiency of an Ig test gene. J Immunol 146:2826-2835

Engler P, Haasch D, Pinkert CA, Doglio L, Glymour M, Brinster R, Storb U (1991b) A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci. Cell 65:939-947

Engler P, Klotz E, Storb U (1992) N-region diversity of a transgenic substrate in fetal and adult lymphoid cells. submitted

Feeney AJ (1990) Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J junctional sequences. J Exp Med 172:1377-1390

Feeney AJ (1991a) Junctional sequences of fetal T cell receptor β chains have few N regions. J Exp Med 174:115-124

Feeney AJ (1991b) Predominance of the prototype T15 antiphosphorylcholine junctional sequence in neonatal pre-B cells. J Immunol 147:4343-4350

Meek K (1990) Analysis of junctional diversity during B lymphocyte development. Science 250:820-823.

Schatz DG, Oettinger MA, and Schlissel MS (1992) V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 10:359-383

Regulation of Switch Recombination to the Murine γ1 Gene

W. DUNNICK[1], L. ELENICH[1], J. BERRY[1], D. ALBRECHT[2], J. STAVNEZER[2], and J. LATHAM CLAFLIN[1]

[1]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI and
[2]Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA

INTRODUCTION

The immunoglobulin heavy chain switch, in which a variable region gene is rearranged from association with the Cμ gene to association with a Cγ, Cε, or Cα gene, is mediated by a recombination event between switch (S) regions [reviewed in 1]. Switch regions are 2 to 10 kb segments of simple sequences repeated in tandem and are found upstream of each of the heavy chain C genes, except Cδ [1]. Switch recombination is regulated in a gene-specific way; individual B cells direct recombination to one, or sometimes two, CH genes out of the six possible genes. For example, B cells treated with a mitogen, like lipopolysaccharide (LPS), and moderate amounts of interleukin 4 (IL4) switch almost exclusively to the γ1 gene [2, 3].

This gene specificity is likely to be mediated by changes in the accessibility of the CH gene and switch region [reviewed in 1 and 4]. The change in accessibility is best evidenced by transcription of the germline CH gene and switch region [5, 6]. There is a strict correlation between induction of germline transcription and switch recombination. In B cells activated by LPS or T cells, certain cytokines induce transcription and then DNA rearrangement of a specific heavy chain gene. In a manner similar to other CH genes, IL4-induced transcription of the γ1 gene begins upstream of Sγ1 and continues through the CH gene. The first exon, called Iγ1, is about 400 bp long (with a variable 5′ end due to variable transcription start sites) and is spliced to the C region exons [7, 8]. The promoter region has been extensively characterized by transfections of reporter gene constructs into tissue culture cells [9].

One of our goals is to define the *cis* acting DNA elements that direct γ1-specific accessibility for both transcription and switch recombination. To this end, we have constructed transgenic mice with an intact γ1 gene or parts of the γ1 promoter region linked to reporter genes. To characterize the *trans* acting factors that bind to these DNA elements, we have attempted to immortalize cells making these factors by transformation with SV40 T antigen.

RESULTS AND DISCUSSION

We have prepared three lines of transgenic mice with a construct containing the Sγ1 region, the $C\gamma1^a$ region, and about 4 kb of 5′ and 3′ flanking sequences (total size = 17 kb). Two of the lines (from founders #45 and #46) have three copies of the transgene; the third line, from founder #3 has more than 20 copies of the transgene. We prepared splenic B cells from these transgenic mice and from non-transgenic littermates [10]. Cultures of these B cells were stimulated with LPS or with LPS and IL4 (100 units per ml). After three days, total RNA was prepared from the

cells in these cultures and examined for the presence of Iγ1 transcripts by S1 nuclease analysis [11]. In the Iγ1 exon there are only two positions of nucleotide polymorphism between the endogenous genes and transgenes. Therefore, this S1 analysis measured simultaneously transcription of both types of genes. Nevertheless, the transgenes from the founder #3 line seem to have been expressed, as B cells from these mice made excess Iγ1 transcripts compared to B cells from non-transgenic littermates (32.4 vs. 3.9, Table 1A). To confirm that some of the transcripts detected by the S1 nuclease experiment derived from the transgene, we have amplified them by the polymerase chain reaction. Analysis for transgene and endogenous gene-specific nucleotides [12] revealed that both types of transcripts are found in the population.

Is transcription from the transgene properly regulated? No Iγ1 transcripts were detected in thymus (Table 1A), so the transgene was not transcribed in this IL4-responsive lymphoid tissue. Additionally, Iγ1 transcripts were detected in B cells after LPS + IL4 stimulation, but not after LPS stimulation. Production of these transcripts corresponded to that from the endogenous gene; significant amounts of Iγ1 transcripts were detected only upon LPS + IL4 stimulation of B cells. Therefore, either the transgenes were expressed with proper regulation, or they were not expressed at all. We have strong indications that the transgenes in #3 founder offspring and in #46 founder offspring are expressed, and we are testing by similar technology offspring of the #45 founder.

The results from transgenic mice with the complete γ1 gene and some flanking sequences (Table 1) suggest that these transgenes can be properly regulated. It might be possible for the transgenic heavy chain to be expressed as protein if switch recombination could occur by translocation or by trans-splicing. However, the B cells in these mice do not secrete $\gamma 1^a$ protein from the transgene as assayed by an allotype specific ELISA. At most, 1 in 100,000 γ1 protein molecules arise from the transgene; the remainder are encoded by the endogenous locus. This tiny level of transgene protein expression needs to be substantiated by more sensitive assays. These results contrast with results from transgenic mice with VDJ-Sμ-Cμ transgenes, which seem to recombine with the endogenous locus by

Table 1. Germline γ1 transcription by Iγ1-Sγ1-Cγ1 transgenic mice.

	Founder	Cells and Treatment	Relative Iγ1 transcripts
A.	Non-Tg	Splenic B--LPS	0.6
		Splenic B--LPS + IL4	3.9
	#3	Splenic B--LPS + IL4	32.4
		Thymocytes	0.3
B.	#3	Splenic B--LPS	1.0
		Splenic B--LPS + IL4	81
	#45	Splenic B--LPS	1.0
		Splenic B--LPS + IL4	53
	#46	Splenic B--LPS	1.0
		Splenic B--LPS + IL4	78

In part A, the amount of Iγ1 transcripts (in arbitrary units) as detected by S1 nuclease analysis was corrected by the amount of actin transcripts in the same assay tube. In part B, the amount of Iγ1 transcripts were normalized to S1 protection by the tRNA control.

Table 2. Expression of transgenic luciferase directed by the γ1 promoter.

Splenic B cell treatment	Luciferase activity (light units*)
LPS	34
IL4	115
LPS + IL4	1041

*The value from the no extract control (230 light units) was subtracted from each experimental result.

translocation or perhaps produce VDJ-Cγ transcripts by trans-splicing [13-15]. There are a few differences in the experiments that would explain the different activity of the two types of genes; one is the much greater transcriptional activity of the VDJ-Sμ-Cμ type of transgene [13, 14].

Selsing and his colleagues have shown that switch recombination can occur by chromosomal translocation [13]. However, it is not clear that switch translocations in VDJ-Sμ-Cμ transgenic mice occur at a moderate rate, or occur at a very low rate and are under strong selection due to immunization with an antigen to which the transgenic VDJ binds. In our Iγ1-Sγ1-Cγ1 transgenic mice, switch recombination by translocation between switch regions must occur at a very low rate, if at all. This result re-emphasizes how rare translocations between switch regions and the c-*myc* oncogene must be, even though such translocations are detectable by the dramatic phenotype they cause.

To better define the DNA sequences responsible for γ1-specific transcription, we have constructed mice with 1491 bp 5′ and 200 bp of the Iγ1 exon (using a 1.7 kb *Bgl*II fragment from upstream of the Sγ1 region) fused to a luciferase reporter gene. Preliminary results indicated that luciferase was expressed in B cells only after stimulation with LPS and IL4 (Table 2). The construct may have been slightly responsive to either LPS or IL4, although the level of expression from these transgenes was so low that such questions could not be resolved.

It would be of great utility if one had a cell line that constitutively produced the factors that mediate γ1 accessibility. We have attempted to immortalize such cells with SV40 T antigen. This approach has been used to generate tumors of unusual cell types [16, 17]. Two hundred bp of Iγ1 exon and 2000 bp of 5′ flanking region were fused to T antigen coding sequences, so that T antigen expression would be under control of this promoter region. Four lines of transgenic mice were prepared using this construct. Three of the lines (#237, #273, and #274 founders) made low levels of T antigen transcripts in B cells. In some experiments T antigen transcripts were found in thymocytes, in other experiments they were not (data not shown). Offspring of the fourth line, from the #220 founder, have never expressed T antigen transcripts, even though the founder herself had a very low level of transcripts. We tested

Table 3. Expression of Iγ1 transcripts by T antigen transgenic mice

	Relative level of Iγ1 transcripts expressed*			
Founder: Treatment	#220	#237	#273	#274
LPS	1.2	12.6	3.0	2.4
LPS + IL4	2.4	14.7	8.1	6.6

*The amount of Iγ1 transcript (in arbitrary units) relative to an internal actin probe.

splenic B cells from all four lines of T antigen transgenic mice for expression of Iγ1 transcripts from the endogenous locus (Table 3). In contrast to normal mice (refer to Table 1), mice which expressed the T antigen transgene also expressed significant amounts of endogenous Iγ1 transcripts when stimulated with LPS alone. The level of Iγ1 transcripts can be increased further with IL4 stimulation. However, compare the 6.5-fold induction by LPS and IL4 compared to LPS alone (non-transgenic mouse in Table 1A) with 1.2-fold to 2.7-fold induction in T antigen transgenic mice (Table 3).

In B cells from non-transgenic mice, germline γ1 transcription is easily detected only after activation with both LPS and IL4; we have not detected Iγ1 transcription in normal B cells or other transgenic mice with LPS stimulation alone (for example, Table 1). In B cells with a transgene linking T antigen to the γ1 promoter, abundant germline transcripts of the endogenous γ1 gene are detected after stimulation with LPS alone. We hypothesize that when B cells in these transgenic mice are activated *in vivo* to express endogenous germline γ1 transcripts, coordinate expression of T antigen allows such cells to divide several more times, or perhaps even to become immortalized. The presence of such cells is then detected *in vitro* by LPS-driven expansion of their numbers and/or RNA content. (Iγ1 transcripts might be detectable in fresh B cells from T antigen transgenic mice; we have not investigated this question as yet.) One future goal is to increase the numbers of these cells by more intense or prolonged stimulation of the B cells with IL4, either *in vivo* or *in vitro*. A second goal is to obtain tumors of these cells. Tumors could arise if naturally occurring secondary tumorigenic events occur, or by our providing a second activated oncogene by transgenic technology. We hope that such tumors would express factors that bind to the γ1 promoter and mediate its accessibility and transcription.

We are not sure that T antigen expression is restricted to B cells in the process of antigen-driven differentiation. Some results with the γ1 promoter region we have used in the T antigen transgenic mice suggest that the promoter is active in B cells only when they are activated by LPS and IL4, but not in B cells stimulated with LPS nor in T cells. Other results suggest a less stringent tissue and activator control. Better definition of the *cis* acting sequences important to the production of germline γ1 transcripts might allow us to better target T antigen expression to the appropriate cell at the appropriate time.

ACKNOWLEDGEMENTS:

This work was supported by grants from the National Institutes of Health: CA39068 (to W. D.), AI08546 (to D. A.), AI23283 (to J. S.), and (AI12533 to J. L. C.)

REFERENCES:

1. Lutzker SG, Alt FW (1989) Immunoglobulin heavy-chain class switching. In: D. E. Berg and M. Howe (eds) Mobile DNA. ASM Press, Washington, D C, pp. 693-714
2. Radbruch A, Muller W, Rajewksy K (1986) Class switch recombination is IgG1 specific on active and inactive IgH loci of IgG1-secreting B-cell blasts. Proc Natl Acad Sci USA 83:3954-3957

3. Kepron MR, Chen Y-W, Uhr JW, Vitetta ES (1989) IL-4 induces the specific rearrangement of γ1 genes on the expressed and unexpressed chromosomes of lipopolysaccaride-activated normal murine B cells. J Immunol 143:334-339
4. Esser C, Radbruch A (1990) Immunoglobulin class switching: Molecular and cellular analysis. Ann Rev Immunol 8:717-735
5. Stavnezer-Nordgren J, Sirlin S (1986) Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. EMBO J 5:95-102
6. Yancoupoulos GD, DePinho RA, Zimmerman KA, Lutzker SG, Rosenberg N, Alt FW (1986) Secondary rearrangement events in pre-B cells: VHDJH replacement by a LINE-1 sequence and directed class switching. EMBO J 5:3259-3266
7. Berton MT, Uhr JW, Vitetta ES (1989) Synthesis of germline γ1 immunoglobulin heavy-chain transcripts in resting B cells: Induction by interleukin 4 and inhibition by interferon γ. Proc Natl Acad Sci USA 86:2829-2833
8. Xu M, Stavnezer J (1990) Structure of germline immunoglobulin heavy-chain γ1 transcripts in interleukin 4 treated mouse spleen cells. Dev. Immunol. 1:11-17
9. Xu M, Stavnezer J (1992) Regulation of transcription of immunoglobulin germline γ1 RNA: Analysis of the promoter/enhancer. EMBO J 11:145-155
10. Coffman RL, Carty J (1986) A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. J Immunol 136:949-954
11. Schultz C, Elenich LA, Dunnick WA (1991) Nuclear protein binding to octamer motifs in the immunoglobulin γ1 switch region. Intl Immunol 3:109-116
12. Singer-Sam J, LeBon JM, Dai A, Riggs AD (1992) A sensitive, quantitative assay for measurement of allele-specific transcripts differing by a single nucleotide. PCR Meth Appl 1:160-163
13. Gerstein RM, Frankel WN, Hsieh C-L, Durdik JM, Rath S, Coffin JM, Nisonoff A, Selsing E (1990) Isotype switching of an immunoglobulin heavy chain transgene occurs by DNA recombination between different chromosomes. Cell 63:537-548
14. Shimizu A, Nussenzweig MC, Han H, Sanchez M, Honjo T (1991) Trans-splicing as a possible molecular mechanism for the multiple isotype expression of the immunoglobulin gene. J Expt Med 173:1385-1393
15. Nolan-Willard M, Berton MT, Tucker P (1992) Coexpression of μ and γ1 heavy chains can occur by a discontinuous transcription mechanism from the same unrearranged chromosome. Proc Natl Acad Sci USA 89:1234-1238
16. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, Hanahan D, Baekkeskov S (1988) Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. Proc Natl Acad Sci USA 85:9037-9041
17. Windle JJ, Weiner RI, Mellon PL (1990) Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol Endo 4:597-603

Protein Recognition Motifs of Sγ3 DNA Are Statistically Correlated with Switch Recombination Breakpoints

R. A. WUERFFEL and A. L. KENTER

Department of Microbiology and Immunology, University of Illinois
College of Medicine at Chicago, Chicago, Illinois 60680

1 Introduction

The immunoglobulin (Ig) heavy (H) chain class switch permits expression of a mature variable (VDJ) region with constant (C) regions other than Cμ (for review see [6]). Molecular analysis of IgH loci which have undergone isotype switching has led to the observation that this event results in the deletion of the Cμ gene and the intervening genomic sequences [18, 21]. The looping-out and deletion model for switch recombination predicts that the intervening DNA between switch (S) regions will be excised as a circle [9]. Circular excision products of Ig switch recombination have recently been isolated from mitogen stimulated spleen cells [7, 8, 14, 25]. This finding confirms that switch recombination occurs by the looping-out and deletion mechanism.

Switch recombination focuses on switch DNA, regions of tandemly repetitive sequence located upstream of each C_H gene and produces a new hybrid S DNA combination (Sμ-Sx) [2, 10, 23]. Sequence analyses of switch recombination joints have revealed that both donor and acceptor breakpoints fall within the tandem repeats in all events derived from B cells [7, 8, 14, 25]. There are no obvious consensus recombination signal sequences, though the pentamers, GAGCT, GGGGT, and GGTGG, components of all the S regions, are often found at or close to recombination joints [16]. There are two models on the mechanism of switch recombination. The first emphasizes the sequence divergence of S regions and suggests that there are switch region specific recombinases which are responsible for the recombination [2]. The second highlights the short nucleotide repeats which are common amongst all the switch regions and suggests that switch recombination is due to homologous recombination [10]. Little, however, is known regarding the molecular details of this mitotic recombination event.

Recombination requires that the two recombining DNA helices be in close apposition and that the spatial arrangement of the juxtaposed DNA sequences will determine the product of the recombination event [5, 19, 24, 26]. Formation of DNA-nucleoprotein complexes is a common feature of such processes as transcription, site-specific recombination and the initiation of DNA replication [3]. Switch recombination involves DNA rearrangements between sequences distanced by 50-100 kb of genetic material [22]. Based on these considerations we reasoned that juxtaposition of donor and acceptor switch DNA prior to recombination may be facilitated by sequence specific DNA binding proteins. Activation of normal splenic B cells with LPS

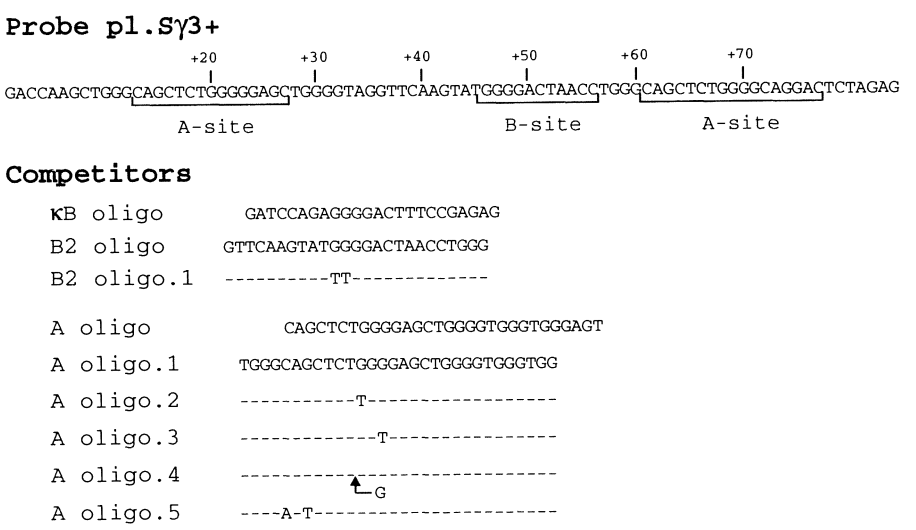


Fig.1. List of probe and competitors. For simplicity, only the coding strands are shown. The A-site and B-site sequences in probe pl.Sγ3+ are bracketed. All oligonucleotide competitors were chemically synthesized and annealed with their complementary strands for use in competition experiments. Dashes indicate positions of identity with the B2 oligo in the case of B2 oligo.1 and with A oligo.1 for the series of A oligos.

causes switching from IgM expression to a high level of IgG3, some IgG2b, but no IgG1 production [11]. We have detected an Sμ sequence specific DNA binding protein in these cells, termed SNUP (switch nuclear μ-binding protein), which recognizes a portion of the Sμ tandem repeat, GAGCTGGGGTGAGCT [29]. We have also identified two DNA binding proteins which specifically interact with two discreet portions of the Sγ3 tandem repeat [28]. The DNA sequences at which recombination occurs in the Sγ3 switch region are statistically correlated with the binding sites of these two switch binding proteins.

2 Experimental Results

To ascertain whether Sγ3-specific binding proteins are present in activated B cells, binding reactions were performed with a probe containing 1.5 repeats of the Sγ3 genomic sequence and nuclear extracts prepared from splenocytes which had been stimulated with LPS/DxS for 44 hours (Fig.1). Analysis by electrophoretic mobility shift assay (EMSA) resulted in resolution of 5 distinct complexes (Fig.2). Two complexes were judged to be non-specific by methylation interference analysis since no footprint was evident for B3 and the binding of B5 is dependent on pUC polylinker DNA [28]. The methylation interference patterns for the other three complexes, B1, B2, and B4, revealed that their binding focuses on Sγ3 sequences.

2.1 Delineation of the SNIP Recognition Motif

The methylation interference analysis showed that the binding sites for B1 and B2 overlap [28]. Extensive competition experiments demonstrated that B1 binding is dependent upon a unique sequence in the probe [28]. The B1 complex does not recognize the Sγ3 consensus tandem repeat and is therefore predicted to bind to only one site in Sγ3. The B2 recognition motif, as it appears in the probe, also varies from the consensus Sγ3 repeated sequence. In

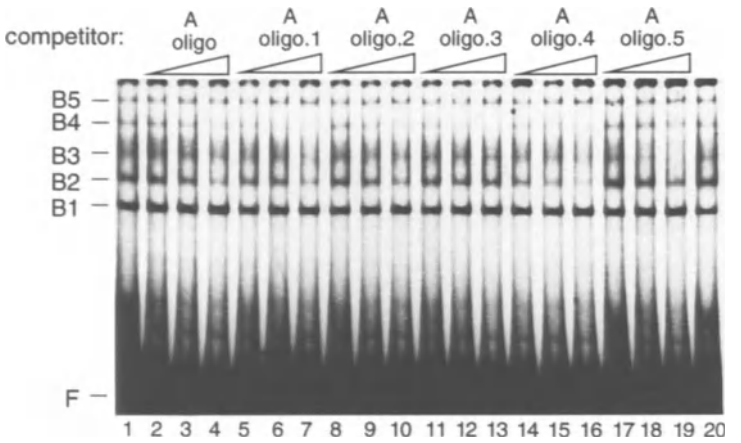


Fig. 2. Competition binding assays to test the A-site specificity of the Sγ3 B4 complex. Probe pl.Sγ3+ (10,000 cpm) and nuclear extract from LPS/DxS stimulated B cells (5 μg) were incubated in binding reactions either in the absence (lanes 1 and 20) or presence of increasing amounts (5.6 ng, 22.5 ng, and 90 ng) of the indicated competitor DNA (lanes 2-19). The complexes were resolved by EMSA on a 6% polyacrylamide gel.

contrast to B1, the B2 factor binds to the consensus sequence. Hence, the B2 protein could potentially interact with many repeats in Sγ3.

Examination of the B2 recognition motif revealed a striking similarity with NF-κB binding sites [13]. It is known that LPS stimulation of B cells causes NF-κB to be activated and translocated to the nucleus [1]. Likewise, the B2 binding protein is found only in the nuclei of mitogen stimulated B cells [28]. In a previous set of studies it was shown that an oligo competitor containing the κB site derived from the intron enhancer of the κ light chain gene effectively competed with the Sγ3 genomic probe for B2 binding [28]. When the κB oligo is used as probe in a competition binding assay the B2 oligo competitor effectively competes for the lower bound complex (Fig.3). The lower bound complex comigrates with the single complex formed with the B2 oligo probe [28]. Moreover, the B2 recognition sequence as found in the consensus Sγ3 repeat also competed for the lower complex, albeit at reduced affinity ([28] and data not shown). B2 oligo.1, containing two mutations in the B2 binding site, does not compete (Fig.1 and Fig.3). These results establish the Sγ3 B2 motif as an NF-κB binding site.

Since a number of different proteins have been shown to bind to κB sites it was necessary to more directly determine the relationship between the B2 binding protein and NF-κB. When an antiserum specific for the p50 subunit of NF-κB was used in supershift assays, the bound complex formed with the B2 oligo probe and the lower bound complex seen with the κB oligo probe were supershifted [28]. No supershifting occurred when preimmune serum was used. This directly demonstrated that the B2 factor contains the NF-κB p50 subunit or a highly related protein. We have designated the B2 binding factor as switch nuclear protein or SNIP/NF-κB. The position of the Sγ3 SNIP/NF-κB recognition motif within the Sγ3 tandem repeat is presented in Figure 4.

2.2 Delineation of the SNAP Recognition Motif

The methylation interference footprint of the B4 complex indicated that it bound to a discreet portion of the Sγ3 tandem repeat. To more finely analyze B4 binding, a new probe, pl.Sγ3.A, was prepared from a recombinant plasmid containing the Sγ3 A oligo (Fig.1) [28].Additional methylation interference and competition assays were performed using partially

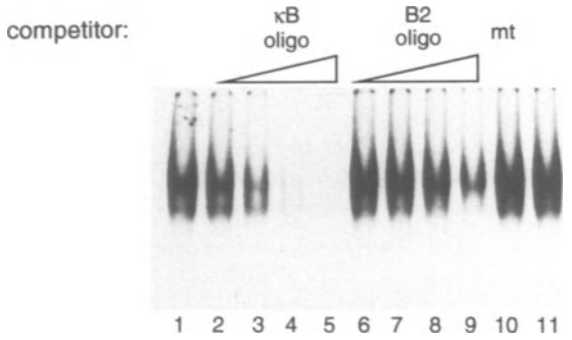


Fig. 3. Competition binding assay to analyze NF-κB recognition of the Sγ3 B-site sequence. End labeled κB oligo was used as probe (10,000 cpm) in binding reactions with nuclear extract from LPS/DxS stimulated B cells (10 μg). The specificities of the bound complexes were tested by competition with increasing amounts (.009 ng, .35 ng, 1.4 ng, and 5.6 ng) of unlabeled κB oligo (lanes 2-5), B2 oligo (lanes 6-9) or 5.6 ng of the mutant (mt), B2 oligo.1 (lane 10). The complexes were resolved by EMSA on a 6% polyacrylamide gel.

purified extracts [28]. These studies resulted in definition of the A binding site (Fig.4). To confirm that the B4 complex is truly specific for the A-site, an EMSA was carried out using the series of A oligos as competitors (Fig.2). The footprint data indicated that the A-site binding factor strongly contacts the G residue of the non-coding strand which is complementary to the first C of the A oligo. It was observed that binding affinity could be increased by moving the A-site to a more centered location within the oligo (compare lanes 2-4 to lanes 5-7). To permit a direct comparison of the effects of mutations within the A-site, oligo.1 through oligo.5 all have centered sites. A oligo.2 has a T substituted for a strongly contacted G (position +12 in the oligo). This single change results in a greater than 4-fold reduction in the ability of this oligo to compete for B4 binding as compared to A oligo.1. A further decrease in competition efficiency is observed with A oligo.5, in which two strongly contacted G residues have been changed to T residues. A oligo.3, in which the G at position +14 in the oligo sequence has been changed to a T, competes for B4 binding nearly as well as A oligo.1. This is consistent with the methylation interference studies which indicated that the G at that position is only weakly contacted by the A-site binding protein. A common, natural variation of the Sγ3 A-site motif has a run of 5, instead of 4, guanines following the CTCT. Efficient competition with A oligo.4, which contains this variant A-site, demonstrates that the insertion of an extra G does not adversely affect B4 complex formation. These data show that the B4 complex formed with the pl.Sγ3+ probe is indeed A-site specific. We have designated this DNA binding factor as switch nuclear A-site protein or SNAP. The SNAP recognition motif, positioned within the Sγ3 tandem repeat, is presented in Figure 4.

Fig. 4. Summary of the SNIP and SNAP recognition motifs. A representative Sγ3 49 bp repeat is presented with the B-site and A-site sequences boxed. G residues which are strongly (*) or moderately (o) contacted by the proteins, as determined by methylation interference, are shown.

3 Sγ3 Recombination Breakpoints Fall in SNIP and SNAP Recognition Motifs

Previous analyses of cloned, recombined switch regions have failed to define switch recombination as either site specific or dependent on extensive homologous base pairing between the donor and acceptor switch regions. It was of interest to re-examine the Sγ3 breakpoints with respect to the SNIP and SNAP binding sites. The sequences of 21 Sγ3 switch recombinants are available [7, 10, 14, 16, 17, 23, 27, 30]. The Sγ3 germline sequences surrounding these breakpoints are presented in Table 1. It is remarkable that of these 21 examples, 10 breakpoints occurred within A-sites which are 15 bp long. Six breaks mapped to B-sites which are 11 bp long. Only 5 breakpoints fell in the remaining 23 bp spacer sequences outside the A or B recognition sites (Fig.5). The distribution of 16 breaks in the A and B sites compared to 5 breaks in the remaining Sγ3 DNA is shown by χ^2 analysis to be significantly non-random with p=.04. It is also evident that the breakpoints are particularly focused to a subregion of the A-site. All 10 A-site breakpoints occurred within the 3'-most two thirds of the A-site sequences. When this region is compared to rest of the Sγ3 49-mer, χ^2 tests result in a high level of confidence that the concentration of breakpoints in this A-site subregion did not arise by chance, p=.002. Thus while the most significant focusing of recombination breakpoints is to A-sites, the B-site and the 4 bp spacer sequences also may serve as recombination substrates. The sequences downstream of the A-sites, in contrast, show a significant under-representation of recombination crossovers, with p=.025.

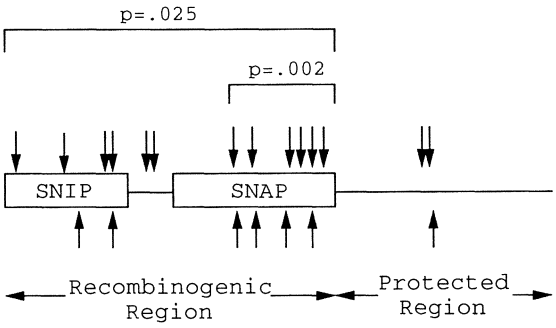


Fig. 5. Schematic diagram showing Sγ3 recombination breakpoints positioned on a single 49 bp repeat. Acceptor and donor breakpoints are indicated by arrows above and below the repeat, respectively. The levels of significance of breakpoint clustering in the bracketed areas, as determined by χ^2 tests, are shown.

Given the concordance of switch recombination breakpoints with the SNIP and SNAP recognition motifs we hypothesized that SNIP and SNAP may be functionally involved in focusing switch recombination to the B and A sites of Sγ3. We then wished to consider whether the sequences in which breakpoints occurred could be bound by SNIP and SNAP. The 21 sequences represent 16 different Sγ3 tandem repeats. All of the A-site sequences and 14/16 B-site sequences are easily recognizable by visual inspection and could presumably serve as binding sites for SNAP and SNIP. Many sites are variants of the consensus sequence and although each variant has not been directly tested for SNIP and SNAP binding, it is predicted that SNIP and SNAP could interact with most if not all these sites. The degenerate B-sites derive from repeats 1 and 44, at the extreme ends of the Sγ3 region where the 49 bp repeated sequence is less well conserved. It is of interest to note that both the B-site and A-site sequences are more highly conserved than the short and long spacer sequences. This may reflect the functional importance of the B an A site sequences in switch recombination.

Since Sγ3 contains multiple binding sites for SNIP and SNAP it is possible that these proteins facilitate the formation of a higher order architecture of the Sγ3 region. This may result

Table 1. Sγ3 Switch Recombination Breakpoints

Sγ3 germline sequences with breakpoints (▼)				Clone	Cell Type	Switch Type	Repeat No.	Ref.
B-site		A-site						
GGGGAACAGGT	TGGA	CAGCTCTGGGGAGC	TAGGGTAGTTGGGGGTGTGG	pCS23	B-cell	μ-γ3	14	7
GGGGACTAGGC	TGGG	CAGCTCTGGGGAGC	TAGGGTGGGTGGGATGTGGG	T2-2	B-cell	γ3-α	15	14
GGGGACCAGGC	TGGGGAG	CAGCTCTGGTGAGC	TAGGGTGGGTGAGGGTGT	pCS35	B-cell	μ-γ3	4	7
TACAGTAGAGC	TGGGG	CAGCCTTGGGGGATC	TGGGGGTAGGTGGGGTGT	pCS29	B-cell	μ-γ3	1	7
GGGAACCAGGC	TGGA	CAGCTCTGGGGAAAC	TGGGGTACATGGGGTTGTG	T2-26	B-cell	μ-γ3	28	30
GAGGACTAGGT	TGGG	CAGCTACAGGTGAGC	TGGGTTGGATGGAAATGTG	pCS19	B-cell	γ3-α	42	7
GGGGGCCAGGC	TGGA	CGGCTCTGGGGGTAGC	TAGGGTAAGTGAGGATGT	L32-52	B-cell	γ3-γ2b	12	14
GGGGGCCAGGC	TGGA	CGGCTCTGGGGGTAGC	TAGGGTAAGTGAGGATGT	L32-21	B-cell	μ-γ3	12	14
GGGGACCAGGC	TGAG	CAGCTCTCAGGGAGC	TGGGGAGGTGGAGCTATGG	T2-7	B-cell	γ3-α	11	14
GGGGACCAGGC	TGGG	CAGCCTGGGGAGC	TAGGGTAGGTGGGATGTGGGG	GAM3-2	B-cell	μ-γ3	4	27
GGGGACCAGGC	TGGG	CAGCTCTCAGGGAGC	TGGGGTGGGTGGGGTTGTG	GAM3-8	B-cell	μ-γ3	39	27
GGGGACCAGGG	TGGA	TAGCTGTGGGGGTGC	TGGGGTAGGTTAGAGCATG	pCS28	B-cell	μ-γ3	13	7
AGGGACCAGGC	TGGG	CAGCTCTGGGGGAGC	TAGGGTAGGTGGAAGTATA	180.2B2	Hybridoma	μ-γ3	8	23
AGGGAGCAGGC	TGGA	CAGCTCTGAGGGGAAGC	TAGAGTAAGTGTGGGTG	470	Hybridoma	μ-γ3	24	17
GGGGACCAGGC	TGGA	CAGCTCTCGGGGGAGC	TAGGGTAGGTTGGAGCAT	470	Hybridoma	γ3-γ1	25	17
GGGGACCAGGC	TGAG	CAGCTCTCAGGGAGC	TGGGGAGGTGGAGCTATGG	59.6C5	Hybridoma	μ-γ3	11	23
AGGAGCAGGC	TGGA	CAGCTCTGGAGGGAGC	TAGGATAAGTGAGGATGTG	198.5C8	Hybridoma	μ-γ3	32	16
GGGGACCAGGC	TGGG	CAGCTCTCAGGGAGC	TGGGGAGGTGGAGCTGTGG	198.5C8	Hybridoma	γ3I-γ3	37	16
GGGGAACAGGT	TGGA	CAGCTCTGGGGAGC	TAGGGTAGTTGGGGGTGTGG	J606	Myeloma	μ-γ3	14	23
TACAGTAGAGC	TGGGG	CAGCCTTGGGGGATC	TGGGGGTAGGTGGGGTGT	MPC11	Myeloma	γ3-γ2b	1	10
GAATAACTGCC	TGAAG	GGGCCACAGGGGAGC	TGGGGCTATCAGATCACA	I.29	Lymphoma	μ-γ3	44	16
GGGGACCAGGC	TGGG	CAGCTCTNGGGGAGC	TGGGGTAGGTTGGGAGTGT	Sγ3 consensus 49-mer				

in greater frequencies of recombination in certain areas of Sγ3. Examination of the 21 examples at hand reveals that 13 breakpoints mapped to the first third (repeats 1-15), 3 mapped to the second third (repeats 16-30), and 5 mapped to the last third (repeats 31-45) of the Sγ3 switch region. Such a distribution is non-random according to χ^2 analysis where p=.002. This confirms and extends earlier observations that breakpoints preferentially occur in the first third of Sγ3 [7, 23]. This finding may allude to a higher order structure of the Sγ3 switch region *in vivo*.

4 Conclusions

When all 21 examples of sequenced switch recombination breakpoints involving Sγ3 are considered, there is a statistically significant over-representation of breaks in the A-site/B-site regions of the tandem repeats. This suggests that these sequences of Sγ3 may serve as recombination substrates in isotype switching. In contrast, resolution of crossovers rarely occurs in the sequences directly downstream of A-sites, as evidenced by the dearth of breakpoints in these areas. Even more striking is the focusing of 48% of the breakpoints to a 10 bp sub-region of A-sites. In addition to serving as recombination substrates, the B-site and A-site sequences of the Sγ3 tandem repeats are recognition motifs for binding proteins, namely SNIP and SNAP. We find that the vast majority of breakpoint sites are at intact binding motifs.

By several criteria SNIP is indistinguishable from NF-κB. Possible roles for NF-κB in switch recombination are suggested by it's well studied characteristics. First, it is a transcription factor [13]. The work of several investigators has demonstrated that transcription across a given C_H region precedes switch recombination to that isotype (reviewed in [4]). The precise functions of this transcription and the sterile transcripts which result from it are still unclear. Perhaps SNIP/NF-κB plays a role in regulating the transcription across the Cγ3 locus. Second, NF-κB is known to bend DNA [20]. The bending of DNA is a critical and essential feature in certain prokaryotic site-specific recombination systems [15]. In bending Sγ3 DNA, SNIP/NF-κB may facilitate the formation of a higher order DNA-protein structure which is required for recombination. Third, NF-κB is composed of rel-related proteins and therefore contains rel dimerization domains [12]. Hence, SNIP/NF-κB could potentially mediate the synapsis of switch regions through protein-protein interactions.

The focusing of breakpoints to SNAP recognition motifs suggests an intimate role for SNAP in switch recombination. The precise functions of SNIP and SNAP in switch recombination must await fuller characterization of the proteins and the development of functional assays.

References

1. Baeuerle PA, Baltimore D (1990) The physiology of the NF-κB transcription factor. In: Cohen P, Foulkes JG (ed) Hormonal Regulation of Transcription. Elsevier-Biomedical, Amsterdam (Molecular Aspects of Cellular Regulation, vol6)
2. Davis MM, Kim SK, Hood LE (1980) DNA sequences mediating class switching in α-immunoglobulins. Science 209:1360-1365
3. Echols H (1986) Multipe DNA-protein interactions governing high-precision DNA transactions. Science 233:1050-1056
4. Esser C, Radbruch A (1990) Immunoglobulin class switching: molecular and cellular analysis. Annu Rev Immunol 8:717-735
5. Gellert M, Nash H (1987) Communication between segments of DNA during site-specific recombination. Nature 325:401-404
6. Gritzmacher CA (1989) Molecular aspects of heavy-chain class switching. CRC Crit Rev Immunol 9:173-200
7. Iwasato T, Arakawa H, Shimizu A, Honjo T, Yamagishi H (1992) Biased distribution of recombination sites within S regions upon immunoglobulin class switch recombination induced by transforming growth factor β and lipopolysaccharide. J Exp Med 175:1539-154

8. Iwasato T, Shimizu A, Honjo T, Yamagishi H (1990) Circular DNA is excised by immunoglobulin class switch recombination. Cell 62:143-149
9. Jack H-M, McDowell M, Steinberg CM, Wabl M (1988) Looping-out and deletion mechanism for the immunoglobulin heavy chain class switch. Proc Natl Acad Sci USA 85:1581-1585
10. Kataoka T, Miyata T, Honjo T (1981) Repetitive sequences in class switch recombination regions of immunoglobulin heavy chain genes. Cell 23:357-368
11. Kearney JF, Lawton AR (1975) B lymphocyte differentiation produced by lipopolysaccaride I. Generation of cells synthesizing four major immunoglobulin classes. J Immunol 115:671-676
12. Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, LeBail O, Urban MB, Kourilsky P, Baeuerle PA, Israel A (1990) The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell 62:1007-1018
13. Lenardo MJ, Baltimore D (1989) NF-κB: A pleiotropic mediator of inducible and tissue specific gene control. Cell 58:227-229
14. Matsuoka M, Yoshida K, Maeda T, Usuda S, Sakano H (1990) Switch circular DNA formed in cytokine-treated mouse splenocytes: evidence for intramolecular DNA deletion in immunoglobulin class switching. Cell 62:135-142
15. MoitosodeVargas L, Kim S, Landy A (1989) DNA looping generated by DNA bending protein IHF and the two domains of lambda integrase. Science 244:1457-1461
16. Petrini J, Dunnick W (1989) Products and implied mechanism of H chain switch recombination. J Immunol 142:2932-2935
17. Petrini J, Shell B, Hummel M, Dunnick W (1987) The immunoglobulin heavy chain switch: structural features of γ1 recombinant switch regions. J Immunol 138:1940-1946
18. Radbruch A, Sablitsky F (1983) Deletion of Cμ genes in mouse B lymphocytes upon stimulation with LPS. EMBO J 2:1929-1935
19. Sadowski P (1986) Site specific recombinases: changing partners and doing the twist. J Bact 165:341-347
20. Schreck R, Zorbas H, Winnaker E-L, Baeuerle PA (1990) The NF-κB transcription factor induces DNA bending which is modulated by its 65-kD subunit. Nucleic Acids Res 18:6497-6502
21. Shimizu A, Honjo T (1984) Immunoglobulin class switching. Cell 36:801-803
22. Shimizu A, Takahashi N, Yaoita Y, Honjo T (1982) Organization of the constant-region gene family of the mouse immunoglobulin heavy chain. Cell 28:499-506
23. Szurek P, Petrini J, Dunnick W (1985) Complete nucleotide sequence of the murine γ3 switch region and analysis of switch recombination sites in two γ3-expressing hybridomas. J Immunol 135:620-626
24. Travers A, Klug A (1987) DNA wrapping and writhing. Nature 327:280-281
25. vonSchwedler U, Jack H-M, Wabl M (1990) Circular DNA is a product of immunoglobulin class switch rearrangement. Nature 345:452-455
26. Wasserman SA, Cozzarelli NR (1986) Biochemical topology: applications to DNA recombination and replication. Science 232:951-960
27. Winter E, Krawinkel U, Radbruch A (1987) Directed Ig class switch recombination in activated murine B cells. EMBO J 6:1663-1671
28. Wuerffel R, Jamieson CE, Morgan L, Merkulov GV, Sen R, Kenter AL (1992) Switch recombination breakpoints are strictly correlated with DNA recognition motifs for immunoglobulin Sγ3 DNA binding proteins. J Exp Med 176:(In Press)
29. Wuerffel RA, Nathan AT, Kenter AL (1990) Detection of an immunoglobulin switch region-specific DNA-binding protein in mitogen stimulated mouse splenic B cells. Mol Cell Biol 10:1714-1718
30. Yoshida K, Matsuoka M, Usuda S, Mori A, Ishizaka K, Sakano H (1990) Immunoglobulin switch circular DNA in the mouse infected with Nippostrongylus brasiliensis: evidence for successive class switching from μ to ε via γ1. Proc Natl Acad Sci USA 87:7829-7833

Regulation of Transcription of the Germline Immunoglobulin α Constant Region Gene

Y.-C. A. LIN, P. SHOCKETT and J. STAVNEZER

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester MA 01655

Different classes of antibodies serve different effector functions and therefore the production of different classes must be regulated during an immune response. Numerous recent studies support the accessibility model for the regulation of heavy chain class switch recombination, as heavy chain (C_H) genes to which cells will switch have been shown to be hypomethylated and to be transcriptionally active (reviewed in Esser and Radbruch, 1990). Each of the unrearranged C_H genes has been shown to be transcribed prior to switch recombination to that particular C_H gene. The structures of all the C_H gene germline transcripts are identical, i.e. they are initiated 5' to the switch (S) regions and are transcribed in the same direction as and terminate near the same poly(A) sites as mature Ig mRNAs (Fig. 1). An exon 5' to the S regions (I or germline exon) is spliced to the normal acceptor site for mature mRNAs. The germline RNAs tend to have multiple initiation sites probably due to the fact that they do not have TATA boxes and at least one (germline α RNA) has multiple splice donor sites for the I exon.

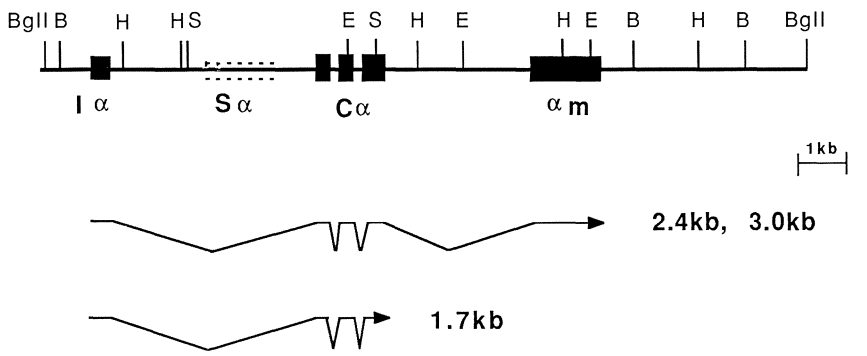


Fig. 1. Map of the unrearranged Ig Cα Gene in I/St mice and diagram of the predominant species of germline α RNA.

Current Topics in Microbiology and Immunology, Vol. 182

Most of the studies in our laboratory use a surface IgM+ B lymphoma cell line (I.29μ) that can be induced by treatment with lipopolysaccharide (LPS) to undergo class switch recombination to IgA (Stavnezer et al. 1985). Isotype switching in this cell line has been demonstrated to occur by classical switch recombination within the tandem repeated sequences located 5' to the Cμ and Cα genes (Stavnezer et al. 1985). I.29μ cells synthesize germline α transcripts and subclones of this line which switch at a high frequency express more of the germline α transcripts than subclones that switch at a lower frequency (Stavnezer-Nordgren and Sirlin, 1986; Stavnezer et al. 1988). These data plus studies in cytokine treated normal B cells have provided strong correlative evidence suggesting that switch recombination is directed by control of transcription. Germline transcripts corresponding to the C_H gene to which cells are directed to switch by cytokines are induced prior to switch recombination (Lutzker et al. 1988; Stavnezer et al. 1988). Thus, it is important to analyze the regulation of transcription of unrearranged C_H genes. We summarize here our analysis of the regulation of transcription of germline α genes in I.29μ cells and the DNA sequence elements and nuclear factors necessary for regulated transcription of the germline Cα gene.

Transforming growth factor β1 (TGFβ1) is a 25-kDa polypeptide synthesized by many cell types. Both human T and B cells have been shown to secrete TGFβ after cellular activation and TGFβ affects proliferation and differentiation of a variety of cell types. TGFβ has been shown to specifically induce IgA+ cells in LPS activated B cell cultures (Coffman et al. 1989; Sonada et al. 1989), and TGFβ also induces expression of germline α transcripts in these cultures prior to appearance of IgA+ cells (Lebman et al. 1990a; 1990b).

TGFβ increases LPS-induced switching to IgA and increases transcription of germline α genes in I.29μ cells. We tested the effect of TGFβ on switching in I.29μ cells. We found that addition of TGFβ (2 ng/ml) at the time of addition of LPS increased the percent of cells which have switched to IgA by greater than 12.5- fold on day 3 after initiation of the cultures. Switching is assayed by immunofluorescence microscopy, scoring switched cells as those which stain in their cytoplasm with both anti-IgM and anti-IgA antibodies. Since cells which express both IgM and IgA in their cytoplasm cannot have been derived by outgrowth of IgA+ cells, this is an excellent assay for actual switch events. We next examined whether TGFβ treatment caused an increase in the level of germline α transcripts prior to the switch to IgA. I.29μ cells were treated with LPS and/or TGFβ for 24 hr, pelleted, and RNA was purified or the cells were put back into culture for an additional day or two days without inducers. RNA was also purified on days 2 and 3. The RNA was analyzed by northern blotting,

hybridizing with a probe specific for germline α transcripts. We found that LPS alone does not induce germline transcripts on any of these days, although it is required for switching to IgA. TGFβ in the absence or presence of LPS induces germline α transcripts by an average of 5.5 fold by 24 hr after addition and this level is maintained to 48 hr, but is greatly reduced by 72 hr (Shockett and Stavnezer, 1991). In nuclear run-on assays, TGFβ (2 ng/ml) was found to increase transcription of germline α genes in I.29μ cells within 4 hr after addition, with a maximal induction of 5-fold at 12 hr (Shockett and Stavnezer, 1991). Thus, TGFβ causes an early increase in transcription and a transient increase in steady state levels of germline α RNA. The stability of the germline α RNA is unaffected by treatment with TGFβ, or by treatment with LPS, having a half-life of about 4 hr in I.29μ cells. Among the known cytokines, TGFβ is the best inducer of switching to IgA that has been thus far identified and these data suggest that TGFβ directs switching to IgA by increasing transcription of germline α genes.

Identification of the promoter for germline α RNA transcription. To determine if the DNA segment on the 5' flank of the two start sites for germline α RNA functions as a promoter for RNA transcription and then to define the regulatory elements functioning in the promoter, a series of Cα-luciferase fusion genes with different lengths of 5' flanking segment were created. The plasmids were transfected transiently into two B lymphoma cell lines: 22D, a surface IgM^+ subclone of I.29μ which expresses germline α RNA, and A20.3, a surface $IgG2a^+$ cell line which has no detectable germline α RNA when cultured with or without TGFβ (Lin and Stavnezer, manuscript submitted). The Cα-luciferase fusion genes are expressed equally well in the two cell lines (Table I). We speculate that repression of endogenous germline α RNA in A20.3 cells is mediated through DNA regions not residing within the 5' flanking segment or by methylation of the endogenous Cα genes in A20.3 cells. It was not necessary to include an additional enhancer in the Cα-luciferase fusion plasmids and addition of the Ig heavy chain intron enhancer or the 3' Cα enhancer does not increase luciferase expression. The activity of the constructs is unaffected by whether the construct has one or both of the two start sites for germline α RNA. The construct containing only 106 nucleotides 5' to the first RNA initiation site is as active as the construct containing 3800 nucleotides, but if the plasmid only contains 98 nucleotides 5' to the first initiation site, 86 to 88% of the activity is lost in both cell lines. When we examined the DNA sequence of this region, we noted an ATF/CRE consensus element residing between -103/-95 relative to the first initiation site. Oligonucleotide-directed mutagenesis was used to create 4 nucleotide substitutions in this motif and this mutated ATF element was inserted into the Cα179-luciferase plasmid.

Table I. Expression of Cα-luciferase plasmids in 22D and A20.3 cells

Cα-luciferase plasmid[1]	% Luciferase Activity Relative to pSV2Luc[2]	
	22D	A20.3
3800	5.83±0.92[4]	9.14±1.22
2-179[3]	7.17±0.22	6.78±0.28
179	5.91±1.47	8.10±0.79
148	5.23±0.90	7.39±1.77
128	5.30±1.39	8.60±0.89
118	5.63±1.72	9.71±0.93
106	5.18±2.11	11.57±1.70
98	0.64±0.11	1.65±0.36
88	0.78±0.27	2.34±0.64
mATF	1.39+0.51	2.20+0.49

1. Numbers refer to the length (bp) of the Cα segment in the plasmids upstream to the first RNA initiation site.
2. pSV2Luc has the SV40 enhancer/promoter driving luciferase expression.
3. Plasmids 2-179 and 3800 have both RNA initiation sites; all others have only the first initiation site.
4. Luciferase activity is normalized to the activity of co-transfected pSV2CAT and the activity of pXP2, the plasmid vector without any Cα promoter sequences, is subtracted. The number given represents the mean of 3-4 experiments±S.D.

Mutation of the ATF element reduces the expression of the Cα179 construct by 76% and 73% in 22D and A20.3 cells, respectively (mATF in Table I). To provide evidence that the ATF consensus element bound a transcription factor, we examined whether a DNA fragment containing the ATF motif binds a nuclear factor by the electrophoretic mobility shift assay (EMSA) (data not shown). The fragment containing the wild type ATF sequence forms 3 retarded bands when incubated with nuclear extracts from a variety of cell types, including I.29μ cells, but the fragment containing the mutated ATF site forms only two of the 3 retarded complexes. Competition experiments demonstrated that the binding is specific. We conclude that the ATF element is binding a transcription factor that is important for basal level transcription of the germline α RNA. Although one member of the family of transcription factors that bind ATF/CRE sites is inducible by cAMP, expression of the germline α promoter fusion plasmids is not inducible by cAMP.

The germline α promoter is inducible by TGFβ. We next examined the TGFβ-inducibility of the Cα–luciferase fusion plasmids in 22D and A20.3 cells by treating transiently transfected cells with TGFβ (1 to 2 ng/ml is optimum), adding it either 7 hr after transfection (22D cells) or immediately after transfection (A20.3 cells). In both cases, cells were harvested 24 hr after transfection for the luciferase assays. In 22D cells, plasmid Cα3800 is the most inducible; TGFβ induces its expression by 3.3±0.7 fold (mean±S.D.) (Table II). Plasmids Cα826 (data

Table II TGFβ-inducibility of Cα-luciferase plasmids in 22D and A20.3 cells

Cα-luciferase plasmid	Fold induction of luciferase activity by TGFβ[1]	
	22D	A20.3
3800	3.30±0.75[2]	21.26±2.64
2-179	2.07±0.25	11.80±5.11
179	2.02±0.19	8.66±1.56
148	2.15±0.19	9.13±1.49
128	2.02±0.19	5.79±1.33
118	1.55±0.31	3.50±0.47
106	0.88±0.39	0.89±0.24
98	0.98±0.17	0.93±0.19
88	1.02±0.17	1.11±0.45
mATF	2.03+0.64	10.27+4.14

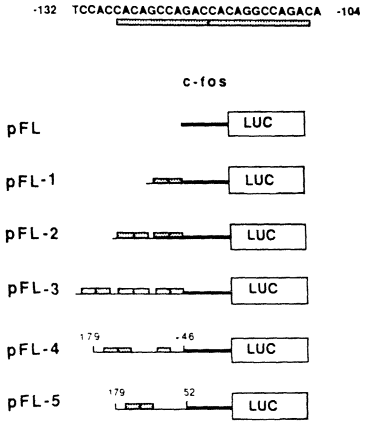
1. Fold induction=light units of induced culture/light units of uninduced culture.
2. The number given represents the mean of 3-4 experiments±S.D. The vector pXP2 is not inducible by TGFβ.

not shown) through Cα128 are induced to about the same extent by TGFβ (average 2 fold, although in later experiments the induction improved to 5-fold), but further deletions result in a loss of TGFβ inducibility. The region from -127 to -105 contains two imperfect tandem repeats of sequence 5' CACAG(G)CCAGAC 3'. The first copy extends from -127 to -117 and the second copy from -116 to -105. Plasmid Cα106, which lacks these repeats is not more inducible than the promoterless vector pXP2, indicating that the repeat sequence is necessary for TGFβ induction. The difference in the amount of induction obtained from Cα128 and Cα106 is statistically significant as determined by an analysis of variance (Student-Newman-Keuls test, $p<0.05$). Plasmid Cα118, which has deleted the more 5' copy of the repeat, shows an intermediate level of induction, suggesting that both repeats contribute to the induction. The repeats are not important for constitutive expression since deletion of nucleotides from -128 to -106 does not affect promoter activity in the absence of TGFβ (Table I). The ATF element is not necessary for TGFβ induction since Cα179 with the mutated ATF element is fully inducible by TGFβ (mATF in Table II). An additional TGFβ-responsive element is present 5' to -826 because Cα3800 is more inducible than all shorter constructs.

We also tested these promoter constructs in A20.3 cells (Table II). There is a greater induction of luciferase acitivity by TGFβ in A20.3 cells than in 22D cells. The greater inducibility may be explained by the fact that 22D cells make TGFβ, whereas A20.3 cells do not (Shockett and Stavnezer, 1991). Again, Cα3800 is the most inducible (21.3±2.6 fold) and Cα through Cα148 are induced to the same extent (about 8 fold) by TGFβ. Deletion of regions between -148/-128, -128/-118 and

-118/-106 all lead to reduction of the TGFβ response. The difference between the inducibility of Cα128 and Cα106 is statistically significant ($p<0.01$). Thus, the DNA segment between -3800 and -826 and the tandem repeats located between -127 and -105 are important for TGFβ induction in both A20.3 and 22D cells. In addition, these results indicate that the segment between -148 and -128 can also contribute to the TGFβ response in A20.3 cells. LPS has no effect on expression of the Cα-luciferase fusion plasmids, as expected from the fact that LPS does not induce transcription of germline α genes (Shockett and Stavnezer, 1991).

The Igα TGFβ-RE oligonucleotide confers inducibility to a heterologous promoter. To determine if the tandem repeats residing at -127/-105 can function by themselves to confer TGFβ inducibility to a heterologous promoter, we inserted a 36-bp oligonucleotide containing the direct repeat of the germline α promoter into the plasmid pFL, which contains a minimal c-fos promoter driving the luciferase gene (Fig. 2). Plasmids pFL-1, -2 and -3 contain one, two and 3 copies of the oligonucleotide in the normal 5' to 3' orientation, respectively. The response of these plasmids to TGFβ is similar in 22D



	Fold induction by TGFβ in 22D	A20.3
pFL	0.82±0.12	1.00±0.27
pFL-1	1.11±0.23	1.16±0.33
pFL-2	5.66±1.51	10.05±0.91
pFL-3	18.19±0.36	26.64±4.22
pFL-4	6.35±2.09	4.69±1.61
pFL-5	3.21±1.99	1.94±0.17

Fig.2. The Igα TGFβ-Response Element is sufficient to transfer TGFβ inducibility to plasmids driven by a minimal c-fos promoter in 22D and A20.3 cells. pFL contains a minimal mouse (-71/+109) c-fos promoter driving luciferase gene expression, and the other plasmids (pFL-1,-2, or -3) contain one, two or 3 copies of an oligonucleotide (-132/-104) containing the direct repeat present in the Cα promoter or they contain segments from the Cα promoter, indicated by the nucleotide positions relative to the first RNA initiation site in the figure (pFL-4, -5). The results given are the means±S.D. of 3-4 experiments. The nucleotide sequence of these segments is reported in Lin and Stavnezer, submitted for publication.

and A20.3 cells. Expression of pFL and pFL-1 are not increased by TGFβ, but luciferase expression of the plasmids containing two and 3 copies of the repeat are strongly induced by TGFβ (6 to 10 fold and 18 to 27 fold, respectively; Fig. 2). These results indicate that the transcription factor(s) which binds to the repeated sequence (Igα TGFβ-RE) functions synergistically to activate transcription and more than two copies of the individual TGFβ-RE are required for TGFβ induction. Consistent with this finding, we noted in the germline α promoter a third copy of the TGFβ-RE located between -41/-30. In addition, we tested two plasmids containing 3 copies of the TGFβ-RE oligonucleotide inserted with the middle copy in the reverse orientation in one and with the 5' copy in reverse orientation in the second. TGFβ induction of these plasmids is consistently about 30% lower than the induction of pFL-2 (data not shown). These results suggest that the nuclear factors which bind to Igα TGFβ-RE interfere with each other when they are closely spaced and bound to DNA in an inverted orientation relative to each other, but they cooperate if in the correct orientation when closely spaced. We tested additional DNA segments from the germline α promoter for ability to confer TGFβ responsiveness to pFL. Plasmid pFL-4, which contains the -179/+46 Cα promoter segment, can be induced by TGFβ by 5 to 6 fold in the two cell lines, whereas pFL-5, which contains the -179/-52 Cα promoter segment, is induced to a lesser extent, by 2 to 3 fold. This result suggests that the additional TGFβ–RE, which is not present in the -179/-52 segment, contributes to TGFβ inducibility.

Nuclear extracts from I.29μ cells contain specific binding activity to TGFβ-RE. To determine if a nuclear factor binds the Igα TGFβ-RE, the double-stranded oligonucleotide containing the direct repeat was end-labeled and incubated with nuclear extracts prepared from 22D cells and analyzed by EMSA. A few retarded bands were detected; the most slowly migrating band appears specific, as it is competed by excess unlabeled TGFβ-RE, but not by an unrelated oligonucleotide. The EMSA pattern is the same when nuclear extracts from TGFβ-treated 22D cells were used. Thus, the TGFβ–RE binds a nuclear factor in a sequence-specific manner but the binding of this factor appears to be unaffected by TGFβ treatment. This is consistent with the fact that the TGFβ signal transduction pathway involves a serine/threonine protein kinase (Ohtsuki and Massague, 1992), since phosphorylation does not always change the DNA binding activity of transcription factors.

The Igα TGFβ-RE is found in the 5' flanks of other genes. Three copies of the Igα TGFβ-RE are present at similar positions relative to the start sites of transcription of the human germline α1 transcript.

A copy of the element is also found in the promoter for both mouse and human TGFβ, a gene whose transcription is induced by TGFβ. The element is located in a region that has not been studied for its effect on TGFβ inducibility (immediately 3' to the first of the two RNA initiation sites), and it is known that an AP-1 binding site is involved in TGFβ induction of transcription from the second initiation site of the TGFβ gene (Kim et al. 1990). Therefore, if this element is involved it would not be the sole TGFβ-RE in this promoter. This element also resides at -560 in the β-actin 5' flank, another gene known to be induced by TGFβ, although again this region has not been examined for its effect on TGFβ-inducibility. The element is also found in the 5' flanks of several other genes that are not known to be induced by TGFβ. TGFβ is known to induce transcription of genes through a few different DNA elements: AP-1 binding sites, NF-I and USF elements. None of these elements are found in the 826 nucleotide 5' flanking region of the germline α promoter.

In conclusion, multiple copies of a novel sequence element are responsible for TGFβ induction of germline α RNA. Further, our results indicate that TGFβ can induce transcription of different genes by a variety of mechanisms. Our results support the hypothesis that TGFβ directs switching to IgA by inducing accessibility of the unrearranged α gene and that TGFβ induces this accessibility by inducing transcription directed by the germline α promoter.

Acknowledgements: This research was supported by NIH, by training grant 1T32-AI07349 and by RO1-AI23283.

REFERENCES

Coffman RL, Lebman DA, Schrader B (1989) Transforming growth factor β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J Exp Med 170: 1039.

Esser C, Radbruch A (1990) Immunoglobulin class switching: molecular and cellular analysis. Ann Rev Immunol 8: 717.

Kim SJ, Angel P, Layyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB (1990) Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. Mol Cell Biol 10: 1492.

Lebman DA, Lee FD, Coffman RL (1990a) Mechanism for transforming growth factor β and IL-2 enhancement of IgA expression in lipopolysaccharide-stimulated B cell cultures. J Immunol 144: 952.

Lebman DA, Nomura DY, Coffman RL, Lee FD (1990b) Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β-induced isotype switching. Proc Natl Acad Sci USA 87: 3962.

Lutzker S, Rothman P, Pollock R, Coffman R, Alt FW (1988) Mitogen- and IL-4-regulated expression of germ-line Ig γ2b transcripts: evidence for directed heavy chain class switching. Cell 53: 177.

Ohtsuki M, Massague J (1992) Evidence for the involvement of protein kinase activity in transforming growth factor-β signal transduction. Mol Cell Biol 12: 261.

Shockett P, Stavnezer J (1991) Effect of cytokines on switching to IgA and α germline transcripts in the B lymphoma I.29μ: Transforming growth factor-β activates transcription of the unrearranged Cα gene. J Immunol 147: 4374.

Sonada E, Matsumoto R, Hitoshi Y, Ishii T, Sugimoto M, Araki S, Tominaga A, Yamaguchi NT, Takatsu K (1989) Transforming growth factor β induces IgA production and acts additively with IL-5 for IgA production. J Exp Med 170: 1415.

Stavnezer J, Radcliffe G, Lin YCA, Nietupski J, Berggren L, Sitia R, Severinson E (1988) Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. Proc Natl Acad Sci USA 85: 7704.

Stavnezer J, Sirlin S, Abbott J (1985) Induction of immunoglobulin isotype switching in cultured I.29 B lymphoma cells: Characterization of the accompanying rearrangements of heavy chain genes. J Exp Med 161: 577.

Stavnezer-Nordgren J, Sirlin S (1986) Specificity of immunoglobulin heavy-chain switch correlates with activity of germline heavy chain genes prior to switching. EMBO J 5: 95.

SαBP/BSAP/NF-Sμ B_1, a Murine and Human B Cell Stage Specific Nuclear Factor with DNA Binding Specificity Implying Roles in Switch-Recombination and Transcription

K. B. MARCU, L. XU and M. G. KIM*

Departments of Biochemistry and Cell Biology, Microbiology and Pathology, SUNY at Stony Brook, Stony Brook, NY 11794-5215
*Present Address: Genetics and Biochemistry Branch NIDDK, NIH, Bldg. 10 Bethesda, MD 20892

Introduction

Immunoglobulin genes undergo a programmed series of DNA rearrangements in B lymphoid cells. Following assembly of a functional variable (V) region gene, an independent series of recombinations determine the heavy chain isotype of the expressed antibody (IgM, IgG, IgE and IgA). Heavy chain class switches result in the deletion and replacement of the C_μ gene with a downstream C_H gene ($C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_ϵ or C_α)(reviewed in Marcu, 1982; Shimizu and Honjo, 1984; Radbruch et al., 1986; Gritzmacher, 1989). These recombinations are mediated by switch (S) regions which are positioned about 2 Kb 5' of every C_H gene segment with the exception of C_δ. Differential regulation of C_H class switching is believed to be manifested by the accessibility of S segments to a B cell specific switch-recombinase activity (Stavnezer and Sirlin, 1986). Switch-recombination occurs at widely spaced positions within S_μ and other downstream S regions and only limited sequence homology between S sequences may be present near the site of recombination (Gritzmacher, 1989; Ott and Marcu, 1989). S regions consist of short tandemly repetitive sequences of varying length and homology which are believed to mediate switch recombination by an illegitimate mechanism. GAGCT and GGGGT sequences are found in all S regions in addition to three other commonly observed pentamers: ACCAG, GCAGC and TGAGC (reviewed in Shimizu and Honjo, 1984; Gritzmacher, 1989). A heptamer consensus motif, YAGGTTG (Y=pyrimidine), has also been found nearby a number of switch recombination sites in plasma cell tumors and hybridoma lines and is also repeated in various S segments (Marcu et al., 1982). The 5' portion of the S_μ region is non-repetitive but does contain interspersed GAGCT, GGGGT and YAGGTTG like sequences while the 3' portion is composed of a simple repetitive block of $(GAGCT)_nGGGGT$ motifs where it ranges from one to seven (Nikaido et al., 1981). The S_μ, S_ϵ and S_α sequences display considerable homology particularly in areas containing repeats of GAGCT sequences while the S_γ regions contain a lower density of these pentamers but are typified by 49mer or 52mer ($S_{\gamma 2a}$) repeats and TGGGG, GCAGC and ACCAG motifs (Kataoka et al., 1981). The overall homology of the S_γ regions correlates with their distance from S_μ (e.g., $S_{\gamma 3}$> $S_{\gamma 1}$>$S_{\gamma 2b}$>$S_{\gamma 2a}$) (Shimizu et al., 1982; Stanton and Marcu 1982).

We have previously employed retroviral vectors to introduce switch-recombination substrates into cells (Ott and Marcu, 1989). These studies demonstrated that: (1) portions of two S regions are sufficient for efficient switch-recombination, (2) switch-recombination is B cell specific, (3) switch repeat motifs may mediate DNA rearrangements but only limited sequence homology resides at the precise sites of recombination and (4) switch-recombination is regulated by multiple factors (presumably contributed in part by the degree of switch region accessibility) in addition to the presence of a B cell specific switch-recombinase.

Here, we describe the properties of a B cell stage specific S region binding factor NFSμ-B_1. B_1 binds to Sμ 5', Sμ3' sequences and to other S regions with varying specificity. Binding site competition assays reveal that NFSμ-B_1 is likely identical to SαBP (Waters et al., 1989) and BSAP (Barberis et al., 1990) which were shown to bind to two sequences upstream of the Sα repeats and within the promoters of sea urchin histone genes respectively. Preincubation of B cell nuclear extracts with protein dissociating agents strongly enhanced NF-SμB_1 binding to switch repeat motifs implying that an inhibitory activity in B cells may alter its DNA binding specificity.

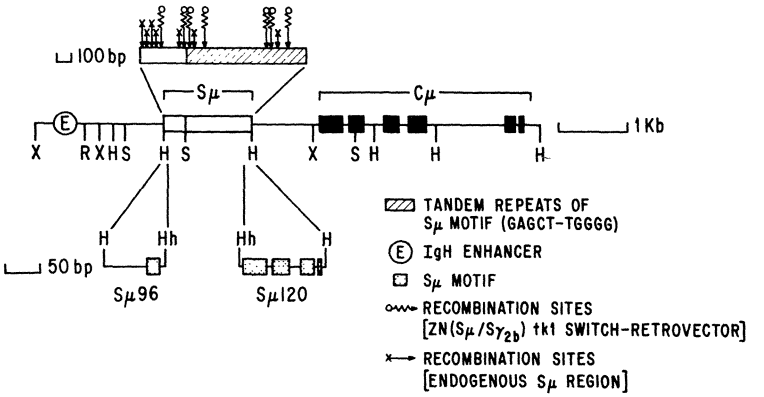


Fig.1. Schematic of the S region and associated flanking sequences. R, EcoRI; H, HindIII; S, SstI; X, XbaI; Hh, HhaI.

Identification of a B Cell Stage Specific S Region Binding Factor

Gel retardation assays were performed with crude nuclear extracts derived from lymphoid and non-lymphoid cell lines and two DNA probes were prepared from the repetitive and non-repetitive portions of the Sμ region respectively (see Sμ96 and Sμ120 in relation to sites of switch-recombination within the Sμ region in Figure 1). Sμ120, a 120 bp DNA fragment containing Sμ tandem repeat motifs revealed a fairly complex pattern of DNA-protein complexes with crude nuclear extracts from different cell types (Figure 2A), but a minor, fast gel shift complex designated NFSμ-B_1, was only detected in nuclear extracts prepared from pre-B and immature surface Ig positive B cells. Sμ96, a 96 bp fragment derived from the 5' non-repetitive portion of Sμ, also gave a fairly complex gel shift pattern, which contained a B cell stage specific complex analogous to that detected with Sμ120. The Sμ96-B_1 and Sμ120-B_1 complexes co-migrated implying, along with their identical B cell stage specific expression patterns, that they are generated by the same DNA binding activity (Figure 2B). A reduced amount of 300-18 pre-B cell crude nuclear extract (5.0 instead of 7.5μg) with the Sμ96 probe only revealed the major ubiquitous complex unique to Sμ96 (NFSμ-U_1) and the B cell specific B_1 band (Figure 3). The Sμ-U_1 band was abolished by an excess of Sμ 5' fragment competitor and only diminished in intensity with other S region competitor DNAs while all repetitive S region fragments competed well for B_1 binding to Sμ96. Sμ5', Sμ3', Sα, Sγ_2a and Sγ_1 sequences were comparable competitors for B_1 binding to Sμ96 while Sγ_3 and Sγ_{2b} DNAs were less efficient (Figure 3). A DNA fragment originating from the 3'portion of Sγ_{2b}, which lacks tandem Sγ repeats, did not show significant competition. Mobility shift competition

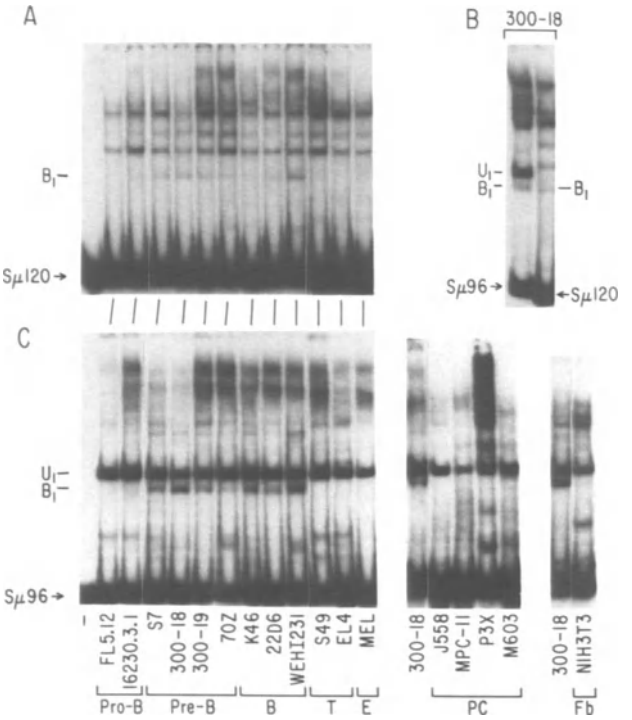


Fig. 2. Gel mobility shift assays of S_μ binding factors with 7.5 μg of crude nuclear extracts and $S_\mu 120$ (panel A) or $S_\mu 96$ (panel C) DNA probes. DNA-protein complexes with $S_\mu 96$ and $S_\mu 120$ are compared in panel B. The $S_\mu 96$ probe is 126 bp due to 30 bp of pBluescript sequences. B and T are mature B and T lines; E, PC and Fb are erythroid, plasma cell tumor and fibroblast lines respectively.

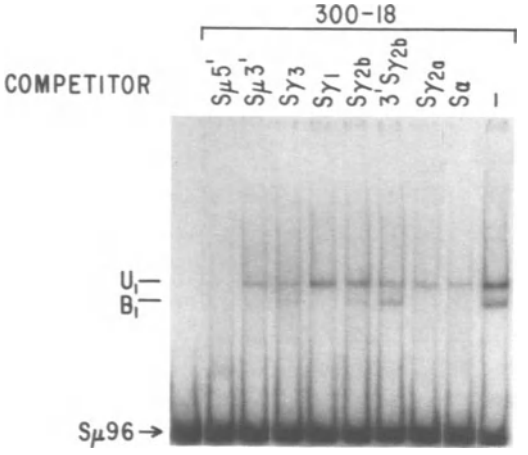


Fig.3. $S_\mu 96$ mobility shifts with 5.0μg of 300-18 extract and a 200 fold excess of unlabeled switch region competitor DNAs. $S_\mu 5'$ and $S_\mu 3'$ are non-repetitive and repetitive portions of the S region. $3'S_{\gamma 2b}$ resides 3' of the $S_{\gamma 2b}$ 49mer repeats. The first lane and (-) contain no extract and S DNA competitor respectively.

assays were performed with 5 μg of 300-18 crude extract and synthetic oligonucleotides derived from different portions of the Sμ96 probe to define the molecular requirements for B_1 binding (Figure 4). U17, only containing the core of the U_1 binding site as revealed by methylation interference footprinting (Xu et al., 1992; data not shown), was a poor competitor for U_1 and B_1 binding. Probes containing additional 3' (UB30) and 5' (YUB65) flanking sequences were better competitors. UB48, encompassing the 3' half of the Sμ96 probe, was the most effective competitor and abolished both B_1 and U_1 binding at a 5-10 fold molar excess. Under these binding conditions the single S motif present near the 3' end of Sμ96 would appear to be a more effective component of the B_1 binding site if it does not reside at the very end of the DNA molecule. Synthetic oligonucleotides containing 3-4 copies of Sμ motifs failed to compete for Sμ-B_1 binding even at a 10^3 fold molar excess while a 30mer Sμ motif oligo comparable in length to UB30 in Figure 4 did compete for Sμ-B_1 but only at a 10^3 fold molar excess (Xu et al., 1992; data not shown). These observations collectively argue that numerous tandem repeats of the Sμ motif (employed in the Sμ3' competition in Figure 3) can be effective competitors for B_1 binding while other sequences can substitute for tandem Sμ motifs.

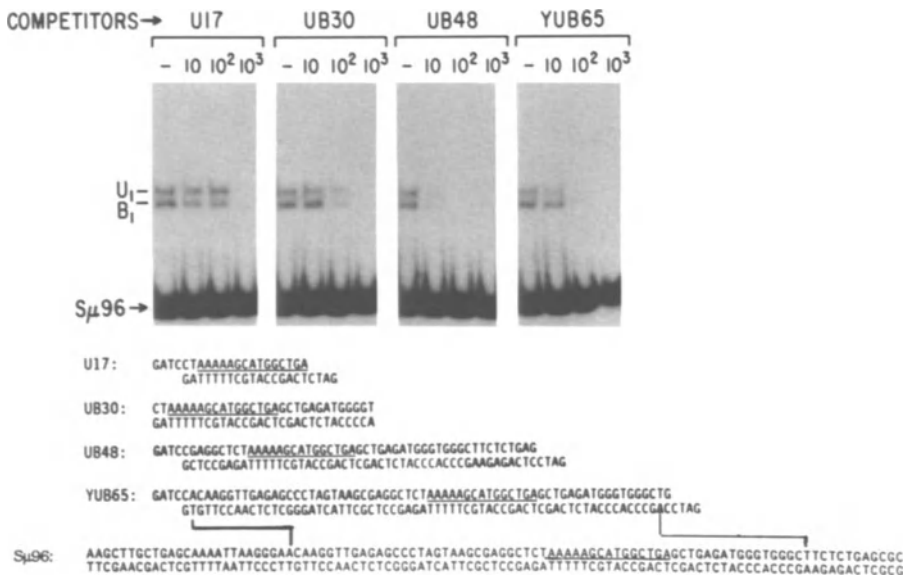


Fig. 4. Sμ96 mobility shifts with 300-18 nuclear extract (5μg) competed by synthetic oligonucleotides derived from the Sμ96 sequence. The underlined sequence in the oligonucleotides corresponds to the ubiquitous (U_1) binding site revealed by methylation interference footprinting (Xu et al., 1992).

NFSμ-B_1 is Likely Identical to SαBP and BSAP

SαBP, a nuclear factor with the analogous B cell stage specific expression pattern as NFSμ-B_1 was reported to bind to at least two different sequences upstream of the Sα switch repeats (Waters et al., 1989). Sequences derived from other switch regions competed to varying degrees for SαBP binding and one of these sites was upstream of the Sμ tandem repeat motifs. In light of these similarities, we compared the binding site specificities of NFSμ-B_1 and SαBP for their respective target sequences. αS-2, an SαBP binding site

oligonucleotide, specifically competed for $S\mu$-B_1 binding to $S\mu$96 (Figure 5A) and $S\mu$96 competed for binding of $S\alpha$-BP to a labeled αS-2 site (Xu et al., 1992; data not shown). NF-$S\mu B_1$/$S\alpha$BP appears to preferentially bind to the αS-2 site under these conditions. $S\alpha$BP bound with similar specificity to two different sequences upstream of $S\alpha$, αS-1 and αS-2, which exhibited about 50% sequence homology (Waters et al., 1989). BSAP, another mammalian B cell stage specific nuclear factor, was reported to bind to a conserved sequence motif within sea urchin histone 2A and 2B gene promoters (Barberis et al., 1990). BSAP like $S\alpha$ BP possesses a B cell stage expression pattern analogous to NF-$S\mu$ B_1. Furthermore, mutation of an invariant C residue in the histone promoter consensus sequence to an A abrogated BSAP binding and promoter function in mammalian B cell lines (Barberis et al., 1990). In Figure 5, a 25 bp synthetic oligonucleotide of the H2A-2.2 promoter is a very effective, competitor for B_1 binding to $S\mu$96 while a mutant H2A-2.2 site is a much poorer competitor. Band shifts with the H2A-2.2 promoter as probe were competed by αS-2 and switch region DNAs and $S\mu B_1$/$S\alpha$BP/BSAP DNA complexes migrated with similar RF values (Xu et al., 1992; data not shown). These cross-competition assays indicate that NF-$S\mu B_1$, $S\alpha$BP and BSAP likely represent the same B cell stage specific DNA binding activity. αS-1, αS-2 and the H2A-2.2 promoter sequences appear to be stronger binding sites for NF-$S\mu B_1$/$S\alpha$BP/BSAP than $S\mu$ probes under these <u>in vitro</u> conditions. A consensus sequence derived from the H2A and H2B gene promoters bears homology to portions of the αS-2 and $S\mu$96 probes and a combined consensus sequence of all these binding sites resembles an $S\mu$ motif (Figure 6B).

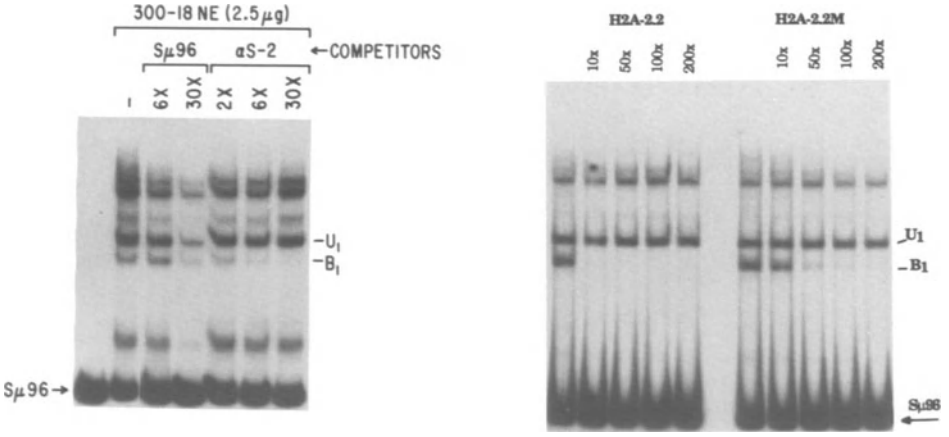


Fig. 5. Cross competition gel retardation assays reveal that NF$S\mu$-B_1, $S\alpha$BP and BSAP likely represent the same DNA binding activities. αS-2 is a 66bp oligo containing one S BP binding site (Waters et al., 1989). H2A-2.2 and H2A-2.2M are 25bp oligos corresponding to wild type and mutant BSAP binding sites (Barberis et al., 1990).

NF$S\mu$-B_1/$S\alpha$BP/BSAP Binding Specificity Maybe Modulated by an Inhibitor

The inclusion of protein dissociating agents such as NP-40, urea or formamide in binding reactions enhanced B_1 specificity for DNA probes containing switch repeat motifs and abrogated ubiquitous factor complexes (Xu et al., 1992; Figures 6 and 7). Representative binding reactions performed with 40% formamide in Figure 6A reveal strong enhancement of NF$S\mu$ -B_1 complex formation on $S\mu$96 in comparison to H2A-2.2. As expected, the $S\mu$ B_1 and BSAP complexes co-migrate and are each effectively competed by an

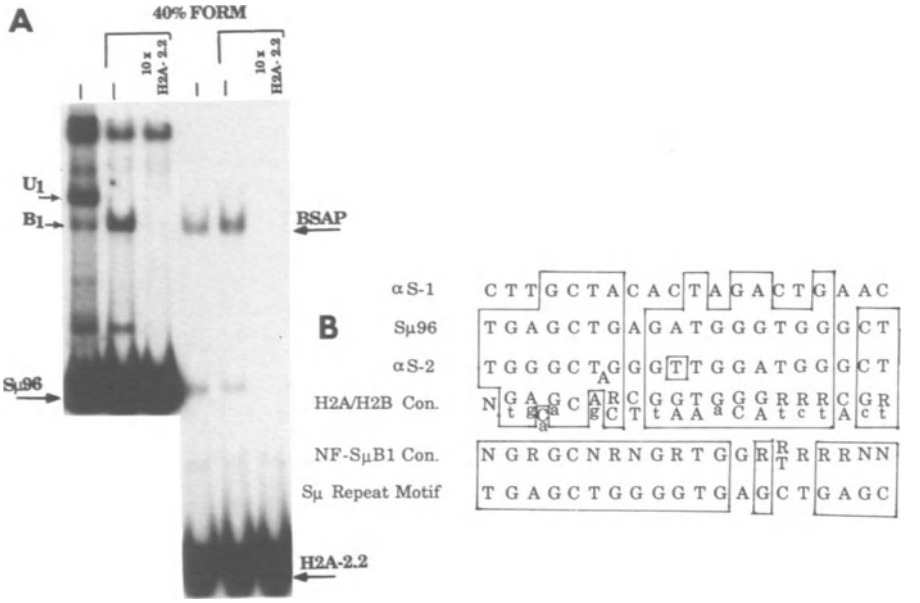


Fig. 6. Panel A: Enhancement of NF-$S\mu B_1$ and BSAP factor binding in the presence of 40% formamide. Panel B: Comparisons of the $S_\mu B_1$/S_αBP/BSAP factor binding sequences. Large case letters indicate the predominant nucleotides and small case letters are only observed once. R= purine and N= all nucleotides observed.

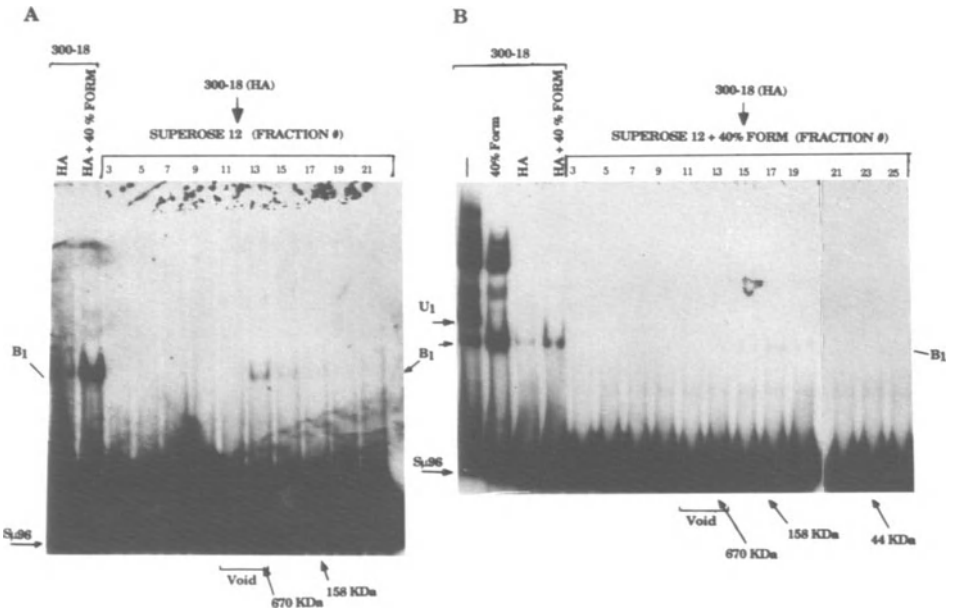


Fig. 7. Superose 12 chromatography of heparin (HA) agarose fractionated 300-18 nuclear extract in the absence (A) and presence (B) of 40% formamide. The position of migration of the S_μ-B_1 complex is indicated relative to other marker polypeptides.

excess of the wild type H2A-2.2 promoter (Figure 6A). However, activation of $S_\mu B_1/S_\alpha BP$ by protein dissociating agents was only demonstrable with pre-B and immature B cell lines indicating that latent inactive forms of $S_\mu B_1/S_\alpha BP$ are not present in fully mature B cells or non-lymphoid cells nor in cytostolic extracts (Xu et al., 1992; data not shown). NFS_μ-B_1 binding activity was partially purified from 300-18 crude nuclear extract and then subjected to superose 12 chromatography in the presence and absence of 40% formamide (Figure 7). Formamide treatment of the heparin agarose fractionated extract revealed strong enhancement of B_1 binding to $S_\mu 96$. In the absence of formamide, the B_1 binding activity migrated in the void volume of a superose 12 column. Inclusion of formamide in the column buffer yielded a B_1 complex of reduced molecular weight. These observations suggest that the B_1 binding activity normally resides in a high molecular weight complex which is disrupted by protein denaturants also causing a concomitant increase in B_1 specificity for a switch repeat probe.

Relationship of NFS_μ-$B_1/S_\alpha BP/BSAP$ to Other S Region DNA Binding Activities and its Potential Role in Switch-Recombination

Two other S region binding activities have been described but are unrelated to $S_\mu B_1/S_\alpha BP/BSAP$. Another S repeat motif DNA binding activity, NF-S_μ ,which appears after mitogen stimulation of splenic lymphocytes generated a single band shift with a $GAGCTGGGGT(GAGCT)_3$ 25 mer and DMS interference indicated that binding centered on the $(G)_4$ run (Wurff et al., 1990). However, no data are available on the B cell specificity of NF-S_μ. Nevertheless, $S_\mu B_1/S_\alpha BP$ only bound to large (>100bp) S motif fragments and as shown here this binding was enhanced by protein dissociating agents. LR1, a B cell specific and LPS inducible S region binding activity, was dependent on phosphorylation for DNA binding (Williams and Maizels, 1991). However, $S_\mu B_1/S_\alpha BP$ binding activity was present in primary splenic lymphocytes without mitogenic stimulation and phosphatase treatments of B cell nuclear extracts did not inhibit $S_\mu B_1$ binding (data not shown). LR1 may play a role in the induction of S region accessibility.

We envision several possible functions for $S_\mu B_1/S_\alpha BP/BSAP$ in class switching which need not be mutually exclusive. Given the ability of $S_\mu B_1/S_\alpha BP$ to preferentially bind to an essential sequence required for sea urchin histone promoter function in mammalian B cells, this DNA binding activity may initially participate in establishing the transcriptional competence and/or accessibility of the C_H locus for switch-recombination. The conversion of $S_\alpha BP/BSAP$ into the $S_\mu B_1$ switch region binding activity could be regulated by inhibitory factors. In the absence of such inhibitory factors, $S_\mu B_1$ would then facilitate the synapsis or alignment of distantly separated switch regions prior to their recombination. This might involve the opening or melting of DNA sequences as a prelude to strand breakage; and the existence of multiple, adjacent $S_\mu B_1$ binding sites amongst the tandemly repetitious sequences in S regions may be involved in its mechanism of action. $S_\mu B_1/S_\alpha BP/BSAP$ may represent a molecular link between transcription and Ig gene recombination.

Acknowledgments

We thank Dr. J. Stavnezer, W. Dunnick and M. Julius for providing αS-2, $S_{\gamma 1}$ and $S_{\gamma 2a}$ DNAs respectively, and Drs. K. Calame and C. Asselin for their generous gifts of nuclear extracts. We acknowledge Karin Bondarchuk for technical assistance, J. Schirmir for illustration work and Margarita Reyes for manuscript and figure preparation. This work was supported by NIH grant GM26939 awarded to KBM.

References

Barberis A, Widenhorn K, Vitelli L, and Busslinger M, (1990) A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. Genes & Dev. 4:849

Dignam JD, Lebovitz RM, and Roeder RG, (1983) Accurate transcription initiation by RNA polymerae II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. 11:1475.

Gritzmacher CA, (1989) Molecular aspects of heavy chain class switching. Crit. Rev. Immunol. 9:173.

Kataoka T, Miyata T, and Honjo T, (1981) Repetitive sequences in class-switch recombination regions of immunoglobulin heavy chain genes. Cell 23:357.

Marcu KB. (1982) Immunoglobulin heavy-chain constant region genes. Cell 29:719-721.

Marcu KB, Lang RB, Stanton LW, and Harris LJ, (1982) A model for the molecular requirements of immunoglobulin heavy chain class switching. Nature 298:87.

Nikaido T, Nakai S, and Honjo T,(1981) Switch region of immunoglobulin C mu gene is composed of simple tandem repetitive sequences. Nature 292:845

Ott DE, and Marcu KB, (1989) Molecular requirements for immunoglobulin heavy chain constant region gene switch-recombination revealed with switch substrate retroviruses. International Immunol.1:582.

Radbruch A, Burger C, Klein S, and Muller W, (1986) Control of immunoglobulin class switch recombination. Immunol. Rev. 89:69.

Shimizu A, Takahashi N, Yaoita Y, and Honjo T, (1982) Organization of the constant-region gene family of the mouse immunoglobulin heavy chain. Cell 24:499.

Shimizu A, and Honjo T, (1984) Immunoglobulin class switching. Cell 36:801.

Singh H, Sen R, Baltimore D, and Sharp P, (1986) A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature 319:154.

Stanton L, and Marcu KB, (1982) Nucleotide sequence and properties of the murine gamma 3 immunoglobulin heavy chain gene switch region:implications for successive C gamma gene switching. Nuc. Acids Res. 10:5993.

Stavnezer J, Sirlin S. (1986) Specificity of immunoglobulin heavy chain switch correlates with activity of germline heay chain genes prior to switching. EMBO J. 5:95-102.

Waters SH, Saikh KU, and Stavnezer J, (1989) A B-cell-specific nuclear protein that binds to DNA sites 5' to immunoglobulin S alpha tandem repeats is regulated during differentiation. Mol. Cell. Biol. 9: 5594.

Williams M, and Maizels N, (1991) LRI, lipopolysaccharide-responsive factor with binding sites in the immunoglobulin switch regions and heavy- chain enhancer. Genes & Dev. 5:2353.

Wuerffel RA, Nathan AT, and Kenter AL, (1990) Detection of an immunoglobulin switch-region specific DNA binding protein in mitogen-stimulated mouse splenic B cells. Mol. Cell. Biol. 10:1714.

Xu L, Kim MG, Marcu KB. (1992) Properties of B cell state specific and ubiquitious nuclear factors binding to immunoglobulin heavy chain gene switch regions. Intern. Immunol. in press.

Multiple Myeloma, Plasmacytomas

Multidrug Resistance of a Continuously Differentiating Monoclonal B Lineage in the Blood and Bone Marrow of Patients with Multiple Myeloma

L. M. PILARSKI[1], C. E. CASS[2], T. TSURO[3] and A. R. BELCH[4]

[1]Department of Immunology,
[2]Biochemistry and
[4]Medicine, University of Alberta, Edmonton, Canada
[3]Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo

Introduction

Although multiple myeloma has long been regarded as a malignancy localized to the bone marrow, recent evidence clearly shows a monoclonal population of B cells in the blood of patients with myeloma both at diagnosis and during therapy. It is difficult however to determine whether or not these circulating tumor-related B cells are in fact malignant or if they are non-malignant relatives of the plasma cells in the bone marrow. Our working hypothesis predicts that circulating monoclonal B lineage cells are the precursors of the bone marrow plasma cells and that, contrary to well established dogma, traffic of myeloma cells travels from the blood to the bone marrow. We predict, and our data support the view, that B lineage cells localized in the bone marrow are predominantly end stage non-proliferating cells which have little or no propensity or capability to migrate, whose future options consist mainly of entry into apoptotic pathways and eventual death. If this view is correct, it predicts that the malignant stem cell in myeloma may be located outside the bone marrow. If the circulating monoclonal B lineage cells in blood are in fact malignant cells, then they either represent the stem cells themselves or are early descendents in the process of trafficking to the marrow where they will terminally differentiate. Spleen might be considered the most likely place of origin for myeloma as plasma cells of normal bone marrow originate in the spleen, migrate as antigen-activated B cells to the bone marrow via the blood and undergo terminal differentiation to antigen-specific plasma cells (1,2), a pattern remarkably similar to that of the monoclonal B lineage in myeloma (3). Also consistent with this view is work indicating that the accumulation of plasma cells in normal bone marrow is the major site of antibody synthesis in the body, and increases with age (1). In myeloma, analysis of autopsy material indicated the least mature cells of the malignant lineage were in the spleen whereas considerably more terminally differentiated B lineage cells were found in blood (Jensen et al, submitted). This highly speculative hypothesis is a difficult one to test as events in the spleen would be predicted to occur in the earliest stages of the disease and cells might become extra-splenic even before diagnosis. Thus the properties and potential of the circulating blood B lineage cells have become an important target of investigation, and an even more crucial consideration in the design of new modes of therapy for myeloma patients.

Monoclonal B Lineage Cells in the Blood of Myeloma Patients

Evidence for Monoclonality

A number of groups have detected monoclonal rearrangements among peripheral blood mononuclear cells (PBMC) from some but not all myeloma patients (reviewed in 3), and initially assumed that these were derived from small numbers of plasma cells in blood. However reports of large numbers (up to 80% of PBMC) of circulating B cells in blood of myeloma patients, together with monoclonal IgH rearrangements, showed that these derived from earlier B lineage cells not from circulating plasma cells (3,4). More recent work from our group indicates that circulating B lineage cells in myeloma express apparently

monoclonal mRNA encoding either κ or λ light chains, consistent with both the type of mRNA expressed by bone marrow plasma cells from the same patient, and with the type of monoclonal Ig found in serum. Light chain mRNA is not detectable among PBMC B cells from normal donors probably because normal PBMC include fewer B cells and those that are present are at a stage of B cell development prior to initiation of Ig secretion. Kappa or lambda light chain mRNA has been detected among PBMC B cells in 12 of 15 patients analyzed using Northern blots (Jensen et al, submitted).

B Lineage Cells in Myeloma Represent a Continuously Differentiating Population

Analysis of PBMC from myeloma patients by multiparameter flow cytometry revealed that B lineage cells in blood are highly heterogeneous, as defined by their expression of CD45 isoforms. Among normal B cells, CD45RA, the high molecular mass CD45 isoform, is expressed by resting B cells from blood and from solid tissues (5), while CD45R0, the low molecular mass isoform, is expressed only by activated late stage B cells (6). Upon terminal B cell differentiation to plasma cells, all CD45 is gradually lost (5). In myeloma PBMC, B lineage cells are predominantly CD45R0, defining them as late stage B cells, but the population of blood B cells usually includes B cells expressing only CD45RA, transitional cells expressing both CD45RA and CD45R0, and late stage B which are CD45R0$^+$. CD45$^-$ plasma cells are found only in the bone marrow except at very late phases of the disease (3).

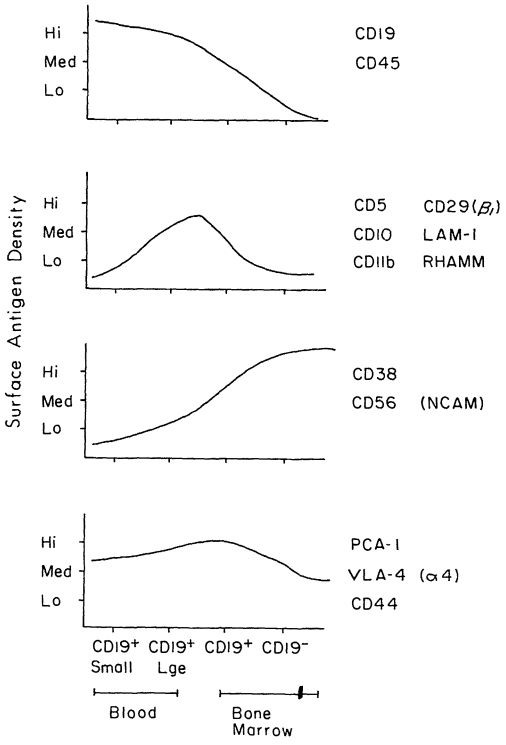


Fig. 1. Blood and bone marrow from a single patient at diagnosis were analyzed for phenotypic patterns on small (low SSC/FALS) CD19$^+$ PBMC B cells, large (high SSC/FALS) CD19$^+$ PBMC B cells, CD19$^+$ BM cells and CD19$^-$ plasma cells. Bone marrow was 80% plasmablasts by morphology. The expression pattern of markers for this patient was representative of that seen for a large number of patients in the aggregate (Pilarski et al, in preparation).

Use of a panel of mAbs allows an even finer discrimination of B lineage stages. During B cell differentiation to plasma cells, signal transduction molecules such as CD45 and CD19 are lost, presumably reflecting the status of plasma cells as an Ig factory, no longer requiring signal transduction. In contrast, the density of molecules such as CD38, VLA-4, CD44 and CD56 increases on plasma cells perhaps indicating a need for homotypic adhesion or for adherence to bone marrow stromal cells. The apparent sequential stages of B lineage development in the blood and bone marrow of a single patient at diagnosis is shown in

Figure 1. This figure assumes that B cell differentiation proceeds from small $CD19^+$ B cells, to larger $CD19^+$ activated cells with enhanced granularity and specialization for Ig secretion, culminating in further B cell differentiation in the marrow and eventual loss of B cell antigens as the terminal plasma cell stage is reached. This figure also assumes as a working hypothesis that cell migration proceeds from the blood to the bone marrow.

If B lineage cells in blood and bone marrow are considered as a sequentially related continuum, Figure 1 shows the loss of both CD19 and CD45, and the acquisition of CD38 and CD56, as B cells differentiate in the bone marrow. Expression of both CD38 , at high intensity, and CD56 at somewhat lower intensity, on the large blood B cells suggests they are already at a very late stage of B development. Other markers which define late stage B cells, such as CD5, CD10 and CD11b, are found predominantly, and at highest intensity, on large blood B cells, and are lost as these cells migrate to the bone marrow and terminally differentiate.

Circulating Monoclonal B Cells Persist Despite Intensive Chemotherapy

Although bone marrow is often heavily depleted of plasma cells during chemotherapy, the population of blood B cells remains at high levels despite chemotherapy of multiple myeloma (3,4, Table 1). These apparently chemotherapy-resistant late stage monoclonal B cells remain phenotypically similar to those detected prior to treatment.

Table 1. Circulating B cells Persist During Chemotherapy

Treatment	$\%CD19^+$ B cells	% of $CD19^+$ B cells that are $CD38^{hi}$	
		CD19Sm	CD19Lge
Untreated	28±13	10±9	45±23
VAD	28±14	18±13	53±13
Melp/Pred	31±18	20±12	32±21
Off Tr	23±9	25±15	58±21
Relapse	35±10	17±15	62±17

Values are the mean ± SD. $CD38^{Hi}$ includes those B cells ($CD19^+$) with an intensity of CD38 staining greater than channel 516.

The P-glycoprotein Multidrug Transporter is Expressed on Circulating B Cells in Myeloma

Because the circulating B lineage cells in myeloma are relatively unaffected by chemotherapy, the expression of p-glycoprotein was examined. P-glycoprotein is a membrane transport protein that appears to mediate ATP-dependent transport of heterocyclic drugs and is implicated as a primary mediator of multi-drug resistance in malignant disease (7,8). It has been detected on plasma cells resident in the bone marrow of myeloma patients (9,10) and treatment of resistant patients with drug in conjunction with verapamil, known to block drug efflux in vitro, can enhance drug sensitivity in vivo (11,12). Mab MRK-16 detects the fourth external domain of p-glycoprotein which is specific to the mdr1 gene product (13,14). Table 2 shows that p-glycoprotein is expressed on both small and large B cells in PBMC of myeloma patients and is frequently detected on a subset of circulating B cells prior to treatment.

Since p-glycoprotein was detectable, often at high intensity, on circulating B cells in newly diagnosed untreated myeloma patients, both blood and bone marrow cells were compared from individual patients (Table 3). A variety of patterns were detected, including expression

of p-glycoprotein on only blood B cells, or on both blood and bone marrow B lineage cells. Overall, p-glycoprotein was detected on circulating B lineage cells from a large proportion of myeloma patients, although the amount and intensity of staining varied considerably (Table 4). However malignancies involving earlier stage B cells such as B chronic lymphocytic leukemia, or later stages such as plasma cell leukemia, appeared to be negative further suggesting stage-specific expression of the mdr1-encoded transporter.

Table 2. Expression of P-glycoprotein on Circulating B Lineage Cells in Multiple Myeloma

Treatment	Proportion of patients with a subset of MRK-16$^+$ CD19$^+$ B cells in PBMC MRK-16$^-$	MRK-16^{lo}	MRK-16^{hi}
Untreated	1/14	5/14	8/14
VAD-treated	2/11	4/11	5/11
Melp/pred	4/17	4/17	9/17
Off tr	5/14	4/14	5/14
Relapse	4/7	1/7	2/7

PBMC from patients were stained with CD19 and MRK-16 in double immunofluorescence. Files were gated to include only CD19$^+$ B cells and histograms of staining by MRK-16 were plotted. In all samples an identically stained and gated sample was stained with an isotype-matched IgG2a control antibody and the staining by MRK-16 evaluated in comparison to this control. Low staining was defined as 20-90 channels above the isotype control staining, and high staining was defined as 100 or greater channels above the control. In many cases MRK-16 staining was 200-250 channels above the control which is approximately 10 fold greater intensity.

Table 3. Expression of P-glycoprotein on B Lineage Cells from Blood and Bone Marrow of Untreated Myeloma Patients

Patient	Percent of population expressing MRK-16 Blood CD19$^+$Sm	Blood CD19$^+$Lge	Bone Marrow CD19$^+$Lge	CD19$^-$Lge
#1	20 Hi	0	0	0
#2	-	86 Lo	0	0
#3	56 Hi	-	0	0
#4	-	29 Hi	63 Hi	-
#5	10 Hi	48 Hi	20 Hi	0
#6	71 Hi	20 Hi	67 Hi	40 Hi
#7	30 Lo	46 Lo	12 Lo	-

Expression of MRK-16 on CD19$^+$ B cells or CD19$^-$ plasma cells was as described above. Plasma cells were defined as CD19$^-$ cells with high side and forward scatter, expressing CD38 and morphologically identified as plasma cells on cytospins. All patients were untreated.

Table 4. Proportion of Malignant B Lineage Cells Expressing P-glycoprotein

Disease	% CD19+ expressing MRK-16	Intensity of MRK-16 Number of Channels
All Myeloma PBMC (40)		
$CD19^+$ Small	24±27	94±26
$CD19^+$ Large	55±36	94±61
Untreated Myeloma (13)		
$CD19^+$Sm PBMC	35±31	118±77
$CD19^+$Lge PBMC	43±35	
Bone marrow	25±24	119±80
Plasma cell leukemia (2)		
PBMC	0	0
Bone Marrow	0	0
B-CLL (7) PBMC	0	0

Intensity is the number of channels above the staining by the isotype matched control. Values are the mean ± SD. The range of values was from 0-100% for nearly all myeloma groups. The number of patients analyzed is indicated in brackets. A shift of approximately 80 channels indicates about 3 fold greater staining than the control, and a shift of 240 channels represents a 10 fold increase.

Functional Activity of P-glycoprotein on Circulating B Cells in Myeloma

To determine if the expression of MRK-16 epitopes on the surface of circulating B cells in myeloma revealed the presence of functional p-glycoprotein, adriamycin accumulation by B lineage cells was evaluated in the presence and absence of verapamil (Figure 2). Studies with cell lines have shown that sensitive cells lines take up adriamycin by diffusion, and lacking p-glycoprotein, do not pump it out again, whereas drug-resistant lines rapidly pump adriamycin resulting in virtually no drug accumulation. Since this efflux is inhibited by verapamil, resistant cells treated with verapamil accumulate drug to the same extent as do sensitive cells. Figure 2 shows adriamycin accumulation by B cells from an untreated patient and increased accumulation in samples treated with verapamil indicating functionally active p-glycoprotein on the surface of these cells. B cells from a second patient also showed a increase in drug accumulation in the presence of verapamil (Figure 2). For both patients a proportion of B cells, about 30%, showed reduced adriamycin uptake which was shifted to high uptake in verapamil-treated samples. This proportion is approximately the same as the proportion of $MRK\text{-}16^+$ B cells (Figure 2).

P-glycoprotein is Expressed at High Intensity on Normal B Cells from Blood and Lymphoid Tissue

Since p-glycoprotein was detectable on PBMC B cells from a large number of patients and was apparently over-expressed on B cells from untreated patients, its expression was assessed on normal B cells from blood, bone marrow and spleen (Table 5). B cells in both blood and spleen expressed a high intensity of MRK-16 reactivity suggesting that p-glycoprotein plays a significant role in normal B cell function. ATP-dependent transport pumps have been implicated in antigen processing and association of nominal antigen with MHC Class I, and these pumps have extensive structural and sequence homology with p-glycoprotein (15). Thus, it seems possible that p-glycoprotein may also be involved in antigen presentation by B cells. In contrast to B cells of blood and spleen, B cells of normal bone marrow do not express appreciable p-glycoprotein.

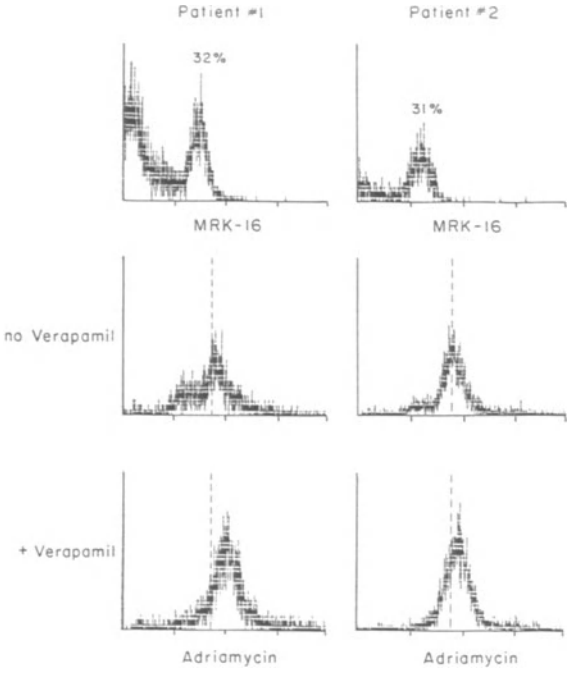


Fig. 2. PBMC from patients were incubated in adriamycin with or without verapamil for 3 hours followed by staining with CD19 to allow identification of B cells, and analysis of drug accumulation by fluorescence flow cytometry. After gating for $CD19^+$ cells, accumulation of drug in B cells was evaluated. The shift in fluorescence intensity by the main peak for these two patients was equivalent to that seen for a sensitive cell line. The shift of the shoulder to the left of the main peak to a higher intensity in the sample treated with verapamil was roughly equivalent to that seen for the intermediate drug-resistant cell lines

Table 5. Expression of P-glycoprotein on normal B cells

Normal Tissue	% of CD19$^+$ B Cells Expressing MRK-16	Intensity of Staining
PBMC (7)	65±30	200 channels
Bone Marrow (5)	8±14	120 "
Spleen (4)	87±21	200 "

Values are the mean ± SD. Two spleens were from organ transplant donors and 2 were from patients with ITP. Bone marrow was from hip joints removed during orthopedic surgery. Normal blood was from the Red Cross Blood Transfusion Service.

Table 6: Expression of MRK-16 is Lost from Stimulated Normal B Cells but Increases on B Cells from Some Patients

Donor	% of CD19+ B cells that are MRK-1+ Time in Culture + PWM		
	Day 0	Day 3	Day 7
Normal (3)	65±30	33±46	0
LSM-2 (VAD)	4	0	
WGA-10 (IFn)	66 Hi		0
FKN-2 (VAD)	90 Med		15 Lo
ALR-4 (VAD)	92 Hi		53 Lo
AWA-6 (VAD)	88 Med		75 Hi
SSZ-7 (Relapse)	84 Med	67 Hi	59 Med
JIS-6 (Relapse)	88 Hi		59 Hi

Values are the mean ± SD. PBMC from patients were cultured with 1/100 PWM for the indicated time period, harvested and analyzed for surface marker expression on CD19$^+$ B cells.

The pattern of MRK-16 staining suggested that this transporter might be differentially expressed during sequential stages of B lymphocyte differentiation and by analogy might appear only transiently during differentiation of the malignant B lineage in myeloma. Experiments utilizing both normal and abnormal B cells were designed to explore this possibility. PBMC B cells were stimulated in culture with pokeweed mitogen (PWM) followed by analysis at various time points to determine the expression of MRK-16 epitopes (Table 6). Expression on stimulated normal B cells, and on B cells from some patients, decreased with time. Expression was not induced on B cells which had been negative prior to culture. However MRK-16 staining increased significantly on MRK-16$^+$ B cells from two patients in relapse, and was maintained for at least 7 days in culture, whereas that on normal B cells was completely lost by day 7.

Conclusions

Although over-expression of the multi-drug resistant phenotype has been widely viewed as a stable genetic change, our work analyzing expression of p-glycoprotein on normal and malignant B cells strongly suggests this represents an inducible transient change. We speculate that p-glycoprotein is expressed as a B cell differentiation molecule required for a normal B cell function, with a role in antigen presentation being an obvious candidate given the sequence homology between p-glycoprotein and a transporter of MHC peptides (15). If this is true, then expression of high density p-glycoprotein on B cells within a malignant

lineage might be restricted to only certain stages of the differentiation pathway. In the case of myeloma, it appears that p-glycoprotein expression predominates on the large B cells which share properties with activated normal B cells, although expression sometimes occurs on earlier stage small B cells and on later pre-plasma cells in the bone marrow. It is possible that expression of p-glycoprotein is actually lost as the malignant cells differentiate to end-stage plasma cells which might have no neeed for a transporter and thus lose it along with the documented loss of CD45 and CD19 (3).

An alternative explanation for the expression of p-glycoprotein on earlier stages of the malignant B lineage involves expression of oncogenes and tumor suppressor genes by these B cells. Recent work has shown that expression of the H-ras gene product and/or of a mutant p53 suppressor gene product (p53mut) stimulates transcription via the mdr1 gene promoter, while expression of a wild type p53 inhibits ras- or p53mut- stimulated transcription via the human mdr1 gene promoter (16). Since synthesis of mRNA encoding p53 is induced in activated normal B cells (17), the effects of increased p53 may account for the loss of p-glycoprotein upon stimulation of normal B cells. One might speculate that for B lineage cells in myeloma, there is either expression of ras and/or a p53mut, or an absence of p53 wild type product, which allows or enhances mdr1 gene transcription. If these events were selective to the circulating B lineage cells in myeloma, a reasonable speculation given their similarity to normal activated B cells, then p-glycoprotein might be transiently over-expressed only during these developmental stages and lost with further differentiation which might also turn off ras/p53 activity. Finally, drug treatment itself stimulates transcription via the mdr1 gene promoter (18) which may selectively up-regulate surface expression of p-glycoprotein on some malignant B lineage cells in vivo.

If expression of p-glycoprotein by B cells is transient, then chemotherapeutic treatment must be viewed in a new light. It might be expected that all end-stage plasma cells would be eliminated by drug while earlier progenitor malignant cells, e.g. those B cells in blood, would be selectively spared. This is consistent with our observation that circulating monoclonal B cells are spared during aggressive chemotherapy which effectively depletes bone marrow myeloma cells. It also raises the possibility that cytoreductive treatment prior to bone marrow transplantation might selectively spare circulating B cells thus compromising any chances of successful disease eradication. The work presented here suggests that emphasis should be placed on design of chemotherapeutic strategies which will target the circulating, perhaps transiently multi-drug resistant, B lineage cells in myeloma patients. This could involve either use of agents such as cyclosporin or verapamil to block p-glycoprotein-dependent drug efflux, and/or might involve procedures to "push" monoclonal B cells located in blood and lymphoid tissues into a drug-sensitive stage of development immediately prior to treatment. At least for myeloma, multi-drug resistance does not appear to be a stable heritable change in tumor phenotype but rather a transient inducible property.

Acknowlegments

Excellent technical assistance was provided by Darlene Paine, Kimberly Howland, Dorothy Rutkowski and Delores Mowles. Clincal collaborators were Drs. M. Mant, B. Ruether, and W. Yakimets. Funding was from the National Cancer Institute of Canada and the Alberta Cancer Board Research Initiatives Program

References

1. Benner R, Hijmans W, Haaijman JJ (1981) The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. Clin Exp Immunol 46:1-8.
2. Benner R, van Oudenaren A, de Ruiter H (1977) Antibody formation in mouse bone marrow. IX. Peripheral organs are involved in the initiation of bone marrow antibody formation. Cell Immunol 34:125-137.
3. Pilarski LM, Jensen GS (1992) Monoclonal circulating B cells in multiple myeloma: A continuously differentiating possibly invasive population as defined by expression of CD45 isoforms and adhesion molecules. Hematology/Oncology Clinics of North America in press.
4. Jensen GS, Mant MJ, Belch AJ, Berensen JR, Ruether BA, Pilarski LM (1991) Selective expression of CD45 isoforms defines CALLA+ monoclonal B lineage cells in peripheral blood from myeloma patients as late stage B cells. Blood 78:711-719.
5. Pilarski LM, Jensen GS (1992) Expression of CD45 isoforms (leukocyte common antigen) and adhesion molecules during normal and abnormal B lymphocyte development. Advances in Molecular and Cellular Immunology 1:in press.
6. Jensen GS, Poppema S, Mant MJ, Pilarski LM (1989) Transition in CD45 isoform expression during differentiation of normal and abnormal B cells. Int Immunol 1:229-236.
7. Chan HSL, Haddad G, Thorner PS, DeBoer G, Lin YP, Ondrusek N, Yeger H, Ling V (1991) P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. New Eng J Med 325:1608-1614.
8. Goldie JH, Ling V (1991) The evolution of drug resistance in tumors. Canad J Oncol 1:1-10.
9. Epstein J, Xiao H, Oba BK, Barlogie B (1989) P-glycoprotein expression in plasma cell myeloma is associated with resistance to VAD. Blood 74:174-179.
10. Carulli G, Petrini M, Marini A, Ambrogi F, Ucci G, Riccardi A, Luoni R, Grassi B (1990) P-glycoprotein expression in multiple myeloma. Haematologica 75:288-290.
11. Salmon SE, Dalton WS, Grogan TM, Plezia P, Lehnert M, Roe DJ, Miller TP (1991) Multidrug-resistant myeloma: laboratory and clinical effects of verapamil as a chemosensitizer. Blood 78:44-50.
12. Dalton WS, Grogan TM, Meltzer PS, Scheper RJ, Durie BGM, Taylor CW, Miller TP, Salmon SE (1989) Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of p-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J Clin Oncol 7:415-424.
13. Hamada H, Tsuro T 0(1986) Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. Proc Nat Acad Sci USA 83:7785-7789.
14. Cumber PM, Jacobs A, Hoy T, Whittaker JA, Tsuro T, Padua RA (1991) Increased drug accumulation ex vivo with cyclosporin in chronic lymphocytic leukemia and its relationship to epitope masking of p-glycoprotein. Leukemia 5:1050-1053.
15. Spies T, Bresnahan M, Bahram S, Arnold D, Blanck G, Mellins E, Pious D, DeMars R (1990) A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. Nature 348:744-747.
16. Chin K-V, Ueda M, Pastan I, Gottesman MM (1992) Modulation of activity of the promoter of the human MDR1 gene by ras and p53. Science 255:459-462.
17. Smeland EB, Blomhoff HK, Ohlsson R, de Lange Davies C, Funderland S, Boye E (19880) Transcription of protooncogenes during stimulation of normal human B lymphocytes. Eur J Immunol 18:1847-1850.
18. Kohno K, Sato S-I, Takano H, Matuso K-I, Kuwano M (1989) The direct activation of human multidrug resistance gene (MDR1) by anticancer agents. Biochem Biophys Res Comm 165:1415-1421.

Expression of Multiple Adhesion Molecules on Circulating Monoclonal B Cells in Myeloma

G. S. JENSEN[1], A. R. BELCH[2], M. J. MANT[1], B. A. RUETHER[3] and L. M. PILARSKI[1]

[1]Departments of Immunology and Medicine, University of Alberta, Edmonton,
[2]Cross Cancer Institute, Edmonton,
[3]Department of Medicine, University of Calgary, Alberta.

Introduction

Multiple myeloma is a tumor of the B cell lineage, with characteristic monoclonal end stage plasma cells in the bone marrow, responsible for producing large quantities of monoclonal immunoglobulin present in serum, and for triggering osteoclasts to produce lesions of the bone, leading to high blood calcium, the possibility of kidney failure, and severe pathological damage. The proliferative potential of these plasma cells is low, and they express very few surface molecules involved in locomotion and migration. Candidates for spreading of the disease are circulating, monoclonal B cells, which have been reported by us (1,2) and others (3-8). We have described a large subset of circulating cells belonging to the same clone as the bone marrow plasma cells in myeloma, which represent late stages of B cell differentiation towards pre-plasma cells, predominantly expressing the CD45R0 isoform (1), only found on activated late stage B cells (9). This subset of monoclonal B cells in the peripheral blood was observed among newly diagnosed patients, as well as patients undergoing intermittent chemotherapy, or at stable phases of disease (1), and thus seems unaffected by conventional therapy (see also Pilarski et al., this issue). In contrast to the bone marrow plasma cells, the blood B cells comprise a proliferating subpopulation (2). The monoclonal B cells in the blood of myeloma patients are at a late stage of B cell differentiation, and appear to be continuously progressing towards the plasma cell stage. Significant numbers of plasma cells, however, are only found in the bone marrow, except in terminal or highly aggressive myeloma, where they may be present in the blood as well. These observations suggest that if the blood B cells are, or include, a precursor population for the bone marrow plasma cells of the same clonality, the blood B cells must be capable of extravasating and migration to the bone marrow. It further suggests that in myeloma patients, the blood does not provide the microenvironmental stimuli to support the terminal differentiation to end stage plasma cells. The myeloma blood B cells express multiple adhesion molecules, including CD11b, L-Selectin, CD44, $\alpha 2\beta 1$, and $\alpha 6\beta 1$, in contrast to the plasma cells in the myeloma bone marrow. This strongly indicates that the circulating subset is indeed potentially more motile and capable of extravasating and homing to the bone marrow.

The Circulating Monoclonal B Cells in Myeloma Express High Densities of CD11b

Using multi-parameter flow cytometry, it was demonstrated that the large subset of monoclonal B cells in peripheral blood of myeloma patients express the surface molecule CD11b. CD11b is a member of the leukocyte integrin family, together with LFA-1 and P150,95 (10-12), and is involved in leukocyte adherence to endothelium, and transendothelial migration (13,14). A majority of the B cell population in myeloma blood expressed CD11b at very high densities (beyond 10^2 on a log scale). Normal blood B cells do not express CD11b. After in vitro activation of normal PBMC B cells for 3-7 days, CD11b was expressed at densities comparable to myeloma blood B cells (Fig. 1). This

suggests that CD11b may be involved in migration and extravasation of normal activated B cells, as well as their malignant counterparts in myeloma.

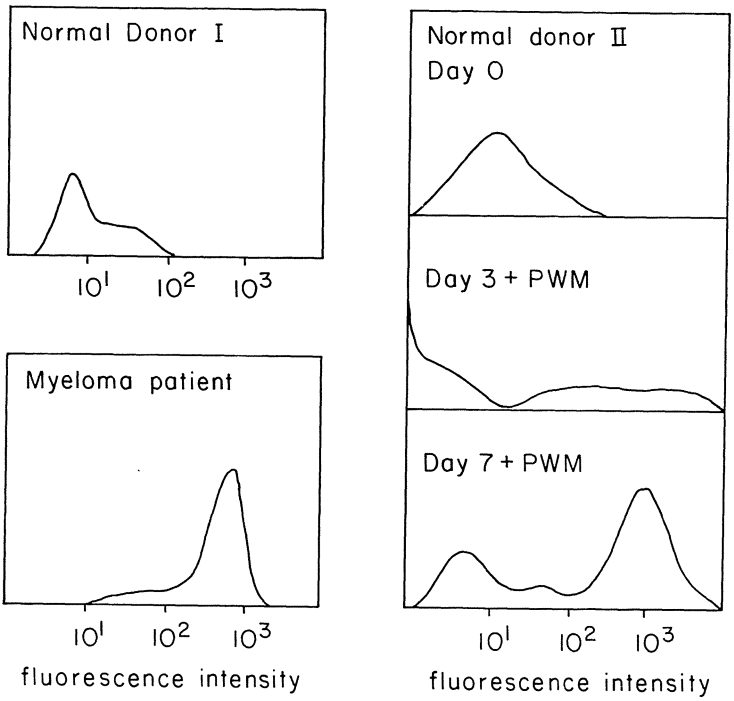


Fig. 1. Expression of CD11b on normal, activated, and myeloma blood B cells. Two-color immunofluorescence was used to analyze the CD11b expression on blood B cells. Gating for CD19 (B4) was used to define the B cell population, and CD11b (Mo-1) expression was evaluated on the B cells. Fluorescence intensity is on a logarithmic scale, where 100 and beyond is considered high density. Normal blood B cells were activated in vitro with Pokeweed Mitogen. The expression of CD11b was assessed at day 0, 3, and 7 by two-color immunofluorescence

The CD11b Expression is Higher on Myeloma Blood B Cells than on Bone Marrow Plasma Cells from the same Patients

Comparing the percentage of CD11b$^+$ B cells in blood and bone marrow from the same myeloma patients, there is a much higher percentage of CD11b on B cells in peripheral blood (Fig. 2). Analysis with other markers, including CD45 isoforms CD45RA and CD45R0, have shown that the B cells in blood are at earlier stages of differentiation than the bone marrow cells (1). The lower expression of CD11b on the more differentiated bone marrow cells indicates that this surface molecule is necessary during the migration from blood to bone marrow, after which it is reduced or lost.

Table 1. Expression of CD11b on blood and bone marrow B cells in myeloma.

	CD11b on CD19+ B cells	Number of patients
Untreated MM	62 ± 4.7 % *	21
Treated MM	75 ± 2.7 %	21
MM bone marrow	33 ± 6.5 %	13

MM: multiple myeloma; CD11b: Mo1 antibody; CD19: B4 antibody;*Mean± SE. Two-color immunofluorescence was performed as described in legend to Fig. 1.

The Circulating Monoclonal B Cells in Myeloma Express Multiple Integrins from the $\beta 1$ Family.

The $\beta 1$ integrins belong to a family of heterodimeric molecules which all share a common β chain ($\beta 1$, CD29), combined with any of the six α chains ($\alpha 1$-$\alpha 6$, CD49a-f). The function of these molecules is to integrate the cytoskeletal elements to components of the extracellular matrix (collagen, laminin, and fibronectin), either as a part of tissue anchoring or during migration. Normal blood B cells only express $\alpha 4\beta 1$ (15,16), which has affinity for the vascular cell adhesion molecule (VCAM), which is induced on endothelial cells by inflammatory mediators. Thus, the $\alpha 4\beta 1$/VCAM interaction provides a mechanism for leukocyte/endothelium interaction different from and independent of the one mediated by $\beta 2$ integrins (12,15,17,18). The $\alpha 5$ is expressed on early B cells and in some cases of B cell activation, but not on normal peripheral blood B cells (16,19-21).

The circulating B cells from myeloma patients express high densities of $\alpha 4$, medium densities of $\beta 1$, and surprisingly, low but significant amounts of $\alpha 2$, $\alpha 5$, and $\alpha 6$, which are not expressed on normal blood or tissue B cells. Figure 3 shows a representative example of expression of $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\alpha 6$ from a myeloma patient. Frequently, only a subpopulation of the B cells express a given integrin, and the rest of the B cell population is negative for that particular integrin. The density of $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\alpha 6$, when present, is relatively low.

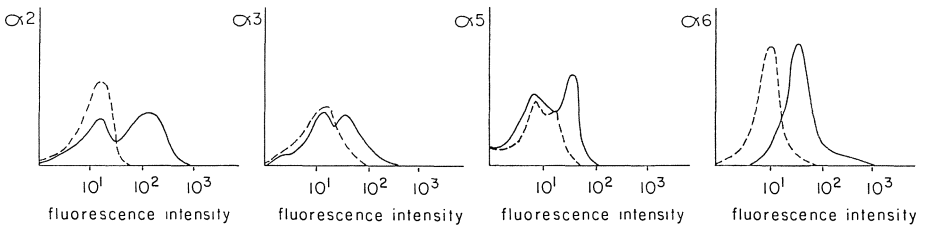


Fig. 2. Expression of $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\alpha 6$ on myeloma blood B cells. Two-color immunofluorescence was used to determine the expression of members of the $\beta 1$ integrin family on the surface of circulating myeloma B cells. CD19 (B4) was utilized to define the B cell subset, and the antibody JBS2 (from Dr. J. Wilkins) was used to define $\alpha 2$ (CD49b), P1B5 (from Dr. E. Wayner) for $\alpha 3$ (CD49c), B1E5 (from Dr. C. Damsky) and JBS5 (from Dr. J. Wilkins) for $\alpha 5$ (CD49e), and J1B5 (from Dr. C. Damsky) for $\alpha 6$ (CD49f). The dashed lines represent the isotype control (IgG1 for $\alpha 2$, $\alpha 3$, and $\alpha 5$, and IgG2b for $\alpha 6$) the CD19+ B cell population, and the solid lines represent the expression of the antibody. Fluorescence intensity is on a logarithmic scale

The $\beta 1$ integrins $\alpha 2\beta 1$ and $\alpha 6\beta 1$ are absent from bone marrow plasma cells.

The myeloma bone marrow B lineage cells represent later stages of differentiation, compared to the circulating B cells from the same patient. The bone marrow B lineage cells express lower densities of CD19, or no CD19, lower densities of CD45 (1), and have medium densities of PCA-1 and high density of CD38, indicating their close resemblance to terminally differentiated plasma cells. In contrast to their blood counterparts, the B lineage cells in myeloma bone marrow do not express $\alpha 2$, $\alpha 3$, or $\alpha 5$, and very little $\alpha 6$ (Fig. 3).

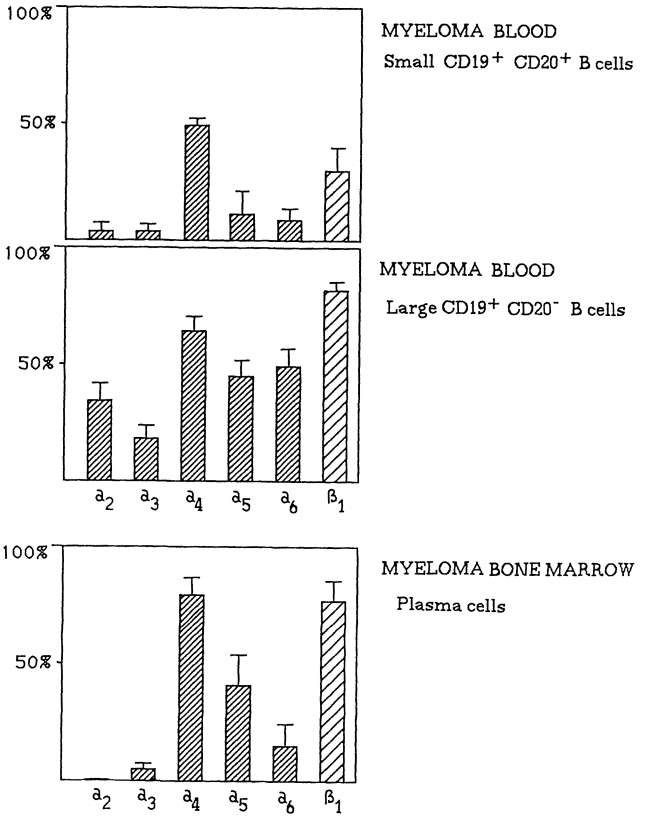


Fig. 3. Expression of $\beta 1$ integrins on myeloma blood B cells and bone marrow plasma cells. The bars in the histogram represent the percentage of the B cell population expressing the integrin in question, and results are presented as the mean value ± S.E.
A. The small B lymphocytes in myeloma blood were defined as cells with a low forward angle light scatter, and being positive for CD19 and CD20. The subset was negative for CD4 and CD8, and predominantly expressed the CD45RA isoform, present on normal mature resting B cells. (8 patients analyzed)
B. The large B cells/pre-plasma cells were defined as cells with a high forward angle light scatter, positive for CD19 but negative for CD20. The subset were negative for CD4 and CD8, and mainly expressed the CD45R0 isoform, normally expressed on late stage B lymphoblasts. (22 patients analyzed)
C. Plasma cells in myeloma bone marrow were defined as large cells (high forward angle light scatter) being negative for CD4 adn CD8, and with little or no CD19 on the cell surface (9 bone marrow samples analyzed)

The Circulating B cells from Myeloma Express L-Selectin and CD44

The L-selectin mediates binding of circulating lymphocytes to the high endothelial venules of peripheral lymph nodes (22,23), and is expressed on mature CD20 + B cells (24,25). L-selectin is expressed on the myeloma blood B cells at a wide range of densities, from low to medium-high, whereas it is expressed at low densities, when present on bone marrow plasma cells.

The CD44 surface antigen is the human homologue of the mouse Hermes/pgp-1 homing molecule (12,26-28), and is expressed at high densities on B lineage cells at most stages of differentiation and activation (29). CD44 is expressed at extremely high densities (beyond 10^3) on the myeloma blood B cells, whereas the expression is lower on the bone marrow plasma cells (100-500 times lower density) (see Table 2).

Both surface molecules are expressed on the myeloma blood B lineage cells, potentially reinforcing the capability of these cells to interact with endothelial cells.

Table 2. Expression of L-Selectin and CD44 on circulating myeloma B lineage cells.

	% of CD19+ B lineage cells in blood:	
	L-Selectin	CD44
MM blood	47 ± 9.8 me/hi	82 ± 7.1 very hi
MM bone marrow	47 ± 6.5 lo	92 ± 2.4 me/hi

Expression of L-selectin was evaluated using the TQ1 antibody (Coulter, Hialeah FL), and CD44 by the pgp-1 monoclonal antibody (kindly provided by Dr. I. Trowbridge). Ten blood samples and 8 bone marrow samples from myeloma patients were analyzed.
*Mean ± SE.

Conclusion

Accumulating evidence points towards the circulating monoclonal B lineage cells in myeloma as candidates for proliferation and spreading of the disease, in contrast to the more sessile end stage plasma cells in the bone marrow of the same patients. If this is correct, the blood B lineage cells, or a subpopulation thereof, must have the capability both to adhere to endothelium, and to migrate through extracellular matrix.

The circulating monoclonal B lineage cells in myeloma express various surface molecules involved in adhesion to endothelium and extravasation. The B lineage cells in the blood express high densities of CD11b, a molecule known to mediate leukocyte adherence to endothelium, and migration through layers of endothelial cells. A subset of the blood B cells also express high densities of $\alpha 4\beta 1$, which binds to the vascular cell adhesion molecule (VCAM), thus facilitating adhesion to endothelium. Furthermore, the blood B cells express two other surface antigens mediating binding to specialized endothelial cells, including L-selectin, expressed at medium to high densities, and CD44, expressed at extremely high densities.

In addition, the blood B cells in myeloma comprise a subset expressing one or several of the $\beta 1$ integrins not normally seen on blood or tissue B lineage cells. Only myeloma blood B cells express $\alpha 2$ and $\alpha 6$. These α chains are not found on normal resting or activated B cells. Both $\alpha 2$ and $\alpha 6$ expression has been implicated on circulating tumor cells capable of metastatic invasion, and not on their non-metastatic counterparts (30-32). The myeloma blood B cells also express some $\alpha 5$, found on some stages of B cell differentiation, but not on normal blood B cells. The bone marrow plasma cells, belonging to the same clone, do not express $\alpha 2$, $\alpha 3$, and almost no $\alpha 6$, although they have variable expression of $\alpha 5$. Thus, they appear to be cells incompetent to invade the extracellular matrix. The bone marrow cells express medium to high densities of $\alpha 4$ and $\beta 1$, which may participate in cell-cell

interactions within the bone marrow, possibly between the plasma cell precursors and stromal cells.

Our results are consistent with a working hypothesis that invasive capability in myeloma is to be found within the abnormal monoclonal B cells in peripheral blood, which alone among B cells or plasma cells expresses the $\alpha 2$ and $\alpha 6$ integrin receptors necessary for cellular translocation across the endothelial extracellular matrix, and into extracellular spaces.

Acknowledgements

This study was supported by the Alberta Cancer Board Research Initiative Program and the National Cancer Institute of Canada. GSJ was an Alberta Cancer Board Research Fellow, presently supported by the Danish Cancer Society. Drs. J. Wilkins, C. Damsky, H. Zola, E. Wayner, and I. Trowbridge kindly provided monoclonal antibodies.

References

1. Jensen GS, MJ Mant, AJ Belch, JR Berensen, BA Ruether, LM Pilarski (1991) Selective expression of CD45 isoforms defines CALLA$^+$ monoclonal B lineage cells in peripheral blood from myeloma patients as late stage B cells. Blood 78:711-719
2. Pilarski LM, GS Jensen (1992) Monoclonal circulating B cells in multiple myeloma: A continuously differentiating possibly invasive population as defined by expression of CD45 isoforms and adhesion molecules. Hematology/Oncology Clinics of North America, in press
3. Grogan TM, BGM Durie, C Lomen, C Spier, DP Wirt, R Nagle, GS Wilson, L Richter, E Vela, V Maxey, K McDaniel, C Rangel (1987) Delineation of a novel pre-B cell component in plasma cell myeloma: immunochemical, immunophenotypic, genotypic, cytologic, cell culture, and kinetic features. Blood 70:932-942
4. King MA, DS Nelson (1989) Tumor cell heterogeneity in multiple myeloma: Antigenic, morphologic, and functional studies of cells from blood and bone marrow. Blood 73:1925-1935
5. Berenson J, R Wong, K Kim, N Brown, and A Lichtenstein (1987) Evidence for peripheral blood B lymphocyte but not T lymphocyte involvement in multiple myeloma. Blood 70:1550-1553
6. van Riet I, C Heirman, P Lacor, M D. Waele, K Thielemans, B van Camp (1989) Detection of monoclonal B lymphocytes in bone marrow and peripheral blood of multiple myeloma patients by immunoglobulin gene rearrangement studies. Brit J Haematol 73:289-295
7. Chiu EKW, K Ganeshaguru, AV Hoffbrand, AB Metha (1989) Circulating monoclonal B lymphocytes in multiple myeloma. Brit J Haematol 72:28-31
8. Berenson JR, AK Lichtenstein (1989) Clonal rearrangement of immunoglobulin genes in peripheral blood of multiple myeloma patients. Brit J Haematol 73:425-427
9. Jensen GS, S Poppema, MJ Mant, LM Pilarski (1989) Transition in CD45 isoform expression during differentiation of normal and abnormal B cells. Int Immunol 1:229-336
10. Kuypers TW, D Roos (1989) Leukocyte membrane adhesion proteins LFA-1, CR3, and p150,95: A review of functional and regulatory aspects. Res Immunol 140:461-486
11. Larson RS, TA Springer (1990) Structure and function of Leukocyte Integrins. Immunol Reviews 114:181-217
12. Springer TA (1990) Adhesion receptors of the immune system. Nature 346:425-434
13. Van Epps DE, J Potter, M Vachula, CW Smith, DC Anderson (1989) Suppression of human lymphocyte chemotaxis and transendothelial migration by anti-LFA-1 antibody. J Immunol 143:3207-3210
14. Oppenheimer-Marks N, LS Davis, PE Lipsky (1990) Human T lymphocyte adhesion to endothelial cells and transendothelial migration. Alteration of receptor use relates to activation status of both the T cell and the endothelial cell. J Immunol 145:140-148
15. Hemler ME, MJ Elices, C Parker, Y Takada (1990) Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. Immunol Rev 114:45-66

16. Klingemann H-G, S Dedhar (1989) Distribution of integrins on human peripheral blood mononuclear cells. Blood 74:1348-1354
17. Campanero MR, R Pulido, MA Ursa, M Rodriguez-Moya, MO de Landazuri, F Sanchez-Madrid (1990) An alternative leukocyte homotypic adhesion mechanism, LFA-1/ICAM-l-independent, triggered through the human VLA-4 integrin. J Cell Biology 110:2157-2165
18. Elices MJ, L Osborn, Y Takada, C Crouse, S Luhowskyj, ME Hemler, RR Lobb (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell 60:577-584
19. Stupack DG, S Stewart, WG Carter, EA Wayner, JA Wilkins (1991) B lymphocyte fibronectin receptors: Expression and utilization. Scand J Immunol 34:761-769
20. Kohn FR, ME Grigg, H-G Klingemann (1991) Fibronectin receptor subunit (a5, a4, and b1) mRNA and cell surface expression in human peripheral blood B lymphocytes. Immunology Letters 28:27-30
21. Ballard LL, EJ Brown, VM Holers (1991) Expression of the fibronectin receptor VLA-5 is regulated during human B cell differentiation and activation. Clin Exp Immunol 84:336-346
22. Spertini OS, GS Kansas, JM Munro, JD Griffin, TF Tedder (1991) Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. Nature 349:691-694
23. Tedder TF, CM Isaacs, TJ Ernst, GD Demetri, DA Adler, CM Disteche (1989) Isolation and chromosomal location of cDNAs encoding a novel human lymphocyte cell surface molecule, LAM-1. Homology with the mouse homing receptor and other human adhesion proteins. J Exp Med 170:123-133
24. Kansas GS, GS Wood, EG Engleman (1985) Maturational and functional diversity of human B lymphocytes delineated with anti-Leu8. J Immunol 134:3003-3006
25. Kansas GS, MO Dailey (1989) Expression of adhesion structures during B cell development in man. J Immunol 142:3058-3062
26. Stamenkovic I, M Amiot, JM Pesando, B Seed (1989) A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56:1057-1062
27. Coombe DR, CC Rider (1989) Lymphocyte homing receptors cloned - a role for anionic polysaccharides in lymphocyte adhesion. Immunol Today 10:289-291
28. Gallatin WM, IL Weissman, EC Butcher (1983) A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 304:30-34
29. Haynes BF, MJ Telen, LP Hale, SM Denning (1989) CD44 - A molecule involved in leukocyte adherence and T cell activation. Immunol Today 10:423-428
30. Chan BMC, N Matsuura, Y Takada, BR Zetter, ME Hemler (1991) In vitro and in vivo consequences of VLA-2 expression on rhabdosarcoma cells. Science 251:1600-1602
31. Klein CE, T Steinmayer, D Kaufmann, L Weber, E Brocker (1991) Identification of a melanoma progression antigen as integrin VLA-2. J Invest Dermatol 96:281-284
32. Dedhar S, R Saulnier (1990) Alterations in integrin receptor expression on chemically transformed cells: Specific enhancement of laminin and collagen receptor complexes. J Cell Biol 110:481-489

Circulating Peripheral Blood Plasma Cells in Multiple Myeloma

T. E. WITZIG, R. A. KYLE and P. R. GREIPP

Division of Internal Medicine and Hematology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Introduction

The proliferation of an abnormal clone of plasma cells can produce a wide variety of human diseases such as monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), active multiple myeloma (MM), amyloidosis, non-Hodgkin's lymphoma, Waldenström's macroglobulinemia, and osteosclerotic myeloma (POEMS syndrome). For the patient to be optimally managed, the clinician must correctly classify the patient as MGUS, SMM, active MM, and relapsed MM (RMM). Patients with MGUS have a small population of monoclonal plasma cells; however, a recent study of 202 patients with MGUS followed for a median of 22 years found that only 19% (39/202) developed multiple myeloma [4]. Patients with SMM meet the criteria for MM but have a normal complete blood count and serum chemistries and can have a prolonged stable course without the need for chemotherapy [5]. Patients with MM and RMM have active disease and require chemotherapy.

These diseases are considered as a proliferation of bone marrow plasma cells and routine peripheral blood smears usually fail to detect any circulating malignant plasma cells. The evidence documenting that some patients with MM have circulating peripheral blood cells that are part of the malignant clone comes from various studies. Early reports utilizing anti-idiotypic antibodies have identified B-cells in the blood of patients with MM that have idiotypic determinants on the cell surface or in the cytoplasm [1,6,8]. Examining cell-surface light chains, Pettersson et al. found an increased number of circulating blood lymphocytes with the same light chain isotype as the patient's monoclonal protein (M-protein) which produced an altered κ:λ ratio that had a good correlation with the number of idiotype-bearing cells [8]. Using flow cytometry, Morgan et al. [7] demonstrated an aneuploid mononuclear cell population in the blood that had a DNA index similar to that of the marrow plasma cells, suggesting that the circulating cells were a part of the malignant clone. Clofent et al. [2] demonstrated immunoglobulin gene rearrangements from peripheral blood cells in patients with documented circulating plasma cells.

Methods

To further evaluate this circulatory phase of myeloma, we studied the blood of patients with various plasma cell proliferative disorders for the presence of circulating monoclonal plasma cells using a two-color immunofluorescence bromodeoxyuridine labeling index technique as previously described [10]. Briefly, 10 mL of peripheral blood is collected into tubes containing lyophilized bromodeoxyuridine and fluorodeoxyuridine; incubated at 37°C for one hour, centrifuged over Ficoll-Hypaque, and the mononuclear cell layer isolated. The sample is then depleted of T-cells with magnetic beads conjugated to antibody to CD2. The B-cell fraction is spun onto cytospin slides and stained with anti-bromodeoxyuridine monoclonal antibody (BU-1). The slides are then stained with fluorescein (FITC) labeled goat anti-human κ or λ monospecific reagent to detect

cytoplasmic immunoglobulin (cIg) and goat anti-mouse IgG labeled with rhodamine isothiocyanate (RITC) to detect BU-1 antibody and read with an epi-illumination fluorescence microscope with FITC and RITC filters. This technique allows identification of monoclonal plasma cells by the cell morphology and monoclonal cytoplasmic light chain staining. A labeling index (LI), which indicates the proliferation rate of the cIg+ cells, can also be obtained. Small numbers of plasma cells can be detected that are not seen on routine Wright-stained peripheral blood smears.

Results

This report summarizes the results obtained from an analysis of the peripheral blood for circulating plasma cells and their LI. The patients were categorized by review of their clinical and laboratory features into either an inactive disease (MGUS, SMM) or active disease (new MM or relapsed MM) group.

Inactive Disease Group

Our initial studies included 29 patients with either MGUS or SMM and examined the LI of the circulating cells that expressed the same light chain isotype as the patient's M-protein (**Table 1**) [11]. The median peripheral blood LI in this group was low at 0.2%. Four of the 29 patients had a high LI (≥0.5%) and circulating monoclonal plasma cells and required institution of chemotherapy within 6 months. This finding that the presence of circulating proliferating plasma cells may indicate which patients have active disease when other clinical parameters appear inactive led to additional studies on 23 patients with inactive disease (**Table 2**) [3]. We found median of 0.0 x 10^6/L circulating plasma cells and all patients had an LI <0.5%.

Table 1: The relationship between the peripheral blood labeling index and disease activity.

Category (N)	Labeling Index (%)		
	Mean	**Median**	**Range**
Inactive Disease (29)	0.25	0.20	0.0 - 1.20
Active Disease (76)			
New Myeloma (35)	3.10	0.80	0.0 - 23.50
Relapsed Myeloma (41)	3.40	1.70	0.60 - 15.90

We recently studied the blood of 41 patients who clinically appeared to have SMM and correlated the results with clinical follow-up for ≥1 year [9]. The absolute number of peripheral blood plasma cells (PBPC) x 10^6/L was calculated and correlated to disease status at 6 and 12 months after the diagnosis of SMM. The results, which are summarized in **Table 3**, suggest that the presence of ≥3 x 10^6/L circulating PBPC indicates that the patient does not have true SMM and should be followed closely for the development of active myeloma that requires chemotheraphy In this study, eight of the nine patients having ≥3 x 10^6/L PBPC required therapy within 12 months. Conversely, of the 26 patients that remained stable over the following 12 months, of one had ≥ 3 x 10^6/L PBPC.

Table 2: The relationship between the number of circulating plasma cells and disease activity.

Category (N)	# Peripheral Blood Plasma Cells (x 10^6/L)		
	Mean	**Median**	**Range**
Inactive Disease (23)	0.6	0.0	0 - 4.0
Active Disease (61)	139.6	6.6	0 - 3186.7
New Myeloma (35)	210.5	5.4	0 - 3186.7
Relapsed Myeloma (26)	44.1	11.6	0 - 373.2

Table 3: Correlation of peripheral blood plasma cells and disease progression in smoldering multiple myeloma.

Group	N (%)	#PBPC (x10^6/L)		%Patients With ≥3x10^6/L PBPC
		Mean	**Median**	
All patients	41 (100)	10.2	0.39	22
Prog at 6 mo	9 (22)	40.6	9.2	66
Prog at 12 mo	6 (15)	6.1	0.75	33
Stable (No prog)	26 (63)	0.6	0	4

Abbrev: PBPC, peripheral blood plasma cells; Prog, progression

Active Disease

Cell kinetic studies were conducted on cells from the peripheral blood of 76 patients with active disease (35 new MM; 41 relapsed MM) (**Table 1**) [11]. The median LI was 0.8% for the new MM group and 1.7% for the relapsed MM group. Twenty-two of 35 (63%) new MM patients had a high LI. When reading the slides, we almost always observed that the cells in S-phase had the morphology of plasma cells.

Additional studies on 61 patients with active disease revealed the median number of circulating plasma cells to be 6.6 x 10^6/L (**Table 2**) [3]. Within the active group, the median number of circulating plasma cells for new MM was 5.4 x 10^6/L compared to 11.6 ($p=0.17$) for relapsed MM. Therefore, as the disease progresses, the plasma cells appear in greater numbers in the peripheral blood.

Serial Studies

We studied the peripheral blood on multiple occasions throughout the disease course of a single patient and correlated the results with the disease activity (**Table 4**). When the treatment was effective, the number of plasma cells in the peripheral blood decreased.

Table 4: Serial studies of peripheral blood plasma cells in a patient with multiple myeloma.

Time From Diagnosis (Mo)	Status	# Plasma Cells $x10^6/L$	M-Protein (g/dL)	Chemo
0	New Myeloma	6.52	6.0	Mel/Pred
22	Response	0	2.0	Mel/Pred
28	Relapse	1.34	3.3	Restart Mel/Pred
33	Stable on Rx	4.68	3.1	Mel/Pred
43	Relapse	6.08	4.7	VBAP
44	Stable on Rx	3.89	4.2	VBAP
49	Relapsing	4.51	4.8	VBAP
54	Relapsing	82.5	5.2	VBAP

Abbrev: M-protein, monoclonal protein; Mel/Pred, melphalan and prednisone; Rx, treatmen VBAP, vincristine, BCNU, doxorubicin, prednisone

Summary

These studies indicate that monoclonal plasma cells can be detected in the peripheral blood of patients with active myeloma even when they are not detectable by routine WBC differential performed on Wright-stained blood smears. These cells are usually not present in patients with MGUS and true SMM. They are detected in approximately 60% of patients with new, action MM and over 90% of patients with relapsed or refractory MM. If treatment is effective, then tend to decrease or disappear from the blood.

When immunological, molecular, or cytogenetic studies are performed on peripheral blood cells from patients with MM, it must be realized that monoclonal plasma cells may be present and then they can influence the results of these tests. Although monoclonal plasma cells can circulated by the peripheral blood, it is not yet clear whether this cell represents the myeloma stem cell. It is possible that there are precursor cells that do not have plasma cell morphology in the blood cell marrow that then differentiate into plasma cells. This question can only be answered by fully depleting the plasma cells and then examining the remaining B-cells with appropriate immunological and molecular techniques.

References

1. Carmagnola A, Boccadoro M, Massaia M, Pileri A (1983) The idiotypic specificities in lymphocytes in human monoclonal gammopathies: analysis with a fluorescence activated cell sorter. Clin Exp Immunol 51:173-177
2. Clofent G, Klein B, Commes T, Ghanem N, Lefranc M, Bataille R (1989) No detectable malignant B-cells in the peripheral blood of patients with multiple myeloma. Br J Haematol 71:357-361
3. Dhodapkar M, Witzig T, Kyle R, Greipp P (1991) Detection of peripheral blood plasma cells and light chain isotype suppression predicts disease activity in multiple myeloma. Proc AACR 49:1013
4. Kyle R (1991) "Benign" monoclonal gammopathy after 20-35 years of follow-up. Blood 78 (Suppl 1):175a
5. Kyle R, Greipp P (1980) Smoldering multiple myeloma. N Engl J Med 302:1347-1349
6. Mellstedt H, Pettersson D, Holm G (1976) Monoclonal B-lymphocytes in peripheral blood of patients with plasma cell myeloma: relation to activity of the disease. Scand J Haematol 16:112-120
7. Morgan R, Witzig T, Gonchoroff N, Kyle R, Greipp P (1987) Circulating peripheral blood cells in multiple myeloma: an important overlooked compartment? Proc AACR 28:140
8. Pettersson D, Mellstedt H, Holm G (1980) Monoclonal B-lymphocytes in multiple myeloma. Scand J Immunol 12:375-382
9. Witzig T, Garton J, Gertz M, Kyle R, Lust J, Greipp P (1992) The number of peripheral blood plasma cells distinguishes smoldering from active multiple myeloma. Proc ASCO 11:1239
10. Witzig T, Gonchoroff N, Ahmann G, Katzmann J, Greipp P (1991) T-cell depletion using anti-CD2 coated magnetic beads simplifies the detection of peripheral blood plasma cells. J Immunol Methods 144:253-256
11. Witzig T, Gonchoroff N, Katzmann J, Therneau T, Kyle R, Greipp P (1988) Peripheral blood labeling indices are a measure of disease activity in patients with monoclonal gammopathies. J Clin Oncol 6:1041-1046

Circulating B Lymphocytes in Multiple Myeloma Patients Contain an Autocrine IL-6 Driven Pre-Myeloma Cell Population

X. THOMAS[1], H. Q. XIAO[1], R. CHANG[1] and J. EPSTEIN[1,2]

[1]Division of Hematology/Oncology, Department of Medicine, Arkansas Cancer Research Center, University of Arkansas for Medical Sciences and
[2]VAMC, Little Rock, AR 72205.

Introduction

Among the unanswered questions in the development of multiple myeloma are the level of hematopoietic development of the myeloma stem cell,[1] the tissue site of initiation (bone marrow? lymph node?)[2,3] and the role of IL-6 in myeloma development.[4-6] The involvement of B lymphocytes in the bone marrow and blood in myeloma has been suggested initially by identifying B cells displaying concordant isotype and idiotype,[7-9] followed by the demonstration of a circulating clonal B cell population with a concordant J_H rearrangement.[10] Abnormal B cells from the marrow of myeloma patients could be differentiated into the myeloma cells upon stimulation,[11] the first functional demonstration of B cell involvement. Similarly, blood mononuclear cells from myeloma patients cultured with IL-3 and IL-6[12] or co-cultured with autologous bone marrow stromal cells[13] differentiated into plasma cells containing the concordant monotypic immunoglobulin.

In order to identify the involved circulating B cells, the fate of $CD19^+CD11b^+$ cells, a potential pre-myeloma cell compartment reported to be abnormally high in numbers in myeloma patients,[14] in IL-3+IL-6 cultures of blood mononuclear cells was studied.

Identification of circulating pre-myeloma cells

Mononuclear cells, separated from the blood of myeloma patients by ficoll-hypaque centrifugation, contained an average of $0.9\% \pm 0.25$ (median 0.95, range 0.5-1.3) $CD19^+CD11b^+$ cells, analyzed by flow cytometry using a FACScan. Following 6-7 days in culture with a combination of IL-3 and IL-6 (10ng/ml each) at $1x10^6$ cells/ml, the absolute number of $CD19^+CD11b^+$ (calculated as FACScan determined proportion x total cell number) increased by a factor of 8.3 ± 11.7 (median 2.9, range 1.4-39.5). These cells co-expressed CD10. The number of CD10-expressing B cells, also reported to be associated with myeloma,[11,15] increased 4.7 ± 2.8 fold (median 3.9, range 1.5-10). This expansion of the CD19CD11bCD10-expressing cells, determined by three-color flow cytometry, was accompanied by the appearance of $2.24 \pm 1.85x10^5$ plasmacytic cells/ml (median $2.55x10^5$, range $0.55\text{-}8x10^5$), comprising 5-80% of the cultures as determined morphologically. A strong correlation was observed between the proportion of plasmacytic cells and those of $CD19^+CD11b^+$ and $CD19^+CD10^+$ cells (correlation coefficients $r=0.9$ and $r=0.95$, respectively). These results are summarized in Table 1.

Table 1. Expansion and differentiation of circulating pre-myeloma cells

Patient	Percent CD19+CD11b$^+$	Percent CD19+CD10$^+$	Percent Plasmacytic Cells
1	1.9	2.0	5
2	2.5	3.1	9
3	2.6	3.2	13
4	2.6	3.6	15
5	4.5	4.2	16
6	6.9	4.4	16
7	7.6	7.6	20
8	9.5	8.1	25
9	10.0	8.7	27
10	13.1	9.7	27
11	43.5	11.3	42

Myeloma plasma cells are phenotypically distinct among bone marrow cells by their high levels of CD38 and none to intermediate levels of CD45 expression.[16,17] Following a week in IL-3 + IL-6 cultures, the expanded CD19$^+$CD11b$^+$ cells, while still expressing high levels of CD45, expressed mostly CD45RO with only a few cells CD45RA positive, indicating progressive maturation.[18,19] In addition, these cells displayed increasing levels of CD38 expression, changing from low to high. The plasmacytic cells derived in IL-3 + IL-6 cultures did not contain cytoplasmic immunoglobulins, even when maintained in the cultures for up to 4 weeks, suggesting that these conditions can not support their complete differentiation. Still, the observed phenotypic changes combined with the morphological evidence of plasmacytic differentiation indicated that circulating CD19CD11b-expressing cells are capable of differentiating into plasma cells.

Further determination of the ability of blood mononuclear cells from myeloma patients to differentiate into Ig producing plasma cells, was studied using co-cultures with autologous bone marrow stromal cells.[13] After 3 weeks in co-culture, differentiation into concordant, monotypic Ig producing plasma cells was observed in all patients. Similarly, differentiation into Ig producing myeloma cells was routinely observed when cytokine-expanded cultures were transferred to co-cultures with autologous bone marrow stromal cells but not in matching control cytokine cultures nor in cytokine cultures to which stromal cell spent media was added. The requirement for stromal cells for complete differentiation to occur suggests that adhesion to stroma has an important role in myeloma cell development.

Differentiation of CD19$^+$CD11b$^+$ cells co-cultured with bone marrow stromal cells was further studied to demonstrate directly their precursor-result relationship with the myeloma cells. CD19$^+$CD11b$^+$ cells, expanded in cytokine cultures, were sorted onto stromal cell cultures from opposite-sex, allogeneic, unrelated myeloma patients, and their differentiation was studied. After 2-3 weeks in culture, plasma cells containing concordant, monotypic cIg and of the patient's sex, determined by interphase (FISH) or metaphase cytogenetics, were the prominent component among the non-stromal cells. These plasma cells adhered tightly to the fibroblastoid stromal cells and forceful agitation

was required for their removal, underscoring the importance of cell-cell contact for their development.

Thus, the abnormal B cells in the blood of myeloma patients indeed appear to be precursors of myeloma plasma cells. The arsenal of adhesion mulecules they display suggests that they are transitory cells, equipped for extravasation and migration to sites of final differentiation.[14] Recently, the presence of expanded pre-switch B cell populations in the bone marrow of myeloma patients, clonally identical with the myeloma cells, was reported.[20] It would appear that the myeloma precursor cells originate in the bone marrow. From there these progenitors mobilize through the circulation to sites of antigenic stimulation and back to the bone marrow or, occasionally, to soft tissue sites, where they undergo final differentiation. Alternatively, the myeloma progenitor cells may be self-stimulatory, not requiring continuous antigenic stimulation. In this case, the circulating pre-myeloma cells may represent a metastatic process, through which the disease is disseminated to distal bone and soft tissue sites.

Role of Interleukin-6

Although IL-6 is believed to play a major role in the development of multiple myeloma, its sources and its effects on the tumor cells are controversial.[4,5,21] Expression of the IL-6 and IL-6-receptor (IL-6-R) genes by myeloma cells, sorted on the basis of their $CD38^{high}CD45^{negative\text{-}intermediate}$ and light scatter profiles on a FACStar Plus cell sorter, was studied by PCR. Total RNA was extracted from $2x10^4$ cells and used for reverse-transcriptase-PCR amplification. The primers used were purchased from Clonetech. IL-6 mRNA was expressed by myeloma cells from 45% of the patients, IL-6-R by 68%, including all patients whose myeloma cells expressed IL-6. These results are summarized in Table 2.

Table 2. Expression of interleukin-6 and its receptor genes by myeloma cells

IL-6[1]	IL-6-R[2]	%[3]
+	+	45
-	+	23
-	-	32
+	-	0

[1] IL-6 transcripts detected.
[2] IL-6-R transcripts detected.
[3] Percent of samples (n=22).

Whereas TNF-α transcripts were detected in 38% of samples, myeloma cells did not express the IL-1β gene. In contrast, IL-1β transcripts were detected in all preparations of simultaneously sorted monocytes and total non-myeloma cells. In mixing experiments, the presence of even one "contaminating" cell among $2x10^4$ non-expressing cells was routinely detected. PCR data for myeloma and control cells are summarized in Table 3.

Table 3. Gene expression by myeloma and control cells

Gene	Myeloma cells	Control cells	%[1]
IL-6	+	+	45
	-	+	55
IL-6-R	+	+	33
	+	-	35
	-	+	32
TNF-α	+	+	38
	-	+	57
	-	-	5
IL-1β	-	+	100

[1] Percent of samples (n=22).

The consistent differences in IL-1β expression between myeloma and control cell preparations, as well as differences in IL-6, IL-6-R and TNF-α expression, attested to the validity of the PCR data.

Interleukin-6 dependent murine plasmacytoma B9 cells[22,23] were used to determine whether myeloma cells containing IL-6 transcripts also produce IL-6. Of 6 myeloma cell preparations studied, only the 3 containing IL-6 mRNA secreted the cytokine. IL-6 was not secreted by the other samples, nor was it detected in the cell pellets.

While IL-6 expression was always accompanied with IL-6-R expression, myeloma cells from one third of the patients did not express the receptor, 23% expressed IL-6-R but not IL-6 and 45% expressed both IL-6 and its receptor. These findings suggest that IL-6 targets pre-myeloma cells and that responsiveness to the cytokine, indicated by the absence of IL-6-R, is lost with maturation. Such interpretation is compatible with the observation that purified myeloma cells do not respond to IL-6.[17] Comparison of PCR results with myeloma cell expression of CD45 as an indication of cell maturity[10,18,19] revealed strong correlations between CD45 expression and the expression of IL-6 ($r=0.0002$) and IL-6-R ($p<0.005$). The correlation between CD45 and IL-6 expression was also seen in 4 patients whose myeloma cells consisted of $CD45^{negative}$ as well as $CD45^{intermediate}$ subpopulations. In these patients, only the CD45-expressing myeloma cells expressed the IL-6 gene.

Development of multiple myeloma

On the basis of the observations described, it would appear that a population of circulating $CD19^+CD11b^+CD45^+$ cells, which can expand and differentiate *in vitro* into myeloma plasma cells, leave the circulation and return to the bone marrow or extramedullary sites and undergo final differentiation. These cells are a transitory population of tumor cells, circulating on route from lymphatic organs where they underwent antigen-mediated heavy chain switch, or as part of a metastatic process.

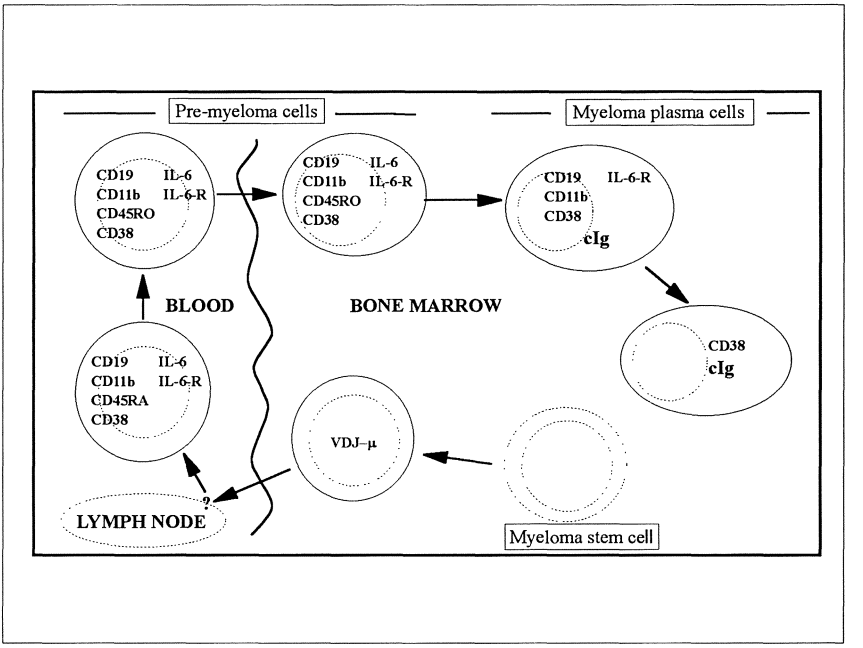


Fig. 1. Development of multiple myeloma. Transitory, circulating pre-myeloma lymphocytes, originating in the bone marrow, undergo spontaneous or antigen-mediated immunoglobulin heavy chain switch. they return to the bone marrow for final differentiation. The antigenic changes delineating the maturation process are delineated.

Maturation of the pre-myeloma cells involves loss of expression of CD11b, CD19 and CD45, and increased levels of CD38. These changes are shown schematically in Figure 1. *In vivo,* these phenotypic changes are seen in patients whose myeloma cells consist of both $CD45^-$ and $CD45^+$ cells. While the $CD45^+$ myeloma cells also express CD19 and CD11b, cells expressing lower levels of CD45 also show lower levels of CD19 and CD11b expression, as well as fewer cells displaying these antigens. The differentiation process is driven by an autocrine IL-6 loop which is functional only in early myeloma cells. Upon maturation of the transitory pre-myeloma cells into myeloma plasma cells, IL-6 expression is turned off, followed by loss of IL-6-R. Heterogeneity in the expression of CD45 by myeloma cells indicates changes in the maturation level of the cells, probably associated with disease progression. This would account for the reported association between elevated serum IL-6 levels and the aggressiveness of myeloma.[24] The association between CD45, a protein tyrosine phosphatase involved in receptor-mediated transmembrane signal transduction[25], and IL-6 expression could suggest a role for CD45 in IL-6 receptor signalling or in induction of IL-6 gene transcription.

Supported in part by Grants CA37161 and CA28771 of the National Institute of Health, Bethesda, MD.

References

1. Epstein J, Xiao H, He XY(1990) Markers of multiple hematopoietic-cell lineages in multiple myeloma. N Engl J Med 322:664-8

2. Epstein, J (1992) Myeloma phenotype-clues to its origin and manifestation. Hematology/Oncology Clinics of North America 6:249-256

3. MacLennan ICM, Chan EUT (1991) The origin of bone marrow plasma cells. In: Obrams GI, Potter M (eds) Epidemiology and Biology of Multiple Myeloma. Springer-Verlag, Berlin, pp 129-135

4. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, Kuramoto A, Kishimoto T (1988) Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature (London) 332:83-5

5. Klein B, Zhang X-G, Jourdan M, Content J, Houssiau F, Aarden L, Piechaczyk M, Bataille,R (1989) Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73:517-26

6. Xiao H-Q, Woodliff J, Epstein J (1992) Autocrine IL-6 production in pre-myeloma cells. Cancer Res. 33:#1372

7. Mellstedt H, Hammerstrom S, Holm G (1974) Monoclonal lymphocyte population in human plasma cell myeloma. Clin Exp Immunol 17:371-84

8. Kubagawa H, Vogler L, Capra JD, Conrad ME. Lawton AR, Cooper MD (1979) Studies on the clonal origin of multiple myeloma. Use of individually specific (idiotype) antibodies to trace the oncogenic event to its earliest point of expression in B-cell differentiation. J Exp Med 150:792-807

9. Bast EJEG, van Camp B, Reynaert P, Wiringa G, Ballieux RE (1982) Idiotypic peripheral blood lymphocytes in monoclonal gammopathy. Clin Exp Immunol 47:677-682

10. Jensen GS, Mant MJ, Belch AJ, Berenson JR, Ruether BA, Pilarski LM (1991) Selective expression of CD45 isoforms defines CALLA+ monoclonal B-lineage cells in peripheral blood from myeloma patients as late stage B cells. Blood 78:711-9

11. Caligaris-Cappio F, Bergui L, Tesio L, Pizzolo G, Malavasi F, Chilosi M, Campana D, van Camp B, Janossy G (1985) Identification of malignant plasma cell precursors in the bone marrow of multiple myeloma. J Clin Invest 76:1243-51

12. Bergui L, Schena M, Gaidano G, Riva M, Caligaris-Cappio F (1989) Interleukin 3 and interleukin 6 synergistically promote the proliferation and differentiation of malignant plasma cell precursors in multiple myeloma. J Exp Med 170:613-8

13. Caligaris-Cappio F, Bergui L, Gregoretti MG, Hiadano G, Goneli M. Schena M, Zallone AZ, Marchisio PC (1991) Role of bone marrow stromal cells in the growth of human multiple myeloma. Blood 77:2688-93

14. Pilarski LM, Jensen GS (1992) Monoclonal circulating B cells in multiple myeloma: a continuously differentiating possibly invasive population as defined by expression of CD45 isoforms and adhesion molecules. Hematology/Oncology Clinics of North America 6:297-322

15. Warburton P, Joshua DE, Gibson J, Brown RD (1989) CD10-(CALLA)-positive lymphocytes in myeloma: evidence that they are a malignant precursor population and are of germinal centre origin. Leukemia Lymphoma 1:11-

16. Terstappen LWMM, Johnsen S, Segers-Nolten IMJ, Loken MR (1990) Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry. Blood 76:1739-47

17. Petrucci MT, Hata H, Xiao H-Q, Kreitman RJ, Pastan I, Hsu SM, Epstein J (1991) Significance of inducible IL-6 gene expression by myeloma cells. Blood 78:115a

18. Caldwell CW and Patterson WP (1991) Relationship between CD45 antigen expression and putative stages of differentiation in B-cell malignancies. American J Hematol 36:111-5

19. Jensen GS, Andrews EJ, Mant MJ, Vergidis R, Ledbetter JA, Pilarski LM (1991) Transitions in CD45 isoform expression indicate continuous differentiation of a monoclonal CD5+ CD11b+ B lineage in Waldenstrom's macroglobulinemia. American J Hematol 37:20-30

20. Vescio RA, Cao J, Lichtenstein AL and Berenson JR (1992) IgM-producing B-lymphocytes are part of the malgnant clone in Multiple Myleoma. Cancer Res 33:229

21. Klein B. Bataille R (1992) Cytokine network in human multiple myeloma. Hematology/Oncology Clinics of North America 6:273-284

22. Aarden LA. De-Groot ER. Schaap OL. Lansdorp PM (1987) Production of hybridoma growth factor by human monocytes. Eur J Immunol. 17:1411-6

23. Hansen MB, Nielsen SE, Berg KH (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203-210

24. Bataille R, Jourdan M, Zhang XG, Klein B (1989) Serum levels of interleukin-6, a potent myleoma growth factor, as a reflection of disease severity in plasma cell dyscrasias. J Clin Invest 84:2008-11

25. Justement, LB, Campbell KS, Chien MC, Cambier JC (1991) Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. Science 252:1839-42.

In Which Cells Does Neoplastic Transformation Occur in Myelomatosis?

I. C. M. MacLennan

Department of Immunology, University of Birmingham Medical School, Birmingham B15 2TT, England.

The physiological equivalent to the neoplastic plasma cells found in patients with myelomatosis

Myelomatosis is strictly a neoplasm of plasma cells in bone marrow. It does not involve other sites of antibody production; thus, even when the monoclonal protein produced by the neoplastic clone is IgA1 or IgA2 the neoplastic cells are not found in the lamina propria of the gut (Leonard et al 1979). Importantly the production of an IgM paraprotein by the neoplastic cells is vanishingly rare, amounting to only 0.2 % of 2011 successive patients admitted to MRC myelomatosis trials. IgG paraproteins were found in 57.2 % (0.15% with IgG with a minor IgM paraprotien of the same light chain isotype), IgA in 27% (0.10% with IgA with a minor IgM paraprotein of the same light chain isotype), IgD in 1.5%, IgE 0.1%. Free light chains alone were produced by the neoplastic cells of 14% of patients and 1.8% had no paraprotein. Physiologically in the established phase of those T cell-dependent antibody responses where B cells are being activated in the spleen antibody production takes place in the bone marrow (Benner et al 1981). This also applies to responses in those lymph nodes which do not receive lymph from mucosal surfaces. It has been shown in rodents that the B cells are activated in the follicles of these secondary lymphoid tissues (Tew et al. 1992) and migrate via the lymph and/or blood to the marrow. Migrant plasmablasts can be found in the blood of healthy humans. Analysis of the life-span of bone marrow plasma cells in rats indicates that IgG-or IgA-secreting plasma cells survive for about a month (Ho et al 1986). Conversely the IgM-producing plasma cells in the marrow like most plasma cells in the spleen and lymph nodes, irrespective of the class of immunoglobulin produced, live for only 3 days (Ho et al 1986). It may be that these IgM-producing cells have been generated by local activation of B cells at the surface of macrophages by T cell-independent antigens (Corbel and Melchers 1983). Normal human bone marrow does not contain obvious secondary lymphoid tissue so it seems likely that the IgG and IgA plasma cells in this tissue are also derived from distant secondary lymphoid tissues. (Studies of plasma cells from the bone marrow from patients with rheumatoid arthritis as a means for investigating the physiological equivalent of neoplastic cells in myelomatosis could be misleading; for the marrow in this disease contains well-developed secondary lymphoid tissue).

Candidate cells in which neoplastic transformation may occur in myelomatosis

Neoplastic transformation in many tumours has been shown to result from a series of genetic alterations. Some occur in

precursor cells long before the final event takes place which results in the emergence of an overtly malignant neoplasm. This is well demonstrated in the plasmacytomas which arise in Balb/c mice if mineral oil is introduced into their peritoneal cavity. The mice have a genetic susceptibility to develop these tumours (see the article by Potter et al in this volume). In addition a translocation between the c-myc gene and an immunoglobulin gene is required but also is insufficient to cause plasmacytoma development (Clynes et al 1988).

Myelomatosis is only very rarely found in closely related individuals (Blattner 1980). There are some variations in the incidence of myelomatosis in different parts of the world but these differences are small compared to those found for other B cell neoplasms including chronic lymphocytic leukaemia and low grade follicular lymphoma. Epidemiological studies have found increased incidence of the disease among Black people in the USA (Devesa 1991). Although these effects are of some importance the evidence for major susceptibility to myelomatosis being transmitted in the germline is lacking. Epstein has correctly pointed out that the incidence of myelodysplasia is more common in myelomatosis than would be expected by chance (Epstein and Hata 1991). Any genetic change, however, which is already present before the time of immunoglobulin light chain gene rearrangement (i.e. up to and including the pre-B cell stage of B lymphopoiesis) can only be associated with a very low susceptibility of going on to develop myelomatosis. This can be deduced from the extreme rarity of biclonal myelomatosis. Only 4 of the 2011 patients of the MRC series cited above had or developed 2 paraproteins with different light chain isotypes during the course of their disease. These were GL with free K, GL with AK, GK with AL and AK with free L. This finding is difficult to reconcile with a report that pre B cells with the same heavy chain variable-region idiotype as that of the neoplastic cells can be found in the marrow of patients with myelomatosis (Kubagawa et al 1979).

The next question to consider is whether neoplastic transformation occurs during the multiple differentiation stages B cells undergo before becoming an IgG or IgA-producing bone marrow plasma cell. These differentiation stages are outlined in the Figure. There is lack of information about whether genetic changes occur in the precursors of migrant plasmablasts. Memory B cell clones generated in T cell-dependent antibody responses have been transferred successively between syngeneic (Askonas et al 1970) or congenic (Gray et al 1986) rodents and in each recipient induced by antigen to give rise both to antibody producing cells and further memory cells. Thus within the context of the life span of rats and mice apparently normal memory B cell clones can be sustained indefinitely, provided antigen is present (Gray and Skarvall 1988). There is, therefore, considerable scope for pre-neoplastic genetic change within these memory clones. It also follows that memory B cells that have not undergone such a change but belong to a clone that

has given rise to neoplastic bone marrow plasma cells may coexist with the neoplastic cells.

Theoretically neoplastic transformation in secondary follicular B blasts or germinal centre cells in follicles of the spleen or peripheral lymph nodes could give rise to the widespread dispersal of neoplastic plasma cells within the bone marrow. Phenotypic evidence has been put forward to suggest that neoplastic plasma cells might be being derived from germinal centre cells (Warburton et al 1989). At some stage the precursors of long-lived bone marrow plasma cells have been centroblasts but there is no direct evidence that neoplastic plasma cell numbers are being maintained by centroblasts. It seems unlikely that they do, for normal centroblasts only appear to have the capacity for self renewal for 3 weeks (Liu et al 1991). Secondary follicular B blasts unlike centroblasts do have the capacity for long term self-renewal and the production both of memory B cells and plasmablasts, provided they are induced to do so by antigen held on follicular dendritic cells (Liu et al 1991). These seem to be better candidates for continued production of neoplastic plasma cells but again there is no direct evidence to support this conclusion.

Solitary plasmacytomas of the red bone marrow suggest that the clinical picture of myelomatosis can develop following neoplastic transformation in a plasma cell or plasmablast which has already localised in the marrow. By definition these patients do not have neoplastic cells spread throughout the marrow at presentation. Consequently it is difficult to see that the terminal event leading to tumour formation did occur in a cell which had already arrived in the bone marrow or in a migrant plasmablast committed to going there. The majority of these patients, however, go on to develop a clinical picture identical to myelomatosis (Wiltshaw 1976). The neoplastic clone at this stage produces the same monoclonal protein as the cells of the original tumour. This occurs despite local treatment of their plasmacytoma, but there is sometimes a prolonged delay before secondary myelomatosis develops. It is conceivable, however, that the secondary myelomatosis arises not from the original neoplastic plasma cells but by further genetic change in the memory B cells of the clone which gave rise to the plasmacytoma.

A number of workers have found lymphoid cells in the marrow and blood of patients with myelomatosis which can be induced <u>in vitro</u> to differentiate to become plasmablasts. These cells appear to belong to the neoplastic clone (see the article by Pilarsky et al in this volume). The phenotype of these cells is unusual and it seems at least possible that they are the progeny of neoplastic plasmablasts rather than a precursor which can give rise to, but has never been, a bone marrow plasmablast. Further work is required to distinguish between these two possibilities; although in either case the potential of these cells to form a reservoir of neoplastic cells exists. Neoplastic cells in the bone marrow of patients with clinically-defined MGUS (monoclonal gammopathy of uncertain significance) unlike patients with progressive myelomatosis

have a minimal proportion of neoplastic cells in active cell cycle. A similar effect is seen in patients brought into plateau phase with chemotherapy. (Plateau phase is defined as a stable clinical and serological state associated with no more than minimal symptoms which persist despite stopping chemotherapy). Although the proportion of neoplastic plasma cells which are in S phase is markedly lowered in patients in plateau phase, these patients do not necessarily have greatly reduced numbers of plasma cells in their marrow. It is not known to what extent relapse from plateau is associated with further genetic change and the emergence of more malignant subclones. If this is the main reason it is equally unclear if the cells which undergo the secondary change are the plasma cells in the marrow or some precursor. The same applies to relapse occurring in patients who apparently have appeared to achieve a complete response to chemotherapy. At this time it seem prudent to keep an open mind on these matters and to continue to seek ways to test the various possibilities experimentally.

References

Askonas BA, Williamson AR and Wright BG (1970) Selection of a single antibody-forming clone and its propogation in syngeneic mice. Proc Natl Acad Sci 67:1398

Benner R, Hijmans W, Haaijman JJ (1981) The bone marrow: the major source of immunoglobulins but still a neglected site of antibody formation. Clin Exp Immunol 46:1

Blattner WA (1980) Epidemiology of multiple myeloma and related plasma cell disorders: An analytical review. In progress in myeloma Ed Potter M. Elsvier North Holland, New York 1

Clynes R, Stanton LW, Wax J, Smith-Gill S, Potter M and Marcu KB (1988) Synergy of an IgH promotor-enhancer-driven c-myc/v-Ha-ras retrovirus and pristane in the induction of murine plasmacytomas. Curr Top Microbiol Immunol 141:115

Corbel C and Melchers F (1983) Requirement for macrophages of T cell-derived factors in the mitogenic stimulation of murine B cells by LPS. Eur J Immunol 13:528

Deves SS (1991) Descriptive Epidemiology of Multiple Myeloma. In Epidemiology and biology of multiple myeloma. Eds Obrams GI and Potter M, Springer-Verlag, Berlin, 3

Epstein J and Hiroyuki H (1991) Multiple myeloma evolves from a malignant haemopoietic stem cell.In Epidemiology and biology of multiple myeloma. Eds Obrams GI and Potter M, Springer-Verlag, Berlin, 143

Gray D, MacLennan ICM and Lane PJL (1986) Virgin B cell recruitment and the lifespan of memory B cell clones during antibody responses to DNP-hemocyanin Eur J Immunol 16:641

Gray D and Skarvall H (1988) B cell memory is short-lived in the absence of antigen. Nature 336:70

Gu H, Tarlington D, Muller W, Rajewski K and Forster I (1991) Most peripheral B cells in mice are ligand selected J Exp Med 173:1357

Ho F, Lortan J, Khan M and MacLennan ICM (1986) Distinct short-lived and long-lived antibody-producing cell populations Eur J Immunol 16:1297

Kubagawa H, Vogler LB, Cappra JE, Conrad ME, Lawton AR and Cooper MD (1979) Studies of the clonal origin of multiple Myeloma. J. Exp. Med. 150:792

Leonard RFL, MacLennan ICM, Sart Y, Vanhegan RI and Cuzick J (1979) Light chain isotype-associated suppression of normal plasma cell numbers in patients with myelomatosis. Internat J Cancer 24:385

Liu Y-J, Oldfield S and MacLennan ICM (1988) Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones Eur J Immunol 18:355

Liu Y-J, Zhang J, Lane PJL, Chan E Y-T and MacLennan ICM (1991) Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur J Immunol 21:2951

MacLennan ICM and Gray (1986) Antigen-driven selection of virgin and memory B cells. Immunol Rev 91:61

MacLennan ICM, Liu Y-J, Oldfield S, Zhang J and Lane PJL (1990) The evolution of B cell clones. Curr Top Microbiol Immunol 159:37

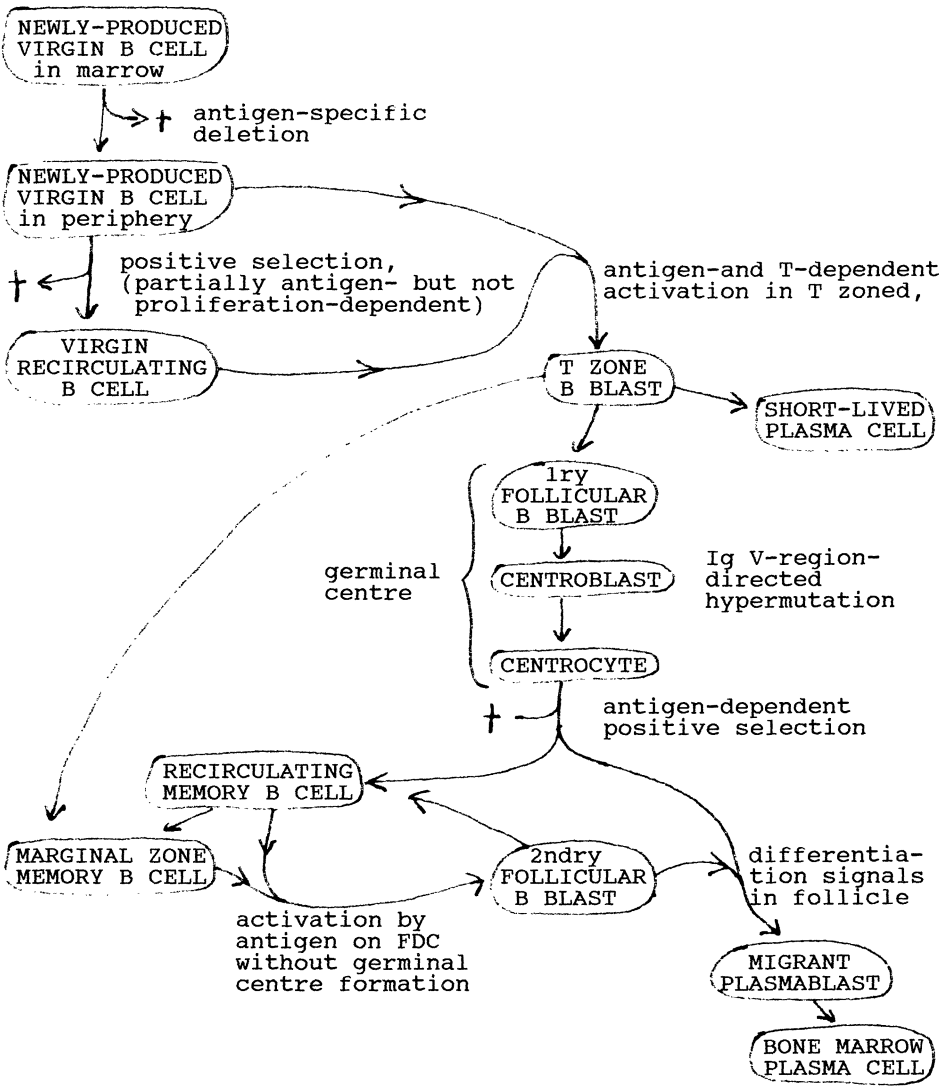
MacLennan ICM, Liu Y-J and Johnson GD (1992) Maturation and dispersal of B cell clones during T cell-dependent antibody responses. Immunol Rev 126:143

Tew JG, DiLosa R M, Burton GF, Kosco M, Kupp LI, Masuda A and Szakal AK (1992) Germinal centres and antibody production in bone marrow. Immunol Rev 126:99

Warburton P, Joshua DE, Gibson J and Brown RD (1989) CD10-(CALLA)-positive lymphocytes in myeloma: evidence that they are a malignant precursor population and are of germinal centre origin. Leuk Lym 1, 1

Wiltshaw E (1976) The natural history of extra medullary plasmacytoma and its relation to solitory plasmacytoma of bone and myelomatosis. Medicine 55:217

Physiological Differentiation Pathways Leading to the Production of Bone Marrow Plasma cells



More detailed dicussion of the evidence for these stages in the differentiation of B cells to plasma cells see MacLennan and Gray 1965, Liu et al 1988, Gu et al 1990, MacLennan et al 1990, Liu et al. 1991, MacLennan et al 1992. FDC=follicular dendritic cell.

Differential Expression of *c-myc*-mRNA and c-*MYC*-Protein During Terminal Neoplastic B-Cell Differentiation

[1]R. GREIL, [2]P. LOIDL, B. FASCHING, and [3]H. HUBER

[1]Laboratory of Molecular Cytology,
[3]Division of Immunhematology and Oncology, Department of Internal Medicine
and
[2]Department of Medical Microbiology, University of Innsbruck, Anichstraße 35,
A-6020 Innsbruck, Austria.

The *c-myc* proto-oncogene encodes for a 64-66 kd helix-loop-helix nucleophosphoprotein, which either alone (Prendergast and Ziff, 1991) or even better in association with the *Max*-protein (Blackwood and Eisenman, 1991) binds to DNA in a sequence-specific manner. The protein may participate in pleiotropic functions such as in DNA-synthesis (Studzinski et al., 1986), transcription of as yet undefined target genes and cell lineage determination (Collum and Alt, 1990). The physiological role of the gene is ill-defined although it essentially contributes to the cell cycle transit of human lymphocytes (Heikkila et al., 1987). The gene is highly expressed in early stages of human myelopoiesis (Kastan et al., 1989), and its inadequate and high level expression may block the differentiation pathway of murine neoplastic erythropoiesis (Birrer et al., 1989) and human leukemic myeloblasts (Baer et al., 1992), thus contributing to the neoplastic transformation. Since the gene is also constitutively overexpressed in many highly mitotic B- and T-cell tumors (Cory, 1986) it is usually deemed an essential "switcher molecule", which in its pathological activation status inhibits terminal differentiation and maintains deregulated proliferative behavior. Nevertheless, it may also contribute to diverse biological functions like resistance of Acute Myeloblastic Leukemia cells to chemotherapy (Preisler et al., 1989) or reversion of the malignant phenotype in *neu*-transformed cells (Suen et al., 1991).

More recently evidence has been provided that the *c-myc* proto-oncogene may also play a role in very mature and terminally-differentiated B-cell tumors: (1) Sümegi and coworkers (1985) and Pegoraro et al. (1989) reported on *c-myc*-amplification and consecutive overexpression of the specific transcripts in human plasma cell leukemias. (2) Selvanayagam et al. (1988) observed *c-myc* mRNA expression in 24% of 40 cases of multiple myeloma with mRNA amounts closely up to that seen in the overexpressing HL-60-AML cell line. (3) Drexler et al. (1989) were able to induce *c-myc* mRNA expression during the phorbol-ester-mediated differentiation pathway of B-Chronic Lymphocytic Leukemia (CLL) cells in a manner independent of cellular proliferation. Since these tumor cells are of extremely low proliferative potential their high *myc* mRNA expression levels might indicate a biological role of the gene different from that of driving tumor cells through the cell cycle. These findings are also important because the *c-myc* gene might serve as a tool for a better determination of the signal transduction pathway specific for the neoplastic B-cell maturation program. However, the above-mentioned studies were hampered by the following disadvantages: (1) The examinations were restricted to the DNA- and RNA level although the *c-MYC* protein should act as the biological effector molecule. (2) Due to the blotting techniques applied these analyses were prone to a biological bias in favor of patients with advanced stage disease, higher tumor load and with highly infiltrated bone marrow. (3) These examinations did not allow to definitely attribute *c-myc* gene expression to either the normal or the neoplastic cell pool, were confined to rather low numbers of cases and did not include a broader spectrum of terminally-differentiated B-cell tumors. For these reasons we were interested in studying the *c-myc* gene expression in mature B-cell tumors in the following setting: (1) The analysis was performed in 23 cases of B-CLL and 19 cases of multiple myeloma on both the mRNA- and the protein level in order to allow a potential insight into the regulation of the gene. (2) The investigation was carried out on the single cell level using anti-sense mRNA *in situ* hybridization and immunocytochemistry. This approach allowed the attribution of *c-myc* gene transcripts and the relevant protein to morphologically defined normal and neoplastic cell types. (3) For a better definition of the clinical role, clinical markers

of tumor load and parameters for cell cycle kinetics were compared to the *c-myc* gene expression.

Materials and Methods

In situ hybridization

In situ hybridization was performed using ^{35}S-labeled anti-sense RNA ($8.5x10^7$ cpm/µg) which was transcribed from a 1.5 kb DNA sequence from exon-2 of the *c-myc* gene inserted into an SP-6 vector system. A nick-translated plasmid sequence of similar specific activity was used as a control and gave negative results in all cases. *In situ* hybridization procedures were performed as recently described in detail (Greil et al., 1989, Greil et al., 1991).

Immunocytochemistry

Immunocytochemistry was carried out using an indirect immunoperoxidase technique (Greil et al., 1988, Greil et al., 1992) and using three antibodies (Abs) directed against the following three different epitopes of the *c-MYC* protein: Amino acid sequence 43-55: Ab 152, 171-188: Ab 155 (both Abs monoclonal; Microbiological Inc., Bethesda, Md); Ab DCP 801 (Cambridge Research Biochemicals) detects a 12 amino acid sequence from the C-terminus of *c-MYC*. Sampling of cells was representative and both *in situ* hybridization and immunocyto-chemistry were highly reproducible as indicated by a median sample/sample and day/day variance of <1% of cells assigned to the individual grading categories (for detail see Greil et al., 1992).

In situ Semiquantitation

In situ semiquantitation of *c-myc* mRNA and protein amounts was performed using a four stage system (Table I) which was derived from the following cell systems with well-defined *c-myc* mRNA amounts per cell. HL-60 (16-32 fold amplification of the gene; Westin et al., 1982), unstimulated and Phytohaemagglutinin (PHA)-activated peripheral blood T-cells (10-20 fold increase in *c-myc* mRNA (Reed et al., 1985) and identical increase in protein amounts (Greil et al., 1992). The "quantitative" character of this grading system and its high reproducibility were further confirmed by their excellent correlation with densitometric scans of Western blotting experiments and results of computer-assisted image analysis of nuclear staining parameters best approximating the absolute amounts of *c-MYC* per cell.

TABLE I: Semiquantitation of *c-myc* mRNA and *c-MYC* protein

	Negative	+ positive	++ positive	+++ positive
mRNA	S/N* < 2.5	S/N* >2.5	confluence of grains to patches	morphological details invisible beneath cytoplasmic silver grains
Protein	Negative	individual nucl. grains	confluence of grains to patches with <50% nuclear area positive	> 50% nuclear area positive independent of staining intensity#

* S/N: Signal to Noise ratio as determined by counting of grains over cells and areas of maximal background (Greil et al., 1989).

Differences in the absolute nuclear size (µm^2) do not significantly influence the value staining intensity x % positive nuclear area x nuclear size as determined by computer-assisted image analysis, which confirms the stability of this evaluation system (Greil et al., 1992).

Results

(1) *c-myc* mRNA and -Protein Expression

c-myc mRNA expression occurs in 92 % (12/13 cases) of multiple myeloma (Fig. 1a) but only in 1/23 (4.3%) cases of B-CLL. Expression levels varied substantially both from case to case and in tumor cell subsets within the same slide. Expression signals ranged between PHA-activated blasts with their 20-40-fold increase over unstimulated peripheral blood lymphocytes and HL-60 cells with their 16-32 fold amplification of the *c-myc* gene (Westin et al., 1982) and their average of 200 mRNA copies/cell (Bakkus et al., 1989) and could definitely be attributed to the neoplastic clone.

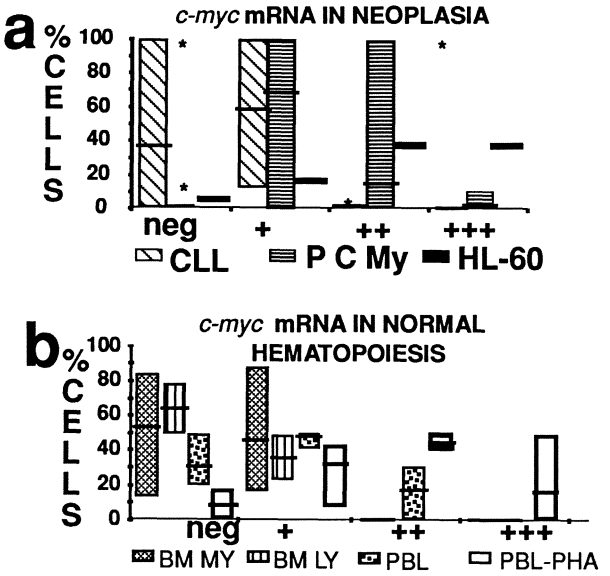


Figure 1: a: *c-myc* mRNA expression as determined in 23 cases of CLL, 13 cases of myeloma and the HL-60 cell line. Results within the four semiquantitation categories (for definition: Greil et al., 1989) are shown as box blot model with the upper and the lower range of the data and the black horizontal line representing the median of the data. CLL cells usually expressed very low amounts of *c-myc* mRNA which may be of questionable biological significance. Plasma cells of myeloma patients, though heterogeneous in their amounts of *c-myc* expressed per cell, were positive in 92% of cases with mRNA amounts similar to those of the HL-60 cell line. **b:** Bone marrow myelopoiesis, as well as the potential precursor cells of myeloma cells (peripheral blood and bone marrow B-cells) and peripheral blood T cells usually remained negative.

(2) *c-MYC* Protein Expression and Comparison with mRNA Analysis

The number of *c-MYC* protein positive cells was usually very similar to or identical with the number of cells positive on the mRNA level with the exception of three cases where mRNA was apparently not translated into protein (Table II: cases R 73, R 75; Fig. 2). Detailed semiquantitation of *c-MYC* protein and epitope mapping by means of three antibodies directed against different epitopes of the *c-MYC* protein revealed: (i) a good general concordance of results in the majority of cases with a defined and characteristic ranking in staining intensity obtained with the three different antibodies (AB 155> AB 152> AB DCP; e.g. cases R 100, R 113). (ii) Inversion in the usual staining hierarchy in at least 2 cases (Table II; e.g. cases R 112, R 88) where brighter staining and higher total number of positive cells were obtained with the DCP as compared with the 152 AB.

Table II: Comparison of *c-myc* mRNA and *c-MYC* Oncoprotein Expression in Special Cases

Case	% negative cells			% + cells			% ++ cells			% +++ cells			% total cells		
	mRNA	*Protein*		*mRNA*	*Protein*		*mRNA*	*Protein*		*mRNA*	*Protein*		*mRNA*	*Protein*	
R 73	0	152	100	60	152	0	40	152	0	0	152	0	100	152	0
		155	100		155	0		155	0		155	0		155	0
		DCP	100		DCP	0		DCP	0		DCP	0		DCP	0
R 75	0	152	100	100	152	0	0	152	0	0	152	0	100	152	0
		155	100		155	0		155	0		155	0		155	0
		DCP	100		DCP	0		DCP	0		DCP	0		DCP	0
R 100	0	152	33.3	100	152	33.3	0	152	33.3	0	152	0	100	152	66.6
		155	0		155	0		155	0		155	100		155	100
		DCP	100		DCP	0		DCP	0		DCP	0		DCP	0
R 113	15.7	152	28	78.7	152	46	5.5	152	26	0	152	0	84.3	152	72
		155	10		155	4		155	64		155	22		155	90
		DCP	50		DCP	50		DCP	0		DCP	0		DCP	50
R 112	0	152	8	100	152	42	0	152	50	0	152	0	100	152	92
		155	14		155	6		155	60		155	20		155	86
		DCP	0		DCP	0		DCP	100		DCP	0		DCP	100
R 88	0	152	28	20	152	39	74.3	152	33	5.7	152	0	100	152	72
		155	43		155	36		155	21		155	0		155	57
		DCP	97		DCP	2		DCP	1		DCP	0		DCP	3

These differences reflect true differences in the amounts of different *c-MYC* protein epitopes present within the neoplastic cells because (i) of excellent concordance of semiquantitation with results of computer-assisted image analysis (ii) their correlation with densitometric scans and concurrently performed Western blotting experiments (Greil et al., 1992) and (iii) their extremely high reproducibility (Greil et al., 1991). In addition, these results (Table II, Fig. 2) may indicate the presence of posttranscriptional and/or posttranslational regulation of the *c-myc* gene in myeloma cells.

(3) Comparison of *c-myc* mRNA and -Oncoprotein Expression with Clinical and Biological Parameters in Myeloma and B-CLL

When the numbers of *myc* mRNA and *MYC* protein positive cells were compared with markers of tumor load (i.e. the number of plasma cells and plasmoblasts in the bone marrow) and the proliferative activity as determined by the Ki-67 antigen expression, the presence of the 152 *c-MYC* epitope alone was clearly associated with a higher tumor load (Table III). The expression of the *c-myc* oncogene, however, was clearly independent of the proliferative activity (mean number of Ki-67 positive plasma cells < 1%).

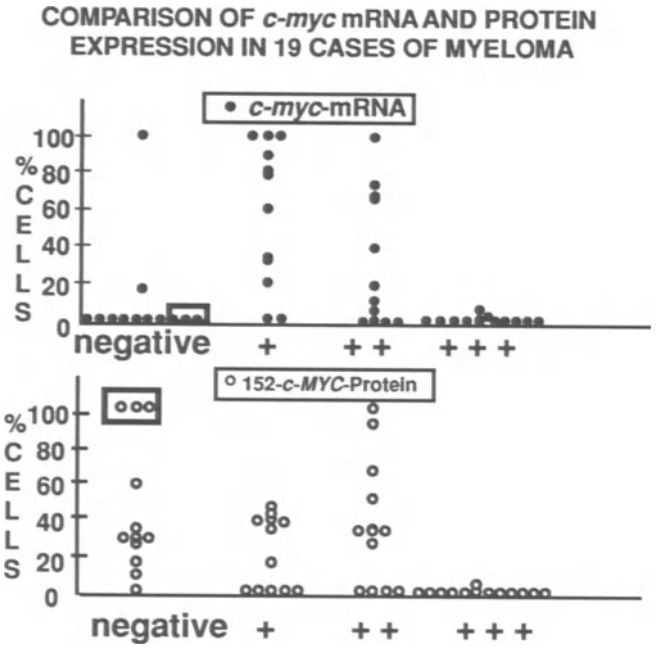


Figure 2: a: *c-myc* mRNA expression in myeloma cells. Each patient is represented by one value within each of the four semiquantitation categories. Extreme heterogeneity in gene dosage becomes apparent. b: Similarly, four semiquantitation categories were defined for immuncocytochemical investigation of *c-MYC* protein amounts expressed per cell (Table I, Greil et al., 1992). Generally, a similar total number of cells positive on the transcript and on the effector molecule level and a similar distribution of *c-MYC* protein and -mRNA amounts within the semiquantitation categories became apparent. However, direct comparison in individual patients reveals that the transcript is not translated into the protein in at least three patients (see boxes for corresponding cases, and Table II) and that the number of cells ++ and +++ for *c-myc* mRNA and protein respectively are not directly correlated (r152AB = 0.046, r155AB = 0.46; p = 0.29). These results may be condidered indicative for posttranscriptional regulation of the gene.

Our results indicate differential expression of *c-myc* mRNA and -protein during the terminal stages of the malignant B-cell differentiation pathway with *c-myc* mRNA expression occurring in up to 92% (12 out of 13 cases) of patients with myeloma but usually lacking in patients with CLL (22 out of 23 cases).*In situ* analysis allowed to definitely attribute the *c-myc* mRNA expression signals to the cells of the neoplastic clone. The interference of *c-myc* gene expression with the malignant B-cell differentiation program may be interpreted as follows: (i) The *c-myc* gene may be directly involved in the neoplastic B-cell differentiation program. Our observation of lack of *c-myc* gene expression in native B-CLL cells is in perfect correlation to results of others (Drexler et al.,1989; Larsson et al., 1987, 1991). In contrast to our results downregulation of the *c-myc* gene expression was reported during cytokine - and growth factor-stimulated plasma cell differentiation of B-CLL cell lines (Larsson et al., 1991). However, B-CLL lines which can be maintained in long-term culture represent a rare biologic selection and it cannot be excluded that the expression of the c-*myc* gene was more directly a result of the stimulation of B-CLL cells by cytokine combinations than of the induced morphological and immunological changes. Furthermore, transition of B-CLL into myeloma does not occur *in vivo,* and stability and biologic behavior of plasma cells originating from B-CLL precursor cells in this *in vitro* model are undefined.

TABLE III: *c-myc* mRNA and Protein Expression and Markers of Tumor Load in Myeloma

	nPlasma cells	n Plasmoblasts	n Ki-67 positive cells*
N 152 ++,+++.pos. Cells	p=0.024 r=0.65	p= 0.09	not significant
N 152 pos. Cells	p=0.043 r=0.64	-	not significant
N mRNA++,+++ pos. Cells	p= 0.240 r=0.16	p=0.19 r=0.42	not significant

* The number of Ki-67 positive cells defines the percentage of cells at the G1/S phase boundary and was consistantly less than 1%.

(ii) Several findings of our investigation, however, are not only in accordance with a differentiation-specific expression of the *c-myc* gene in B-cell neoplasias, but may also explain the *c-myc* gene expression as a result of the neoplastic process *per se* and of the progression of the disease in the multi-step carcinogenetic process. This view is substantiated by the following facts: (a) gene expression was undetectable in potential precursor cells of the malignant plasma cells, namely circulating monoclonal blood and bone marrow B-cells (Fig. 1). (b) A substantial part of the myeloma cells remained negative in several cases (Fig. 3). If *c-myc* indeed plays an essential role during terminal malignant B-cell differentiation, then mature myeloma cells – indistinguishable from the majority of the cells of the neoplastic clone by morphological and immunological means – may also develop via a *myc*-independent pathway. (c) B-CLL cells of a patient with concomitant myeloma remained negative, although plasma cells clearly expressed the gene. (d) *c-MYC* protein expression is undetectable in preneoplastic monoclonal plasma cells of patients suffering from Monoclonal Gammopathy of Undetermined Significance (Greil et al., unpublished observation). (e) Increased *c-MYC* protein amounts were associated with higher tumor load (Table III). Certainly, malignant transformation and neoplastic cell differentiation are overlapping processes and their regulation may even involve a partly identical, though differentially-regulated network of genes. A definite conclusion will require the analysis of *in vivo* and *in vitro* models where the differentiation capacity of normal and neoplastic B cells can be tested. It will also be necessary to investigate the way the *c-myc* gene expression and regulation are influenced by sequential activation of oncogenes during well-defined stages of the multistep carcinogenetic process of plasma cells.

(iii) Although the murine plasmacytoma model is characterized by a translocational activation of the *c-myc* gene and/or the depletion of the *c-myc* plasmacytoma repressor factor (Kakkis et al., 1989), the expression of the gene in the human analogue is not caused by structural alterations of the gene (Neri et al., 1989, Greil et al., 1991) and mechanisms of derepression of the gene have not yet been investigated. Recent examinations of plasmacytoma lines (Hollis et al., 1988) and Acute Myeloid Leukemias (Baer et al., 1992) have shown pathological mRNA stabilization and consecutive accumulation of *c-myc* on the posttranscriptional level in the presence but also in the absence of 5´or 3 ´alterations of the transcript (Baer et al., 1992). Apparently, such a mechanism could be effective in myeloma and account for some of our data. Also, alterations in *c-MYC* protein dimerization partners, or expression of proteins with the capacity for modifying the DNA binding activity *c-MYC* (DePinho et al., 1991, Kerkhoff and Bister, 1991) could not only modify the *c-myc* effects, thus explaining its independence of proliferative capacity as observed in our study (Table III). Modifications in the constitution of the *c-MYC* protein, however, might also account for altered binding affinities of antibodies directed against the C-terminus of the protein (i.e DCP) in at least some cases.

(iv) Finally, the question arises whether *c-myc* gene expression is a central and early point within the transformation process or a consequence of (a) a debalanced cytokine network or (b) a result of activation of other oncogenes with a more upstream localization within the signal transduction pathway. **Ad (a)**: In the murine plasmacytoma model the *c-myc* gene derangement is the result of a pathological recombinase event and a very early step in the carcinogenetic process which is followed by rescue from counterregulatory instabilization of the malignant clone through apoptosis by means of Interleukin-6 (IL-6)-expression (M. Potter, personal communication, NIH-Workshop, 1992). Although the expression of *c-myc* in human myeloma is not due to a structural derangement of the gene on the DNA level (Neri et al., 1989), our results point to a similar role of *c-myc* expression in the human model. *c-myc* overexpression is toxic for many human cell types (Askew et al., 1992) and rescue from apoptosis might be induced by both IL-6- and/or *bcl-2* overexpression. In this context it is interesting to note that

bcl-2-overexpression in myeloma cells was recently reported (Pettersson et al., 1992). However, a debalanced expression of IL-6 – irrespective of its autocrine (Kawano et al., 1988) or paracrine (Klein et al., 1991) nature – could not only explain upregulation of *c-myc* e.g. by means of NFκB-activation (Tanabe et al., 1988, DePinho et al., 1991) but the very same mechanism may induce the IL-6 gene upregulation, thus contributing to the autocrine model of IL-6 in myeloma (Kawano et al., 1988). However, direct experimental evidence will have to be provided for that model. **Ad (b):** Finally, the question arises whether *c-H-ras* activation cooperates with *c-myc* in the human plasmacytoma model. Both genes cooperate in *in vitro* carcinogenesis assays (Birrer et al., 1988) and activation of *c-H-ras* by critical point mutations with subsequent overexpression of the protein has been demonstrated in myelomas (Neri et al., 1989, Tsuchiya et al., 1988). Although *c-Hras-* and *c-myc* activation may reflect independent and different steps of carcinogenesis in the human myeloma system, point-mutated *c-H-ras* possibly suffices for stimulating *c-myc* upregulation in a Proteinkinase-C-dependent manner by modifying the expression of the *c-fos* and *c-jun* gene expression and the constitution of the *AP-1*-complex (Stacey e al., 1987) which can bind to relevant motifs of the *c-myc* gene (DePinho et al.,1991). In this way, the *c-myc* gene deregulation – theoretically – could be explained as a direct consequence of the pathological derangement of the signal transduction pathway. These models are currently under investigation in our laboratory.

ACKNOWLEDGEMENT

This work was supported by the FWF grants P-7042 Med (R.G.) and P 8947 (R.G.) and the Legerlotz Foundation. B.F. is a postgraduate student in the Laboratory of Molecular Cytology. Reprint requests should be addressed to R. Greil, M.D., Department of Internal Medicine, Laboratory of Molecular Cytology, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria.

REFERENCES:

Askew DS, Ashmun RA, Simmons BC, Cleveland JL (1992) Constitutive *c-myc* expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 6: 1915-1922.

Baer MR, Augustinos P, Kinninburgh AJ (1992) Defective *c-myc* and *c-myb* mRNA turnover in acute myeloid leukemia cells. Blood 79: 1319-1326.

Bakkus MHC, Karin MJ, Peer B, Adriaansen HJ, Wierenga-Wolf AF, Akker TW, Dicke-Evinger MJ, Benner R (1989) Detection of oncogene expression by fluorescent *in situ* hybridization in combination with immunofluorescent staining of cell surface markers. Oncogene 4: 1255-1262.

Birrer MJ, Raveh L, Dosaka H, Segal S (1989) A transfected L-*myc* gene can substitute for *c-myc* in blocking murine erythroleukemia differentiation. Mol Cell Biol 9: 2734-2737.

Birrer MJ, Segal S, DeGreeve JS, Kaye F, Sausville EA, Minna JD (1988) *L-myc* coperates with *ras* to transform primary rat embryo fibroblasts. Mol Cell Biol 8: 2668-2673.

Blackwood EM, Eisenman RN (1991) Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with *myc*. Science 251: 1211-1217.

Collum RG, Alt FW (1990) Are *myc* proteins transcription factors? Cancer Cells 2: 69-75.

Cory S (1986) Activation of cellular oncogenes in hematopoietic cells by chromosome translocation. Adv Cancer Res 47: 189- 234.

DePinho RA, Schreiber-Agus N, Alt FW (1991) *myc* family oncogenes in the development of normal and neoplastic cells. Adv Cancer Res 57: 1-46.

Drexler HG, Janssen JWG, Brenner MK, Hoffbrand V, Bartram CR (1989) Rapid expression of proto-oncogenes *c-fos* and *c-myc* in B-chronic lymphocytic leukemia cells during differentiation induced by phorbol ester and calcium ionophore. Blood 73: 1656-1663.

Greil R, Gattringer C, Fasching B, Cleveland J, Thaler J, Radaskiewicz T, Gastl G, Huber C, Rapp U, Huber H (1988) *abl* oncogene expression in Non-Hodgkin lymphomas: Correlation to histological differentiation and clinical status. Int J Cancer 42: 529-538.

Greil R, Fasching B, Huber H (1989) Methods in Laboratory Investigation: *In situ* hybridization for the detection of low copy numbers of *c-abl* oncogene mRNA in lymphoma cells: technical approach and comparison with results with anti-oncoprotein antibodies. Lab Invest 60: 574-582.

Greil R, Fasching B, Loidl P, Huber H. (1991) *c-myc* proto-oncogene expression in multiple myeloma and chronic lymphocytic leukemia: An *in situ* analysis. Blood 78: 180-191.

Greil R, Fasching B, Weger A, Loidl P (1992) Methods in laboratory investigation: Investigation of nuclear *c-MYC* oncoprotein expression in human hematopoiesis: suitability of a rapid and reliable semiquantitative evaluation system. Lab Invest 62: 251-260.

Heikkila R, Schwab G, Wickstrom E, Loke SL, Pluznik DH, Watt R, Neckers LM (1987) A *c-myc* antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. Nature 328: 445-450.

Hollis GF, Gazdar AF, Bertness V, Kirsch IR (1988) Complex translocation disrupts *c-myc* regulation in a human plasma cell myeloma. Mol Cell Biol 8: 124- 129.

Kakkis E, Riggs KJ, Gillespie W, Calame K (1989) A transcriptional repressor of *c-myc*. Nature 338: 718-721.

Kastan MB, Stone KD, Civin CD (1989) Nuclear oncoprotein expression as a function of lineage, differentiation stage, and proliferative status of normal human hematopoietic cells. Blood 74: 1517-1524.

Kawano M, Hirano T, Matsuda T et al., (1988) Autocrine generation and requirement of BSF/IL-6 for human myelomas. Nature 332: 83-85.

Kerkhoff E, Bister K (1991): *Myc* protein structure: localization of DNA-binding and protein dimerization domains. Oncogene 6: 93-102.

Klein B, Zhang X-G, Jourdan M, Content J, Houssiau F, Aareden L, Piechaczyk M, Bataille R (1991) Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73: 517-526

Kerkhoff E, Bister K (1991) *Myc* protein structure: localization of DNA-binding and protein dimerization domains. Oncogene 6: 93-102.

Larsson LG, Gray HE, Tötterman T, Pettersson U, Nilsson K (1987) Drastically increased expression of *MYC* and *FOS* protooncogenes during *in vitro* differentiation of chronic lymphocytic leukemia cells. Proc Natl Acad Sci USA 84: 223-227.

Larsson LG, Schena M, Carlsson M, Sällström J, Nilsson K (1991) Expression of the *c-myc* protein is downregulated at the terminal stages during *in vitro* differentiation of B type chronic lymphocytic leukemia cells. Blood 77: 1025-1032.

Neri A, Murphy JP, Cro L, Ferrero D, Tarella C, Baldini L, Dalla Favera R (1989) *Ras* oncogene mutation in multiple myeloma. J Exp Med 170: 1715-1725.

Pegoraro L, Malavasi F, Bellone G, Massaia M, Boccadoro M, Saglio G., Guerrasio A, Benetton G, Lombardi L, Coda R, Avanzi GC (1989) The human myeloma cell line LP-1: A versatile model in which to study early plasma cell differentiation and *c-myc* activation. Blood 73: 1020- 1027.

Pettersson M, Jenberg-Wiklund H, Larsson LG, Sundström C, Givol I, Tsujimoto Y, Nilsson K (1992) Expression of the *bcl-2* gene in human multiple myeloma cell lines and normal plasma cells. Blood 79: 495-502.

Prendergast GC, Ziff EB (1991) Methylation-sensitive sequence-specific DNA binding by the *c-myc* basic region. Science 251: 186-189.

Preisler HD, Raza A, Larson R, LeBeau M, Bowman G, Goldberg H, Vogler R, Verkh L, Singh P, Block AM, Sandberg A (1989) Protooncogene expression and the clinical characteristics of acute non-lymphocytic leukemia: A leukemia Intergroup pilot study. Blood 73: 255- 262.

Reed JC, Nowell PC, Hoover RG (1985) Regulation of *c-myc* mRNA levels in normal human lymphocytes by modulators of cell proliferation. Proc Natl Acad Sci USA 82:4221-4224

Selvanayagam P, Blick M, Narni F, Van Tuinen P, Ledbetter DH, Alexanian R, Saunders G, Barlogie B (1988) Alteration and abnormal exprtession of the *c-myc* oncogene in multiple myeloma. Blood 71: 30-35.

Stacey DW, Watson T, Kung HF, Curran T (1987) Microinjection of transforming *ras* proteins induces *c-fos* expression. Mol Cell Biol 7: 523-527.

Studzinski GP, Brelvi ZS, Feldman SC, Watt RA (1986) Participation of *c-myc* protein in DNA synthesis of human cells. Science 234: 467- 470.

Suen TC, Hung MC (1991) *c-myc* reverses *neu*-induced transformed morphology by transcriptional repression. Mol Cell Biol 11:354-362.

Sümegi J, Hedberg T, Bjorkholm M, Godal T, Melstedt M, Nilsson MG, Perlman C, Klein G (1985) Amplification of the *c-myc* oncogene in human plasma cell leukemia. Int J Cancer 36: 367-371.

Tanabe O, Akira S, Kamiya T, Wong GG, Hirano T, Kishimoto T (1988) Genomic structure of the murine Il-6 gene. High degree conservation of potential regulatory sequences between mouse and human. J Immunol 141:3875-3881.

Tsuchiya H, Epstein J, Selvanayagam P, Dedman JR, Gallick G, Alexanian R, Barlogie B (1988) Correlated flow cytometric analysis of *H-ras* p21 and nuclear DNA in multiple myeloma. Blood 72: 796-800.

Westin EH, Wong-Staal F, Gelman EP, Dalla-Favera R, Papas TS, Lautenberger JA, Ev A, Reddy EP, Tronick SR, Aaronson SA, Gallo R (1982) Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc Natl Acad Sci USA 79:2490- 2494.

Genes Expressed Selectively in Plasmacytomas: Markers of Differentiation and Transformation

P. L. BERGSAGEL, C. VICTOR-KOBRIN, L. A. BRENTS, J. F. MUSHINSKI* and W. M. KUEHL

Navy Medical Oncology Branch and
*Laboratory of Genetics, National Cancer Institute, Bethesda, MD 20889-5105.

Introduction

Plasmacytomas differ from B lymphomas in a number of ways: Plasmacytomas have a distinct plasmacytoid morphology, lack expression of certain B cell markers (e.g., Ia), express much greater levels of immunoglobulin and interact with epithelial cells in the secretion of IgA. However, there are few if any, molecular markers unique to plasmacytomas. To characterize the molecular basis for the differences between plasmacytomas and B lymphomas, we have constructed a murine plasmacytoma (MPC11) minus highly differentiated B lymphoma (A20.2J) subtractive cDNA library [1, 2]. This strategy should enrich for two classes of genes: genes differentially expressed in both normal and malignant plasma cells, and genes differentially expressed only in malignant plasma cells. As summarized below, we have identified plasmacytoma specific genes of both types.

Results

We initially used probes from 150 random clones from the subtracted cDNA library to analyze for mRNA expression on Northern blots. From this analysis, we identified fifty apparently unrelated genes, each of which expressed mRNAs in both the parental MPC11 and an unrelated plasmacytoma, but not in the A20 subtractive partner. On initial screening, six of these fifty genes expressed mRNAs in most plasmacytomas, but in no more than one of seven other B lymphomas. We summarize our results (Table 1) for four of these genes, as well as two genes (PC166 and PC315) that are expressed at a higher level in plasmacytomas than in the A20.2J B lymphoma subtractive partner. Four of the six genes (PC70, PC166, EGP314, PC315) proved to be markers of the terminal plasma cell stage of normal B lymphocyte

Table 1 Summary of six plasmacytoma specific genes.

GENE	SEQUENCE HOMOLOGY	FEATURES
	Markers of normal plasma cells	
PC70	placental alkaline phosphatase	also expressed in pre-B lymphoma
PC166	homology to XLR gene	X linked; ? no human homologue
EGP314	pan epithelial glycoprotein	? adhesion molecule
PC315	no known homologies	B cell specific/ IL-6 increases expression
	Markers of malignant plasma cells	
PC251	new member of the hematopoietic growth factor receptor gene family	expressed in all normal tissues
PC326	new member of the ß-transducin repeat "mosaic" protein family	X linked; expressed in testis dysregulated in somatic cell hybrids

differentiation. The other two (PC251 and PC326) appear to relate to the neoplastic phenotype of the plasmacytomas.

Genes that are markers of plasma cells

PC70 encodes a 3.3 kb mRNA that is also expressed in 9 out of 12 plasmacytomas and in all pre-B lymphomas, but only one of eight B lymphomas [2]. Sequence of a 289 bp subtractive cDNA revealed that PC70 is mouse placental alkaline phosphatase. Other investigators have reported that in mitogen-stimulated B lymphocytes, there was an increase in alkaline phosphatase activity restricted to the activated B cells committed to proliferation [3], but there are no reports of its expression in earlier stages of B cell differentiation.

PC166 encodes a 2.0 kb mRNA that is expressed at a high level in all plasmacytomas examined, at a much lower level in 3 out of 8 B lymphomas, and in none of 10 pre-B lymphomas. It encodes a 226-amino acid protein homologous to xlr-1 (**X**-linked, **L**ymphocyte **R**egulated)[4] with 26% amino acid identity and 49% similarity over the entire length. Xlr-1 is an X-linked member of a murine multi-gene family that is expressed as a nuclear protein in the terminal stages of lymphocyte differentiation. Xlr-2, a highly homologous gene, is expressed principally in testicular germ cells. PC166 is also X-linked (C. Kozak, unpublished) and it encodes an mRNA that is most abundant in testis, but is also present at a low level in many other normal tissues. Like xlr-1 and xlr-2, PC166 does not cross-hybridize to human DNA or RNA on Southern or Northern blots. Unlike xlr-1 and xlr-2, which are both acidic, PC166 is quite basic. We propose calling it xlr-3.

EGP314 (identified by subtractive clone 289A) encodes a 1.8 kb mRNA that is expressed in 15 out of 16 plasmacytomas, at a much lower level in 3 out of 8 B lymphomas, and 1 out of 10 pre-B lymphomas [5]. It encodes a 314-amino acid protein that contains a signal sequence and a hydrophobic transmembrane domain. It is the murine homologue of an extensively studied human pan-epithelial glycoprotein, EGP. Although previously thought to be exclusively restricted to epithelial cells, EGP314 is also differentially expressed in B cell development, with increased levels of expression when splenocytes are stimulated by LPS to differentiate into mature plasma cells. The function of EGP314 is not known, but it has some homology to nidogen, a basement membrane adhesion molecule. The co-expression of EGP314 on plasma cells and the basolateral surface of epithelial cells suggests a role in the interaction of these two cell types with a common molecule. For example, it could serve to anchor plasma cells in epithelial tissues or to facilitate the translocation of IgA and secretory component across epithelial cells.

PC315 encodes mRNA species of 2.1 and 6.2 kb. Sequence analysis of the corresponding cDNA molecules indicates that these two mRNA species have an identical open reading frame of 1.3 kb, but differ significantly at their 3' ends as a result of using distinct polyadenylation signals. The alternatively polyadenylated mRNAs are expressed, in approximately equal proportions, at a high level in all plasmacytomas and at a much lower level in some B and pre-B lymphomas. Interestingly, IL-6 induces higher levels of expression of both mRNA species in an IL-6 dependent plasmacytoma cell line (MH60BSF2). Of normal murine tissues examined, PC315 is expressed only in the spleen. Although only the 6.2 kb form is observed in virgin spleen cells, following a five day stimulation of these spleen cells with LPS, similar amounts of both mRNAs are observed. To date no significant homologies have been found at the nucleic acid or protein level between PC315 and sequences in several databases.

A marker of the malignant plasmacytoma phenotype

PC326 encodes 4.8 and 5.2 kb mRNAs that encode a 747-amino acid protein with a highly acidic amino-terminal region containing 7 repeats of a unique, moderately acidic 20-amino acid sequence that is directly flanked by two extremely acidic sequences; and a carboxy-terminal region containing one good copy, and two degenerate copies of the repeat found in ß-transducin [6](Fig. 1). Other proteins that contain this repeat fall into two categories [7]. Category I consists of trimeric G-protein ß subunits, whose entire sequence consists of 7-8

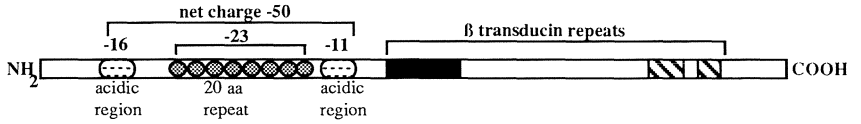


Fig. 1 Diagram of PC326 protein structure.

tandem copies of this repeat. ß-transducin, of the photoreceptor G-protein and STE4, of the yeast pheromone receptor G-protein are examples in this category. Category II consists of "mosaic" proteins whose amino terminal half contains either a charged or mixed charged cluster, and whose carboxyl terminal half contain 5-7 tandem copies of the ß-transducin repeat (Table 2). Many of the proteins in this second category have been shown to localize to the nucleus and some to regulate transcription. E(spl) may interact synergistically with a helix-loop-helix E(spl) nuclear protein to inhibit lateral neurogenic differentiation in the developing Drosophila [8]. TUP1 interacts with SSN6, a TPR protein, and acts as a general repressor of transcription in yeast [9]. It has been postulated that each transducin repeat protein interacts with a given TPR protein: PRP4-PRP6, CDC20-CDC16, SKI3-MAK11, TUP1-SSN6 [10]. PC326 is the first mammalian gene in this second category, but is unique in having only one good copy of the transducin repeat.

Table 2 Examples of proteins containing the ß-transducin repeat.

Category I (trimeric G-protein ß subunits)			
ß-Transducin	48	RRTLRGHLAKIYAMHWATDSKLLVSASQDGKLIVWDSYTTNK	340 aa
Cblp	187	KNNLVGHHGYVNTVTVSPDGSLCASGGKDGIAMLWDLAEGKR	318 aa
STE4	342	TSSSYLDNQGVVSLDFSASGRLMYSCYTDIGCVVWDVLKGEI	423 aa
Category II (charged/mixed charge cluster "mosaic" proteins)			
TUP1	469	NESGTGHKDSVYSVVFTRDGQSVVSGSLDRSVKLWNLQNANN	669 aa
E(spl)	558	VRQFQGHTDGASCIDISPDGSRLWTGGLDNTVRSWDLREGRQ	719 aa
CDC4	563	LYILSGHTDRIYSTIYDHERKRCISASMDTTIRIWDLENIWN	779 aa
Consensus		.....GHs..Δ.....p.g..Δ.sgs.D..Δ.ΔWDΔ.....	
PC326	329	EHVFEGHSGCVNTVHFNQHGTLLASGSDDLKVIVWDWLKKRS	747 aa
	588	YKGHRNNSTVKGVYFYGPRSEFVMSGSGCGHIFIWEKSSCQI	
	631	QFLEADEGGTINCIDSHPYLPVLASSGLDHEVKIWSPIAEPS	

Although PC326 is expressed at a high level (about 30-100 copies of mRNA per cell) in most plasmacytoma cell lines, it does not appear to be expressed in LPS-induced plasma cells or any other normal tissue, apart from a very low level in testis (Table 3). The gene has been localized to the X chromosome (C. Kozak, unpublished). Unlike B-cell-specific genes, it is not suppressed in somatic cell hybrids between plasmacytomas and T cells or fibroblasts. Of interest is that the fibroblast hybrids continue to have a malignant phenotype, even though the rearranged c-myc is not expressed. These features suggests that the expression of PC326 may relate to the malignant phenotype of the plasmacytoma cell lines. Although there is near uniform expression of PC326 in pristane-induced plasmacytoma cell lines, when plasmacytoma

Table 3 PC326 and PC251 mRNA expression patterns.

	PC326	PC251
Plasmacytoma cell lines	13/14	11/12
pre-B/B lymphoma	0/32	0/18
non-B hematopoietic	2/22	0/4
Normal tissues	only testis	all
Plasmacytoma / T cell hybrids	2/2	0/2
BALB/c 3T3	0/1	1/1
Transformed BALB/c 3T3	n.t.	0/2

Table 4 Expression of PC326 in pristane induced plasmacytomas

Accelerating viral oncogene(s)	Latency (days)	# tumors /lines	% PC326 mRNA +
None - cell lines		14	93
- tumors	215	20	85
v-abl	90	22	67
v-raf	85	2	100
v-H-ras + c-myc	90	2	100
v-raf + c-myc	69	4	25
v-abl + c-myc	35	8	13

tumors are induced with pristane and different accelerating viruses, there is variability in the expression of PC326 (Table 4). There is a direct correlation between the mean latency with which the tumors develop and the frequency of PC326 expression; i e., for tumor induction methods that require longer for tumor development there is a greater likelihood of PC326 expression.

The apparently dysregulated pattern of expression in plasmacytomas and intriguing sequence homologies suggest that PC326 may contribute to plasmacytomagenesis. Its expression is more common in plasmacytomas that arise more slowly, suggesting that PC326 expression may be a late event in a multi-step process of neoplastic changes, but one that can be circumvented when tumor development is accelerated by additional exogenous oncogenic factors.

A new member of the hematopoietic growth factor receptor gene family

PC251 encodes alternatively polyadenylated mRNAs of 2.7 and 4.4 kb that encode a 439-amino acid protein with a signal peptide and a hydrophobic transmembrane domain. The protein is most homologous to the IL-5 receptor α chain and has features characteristic of the hematopoietic growth factor receptor superfamily (Fig. 2) [11, 12]. In addition to IL5Rα, PC251 has significant similarity to IL3Rα and GM-CSF-Rα. All three of these receptors use a common ß chain to constitute a high-affinity receptor. Although the expression of PC251 in cell lines is restricted to plasmacytomas, we were surprised to find a uniform, similarly low level of expression (approximately 3-5 copies of each species of mRNA per cell) in all normal tissues (Table 3). Like B-cell-specific genes (e.g., Ig), it is suppressed in somatic cell hybrids between plasmacytomas and T cells [13](Table 3). Interestingly, PC251 is expressed in the A31 BALB/c 3T3 fibroblast cell line, but not in two transformed clones (transformed respectively with v-mos and K-ras retroviruses).

Since the expression of PC251 appears to be ubiquitous but selectively lost from cells when they undergo malignant transformation, it may be a suppressor factor receptor for most cells. Plasmacytomas may be unusual in this context, since PC251 continues to be expressed

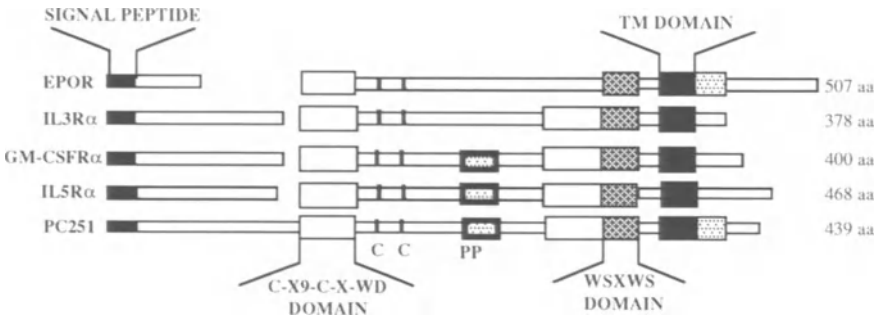


Fig. 2 PC251 homology to hematopoietic growth factor receptors

in these tumors. It is our working hypothesis that PC251 resembles oncostatin-m (OSM) [14], which is growth inhibitory to many cells, but is growth stimulatory for plasmacytomas and Kaposi's sarcoma cells [15, 16]. OSM has been shown to be a member of a cytokine family that includes IL6, LIF and G-CSF, and to use the LIFRα and IL6 gp130 receptor to constitute a high affinity receptor.

Summary

We have analyzed a murine plasmacytoma minus highly differentiated B lymphoma subtractive cDNA library and identified eight genes that are expressed in most plasmacytomas but at a much lower level, or not at all, in most B lymphomas.

Four of the genes are markers of the terminal differentiation of B lymphocytes into plasma cells:

- **placental alkaline phosphatase**, also expressed in pre-B lymphomas
- **xlr-3**, a new X-linked member of the xlr multi-gene family
- **EGP314**, a pan-epithelial glycoprotein with sequence features of an adhesion molecule
- **PC315**, a gene that is up-regulated by IL6, but without obvious sequence homologies.

Two of the genes are not clearly related to normal B cell differentiation, appearing to be associated with malignant transformation of plasma cells:

- **PC326** is a new member of the ß-transducin mosaic protein gene family. It is an X-linked gene, expressed at a very low level in testis, but in no other normal tissue, including LPS- or IL6-induced plasma cells. It has a high level of expression (apparently dysregulated) in most (>85 %) mineral oil induced plasmacytomas. However the likelihood that PC326 is expressed decreases as the tumor latency decreases when different retroviral agents are used to accelerate mineral oil induced plasmacytomagenesis. This suggests that PC326 expression may be a late event in a multi-step process of tumorigenesis.
- **PC251** a new member of the hematopoietic growth factor receptor family, most homologous to IL5Rα. It is expressed at a uniformly low level in all normal tissues and non-malignant cell lines; but,with the exception of plasmacytomas, it is not expressed in tumor cell lines. Like oncostatin-m, it may be the receptor for a cytokine that is growth inhibitory for many cells, but growth stimulatory for plasmacytomas.

References

1. Timblin C, Battey J and Kuehl WM (1990) Application for PCR technology to subtractive cDNA cloning: identification of genes expressed specifically in murine plasmacytoma cells. Nucleic Acids Res 18:1587-93
2. Timblin C, Bergsagel PL and Kuehl WM (1990) Identification of consensus genes expressed in plasmacytomas but not B lymphomas. Curr Top Microbiol Immunol 166:141-147
3. Kasyapa CS and Ramanadham M (1992) Alkaline phosphatase activity is expressed only in B lymphocytes committed to proliferation. Immunol Lett 31:111-6
4. Garchon HJ (1991) The Xlr (X-linked lymphocyte regulated) gene family (a candidate locus for an X-linked primary immune deficiency). Immunodefic Rev 2:283-302
5. Bergsagel PL, Victor-Kobrin C, Timblin CR, Trepel J and Kuehl WM (1992) A murine cDNA encodes a pan-epithelial glycoprotein that is also expressed on plasma cells. Journal of Immunology 148:590-596

6. Bergsagel PL, Timblin CR, Eckhardt L, Laskov R and Kuehl WM (1992) Sequence and expression of a murine cDNA encoding PC326, a novel gene expressed in plasmacytomas but not normal plasma cells. Oncogene (in press)
7. Duronio RJ, Gordon JI and Boguski MS (1992) Comparative analysis of the ß transducin family with identification of several new members including PWP1, a nonessential gene of *Saccharomyce cerevisiae* that is divergently transcribed from NMT1. Proteins Struct Func Genet 13:41-56
8. Delidakis C, Preiss A, Hartley DA and Artavanis TS (1991) Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of Drosophila melanogaster. Genetics 129:803-23
9. Keleher CA, Redd MJ, Schultz J, Carlson M and Johnson AD (1992) Ssn6-Tup1 is a general repressor of transcription in yeast. Cell 68:709-19
10. Goebl M and Yanagida M (1991) The TPR snap helix: a novel protein repeat motif from mitosis to transcription. Trends Biochem Sci 16:173-7
11. Tavernier J, Devos R, Cornelis S, Tuypens T, Van, der, Heyden, J, Fiers W and Plaetinck G (1991) A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. Cell 66:1175-84
12. Cosman D, Lyman SD, Idzerda RL, Beckmann MP, Park LS, Goodwin RG, and March CJ (1990) A new cytokine receptor superfamily. Trends Biochem Sci 15:265-70
13. Yu H, Porton B, Shen LY and Eckhardt LA (1989) Role of the octamer motif in hybrid cell extinction of immunoglobulin gene expression: extinction is dominant in a two enhancer system. Cell 58:441-8
14. Horn D, Fitzpatrick WC, Gompper PT, Ochs V, Bolton HM, Zarling J and Malik N (1990) Regulation of cell growth by recombinant oncostatin M. Growth Factors 2:157-65
15. Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J and Brasher KK (1992) The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. Science 255:1434-7
16. Rose TM and Bruce AG (1991) Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. Proc Natl Acad Sci U S A 88:8641-5

PCS, a Gene Related to the Immunoglobulin Super Family of Axonal Glycoproteins is Expressed in Murine Plasma Cell Tumors

M. A. CONNELLY, R. C. GRADY, J. F. MUSHINSKI@ and K. B. MARCU

Departments of Biochemistry and Cell Biology, Microbiology and Pathology, SUNY at Stony Brook, Stony Brook, NY 11794-5215;
@Laboratory of Genetics, National Cancer Institute, NIH, Bethesda, Maryland 20892

Introduction

Intraperitoneal plasma cell tumors (PCTs) are induced by pristane (tetramethylpentadecane), a branched-chain alkane paraffin oil, in BALB/cAn and other susceptible mouse strains with minimal to mean latencies of 120 to 210 days respectively (Potter, 1972). PCTs harbor one of two reciprocal chromosome translocations always involving band D2/3 on chromosome 15 and either the immunoglobulin heavy chain (IgH) locus on 12 or the kappa light chain locus (IgK) on 6 (Ohno et al., 1979). Band D2/3 was subsequently found to be the c-myc gene locus and the activation of this proto-oncogene by such chromosomal rearrangements has been shown to be a contributing factor for plasmacytomagenesis (reviewed by Mushinski, 1988; Marcu et al., 1992). Plasmacytomagenesis was rapidly accelerated (50-120 day mean latencies) upon injection of oncogene-containing retroviruses (v-abl, myc and raf or myc and ras) along with pristane (Potter et al., 1973; Ohno et al., 1984; Potter et al., 1987; Clynes et al., 1988; Troppmair et al., 1989) and viruses-containing an activated myc oncogene induced PCTs without c-myc associated chromosome translocations (Potter et al., 1987; Clynes et al., 1988; Troppmair et al., 1989). Recently, a retrovirus harboring v-abl and c-myc was found to be the most potent oncogene combination inducing PCTs in 100% of adult BALB/c mice with or without a wild type Moloney helper virus and also in the absence of pristane (Weissinger et al., 1991). These observations would collectively argue that multiple genetic events in addition to c-myc activation are required for malignant plasma cell tumor formation. Indeed, at least three recessive genetic loci are believed to confer resistance to PCT formation in non-sensitive mouse strains but the identities of these genes and other dominant acting ones in addition to myc remain unknown.

Plasma cell tumors have also been known for sometime to express abundant quantities of intracisternal A-type particles (IAP) budding from the endoplasmic reticulum (Dalton et al., 1961; Kuff et al., 1972; Kuff and Lueders 1988). IAPs are defective retrovirus-like particles which are expressed in a number of murine tumor cells and also in the developing murine embryo (Kuff et al., 1972; Calarco and Szollose, 1973; Biczysko et al., 1973; Kuff and Lueders, 1988). The mouse genome harbors several thousand IAP proviral DNAs including a variety of deleted forms (Lueders and Kuff, 1977; Ono et al., 1980). IAP proviruses exist in two major classes. Type-I proviruses are the most abundant variety and type-II forms are about 2 kb smaller and contain other distinguishing sequences (Shen-Ong and Cole, 1982). PCTs express abundant quantities of RNAs derived from type-II IAP proviruses (Cole et al., 1982). IAP proviruses

Current Topics in Microbiology and Immunology, Vol. 182

have been found to act as insertional mutagens in plasma cell tumors having both negative (Hawley et al., 1982) and positive (Canaani et al., 1983) effects on the expression of other cellular genes. IAP proviral insertions within and nearby the c-mos proto-oncogene have been documented in two plasma cell tumors (Canaani et al., 1983; Cohen et al., 1983) and also 3' of a translocated c-myc gene (Greenberg et al., 1985). Though it would be an attractive notion, a more general role for IAPs in plasmacytomagenesis has not been established.

Here, we report the cloning and partial characterization of a PCT specific gene (denoted PCS). About 60-70% of PCTs were found to express PCS. Remarkably, the brain is the normal site of PCS expression; and it encodes a member of a family of polypeptides containing both immunoglobulin and fibronectin-like domains, which are known to promote the growth of neurons (Jessel, 1988; Furley et al., 1990).

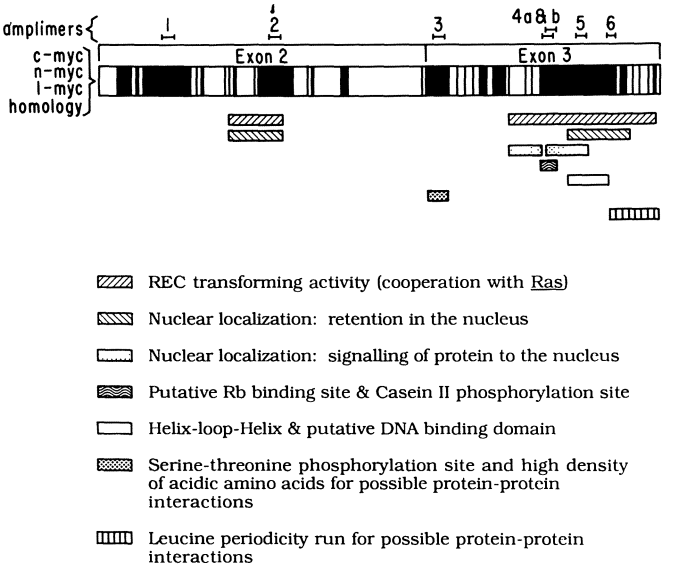


FIGURE 1: Composite of homologous regions (inblack) of the amino acid sequences of c-, l- and n-*myc*. Functional domains of the proteins and the locations of PCR primers are indicated.

PCS was Identified by a PCR Strategy Originally Designed to Identify Myc-Like Genes

We began this study from the perspective of cloning and characterizing novel genes which shared some similarities with the c-myc class of helix-loop-helix proteins. Synthetic oligonucleotides corresponding to two highly conserved blocks of amino acids within the c-myc third exon (primers 3 and 6 in Figure 1) were employed in a PCR based cloning strategy. The 3' primer (#6) was initially used to prepare first strand cDNA from poly A+ RNA and the cDNA was subsequently amplified with primers 3 and 6 by PCR. The PCR products were analyzed on a 6% polyacrylamide gel shown in Figure 2. The band corresponding to c-myc

is indicated and was confirmed by DNA sequence analysis following subcloning in pBluescript. Two other major bands smaller than c-myc are also observed amongst the PCR products. The faster of these later two bands is about 220 bp in size and appears to be expressed in the three plasma cell tumors examined (MPC-11, J558 and 7149) and also in BALB/c liver and spleen. This 220 bp species corresponds to a ubiquitously expressed mRNA of ~4.0 kb which encodes a polypeptide with a helix-loop-helix domain (Connelly and Marcu, unpublished results). In contrast, the upper band of about 240 bp was only amplified from the plasma cell tumor mRNAs. The latter PCR product was denoted PCS for plasmacytoma specific and is the subject of this paper. Nucleotide sequence analysis revealed that PCS

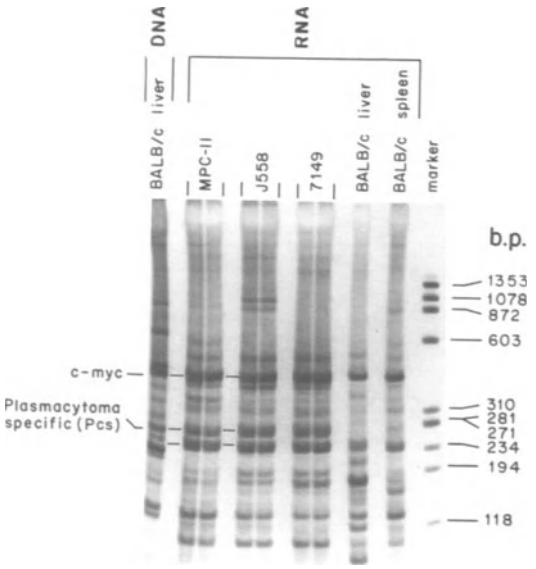


Fig. 2. Analysis of PCR products obtained with c-myc amplimers 3 and 6. The locations of amplimers 3 and 6 within the c-myc third exon are shown in Figure 1. Duplicate 1μg samples of poly A+ RNAs were initially converted into first strand cDNA with AMV reverse transcriptase and primer #6. PCR was then initiated for 5 cycles at 37°C followed by 50 cycles at 55°C with primers 3 and 6 and the resultant amplification products were revealed by ethidium bromide straining.

contained no homology with c-myc or other helix-loop-helix proteins and its amplification resulted from a limited amount of homology with the two PCR amplimers. The PCS PCR product detects a 3.6 kb transcript on Northern blots of the plasma cell tumor RNAs and was subsequently used as a probe to clone a near full length clone from an MPC-11 cDNA library. The 5' end of the MPC-11 PCS cDNA clone contained a 112 bp sequence which was identical to the 3' portion of a variety of IAP proviral LTRs. As expected, the 5' end of the PCS clone corresponding to the IAP LTR sequence was highly repeated in the mouse genome upon Southern blot analysis (data not shown). The remainder of the PCS cDNA's nucleotide sequence revealed a unique open reading frame specifying a 1029 amino acid polypeptide with remarkably strong similarities to a class of proteins

normally involved in neuronal development and axon migration (Figure 3); coupled in vitro transcription/translation revealed a PCS encoded polypeptide with an apparent size of 113 KDa (Connelly et al., manuscript in preparation). Axonal glycoprotein (TAG-1) (Furley et al., 1990) was most related to PCS with the two polypeptides displaying almost 50% identity over their entire length (Figure 3). If conservative amino acid replacements are considered PCS and TAG-1 are 60-70% homologous. TAG-1 contains six amino terminal immunoglobulin-like domains and four carboxy terminal fibronectin-like repeats (Furley et al., 1990). PCS lacks the first three immunoglobulin-like domains of TAG-1.

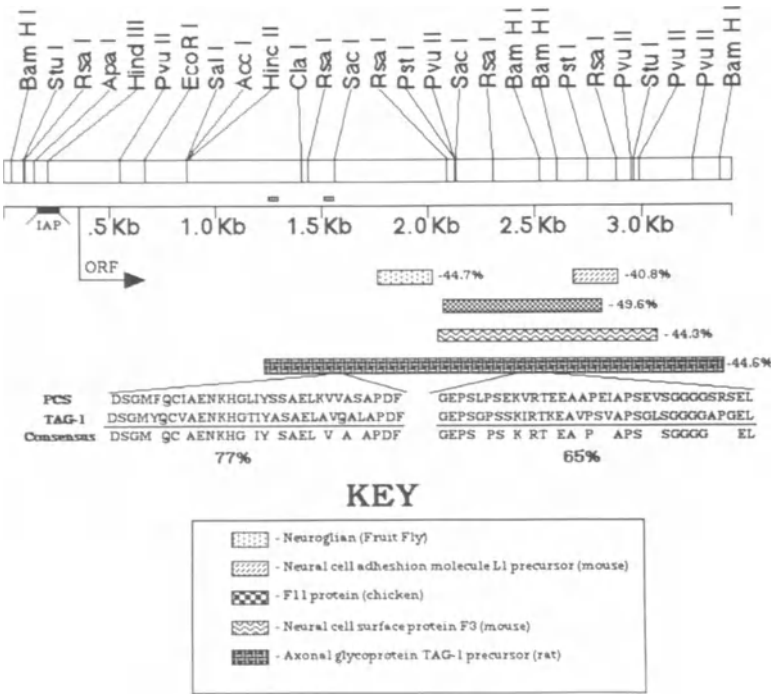


Fig. 3. Structural features of an MPC-11 PCS cDNA clone. The location of a 121 bp IAP LTR sequence, the binding sites of PCR amplimers 3 and 6 and the beginning of the longest open reading frame (ORF) are all indicated just beneath the PCS restriction map. Portions of the PCS ORF bearing significant homologies to several conserved polypeptides associated with neuronal development are indicated. Neuroglian (Bieber et al., 1989), L1 (Moos et al., 1988), F11 (Brümmendorf et al., 1989), F3 (Gennarini et al., 1989), TAG-1 (Furley et al., 1990).

Table I. Survey of PCS expression by Northern blotting

Cell Lines	Cell Type	PCS Expression (kb)
MPC-11	plasma cell tumors	++++(5.0, 3.6, 2.2 kb)
J558	"	++++ (3.6 kb)
7149	"	++++ (3.6 kb)
TEPC 1017	"	++++ (8.3, 5.0, 3.6, 3.0, 1.8 kb)
TEPC 1165	"	+ (3.6 kb)
TEPC 1196	"	++ (3.6 kb)
MOPC 104E	"	++++ (8.3, 5.0, 3.6, 2.2 kb)
ABPC 22	"	++ (3.6 kb)
ABPC 26	"	+/- (3.6 kb)
RIM PC2186	"	-
ABPC 4	"	-
ABPC 20	"	-
ABPC 47	"	-
ABPC 105	"	-
TEPC 1033	"	-
AJ9	mature B	-
WEHI 231	mature B	-
K46	mature B	-
NFS1	mature B	-
18-81	pre-B	-
38B9	pre-B	-
300-18	pre-B	-
70Z3	pre-B	-
FDCP1	myeloid	-
HAFTL3g4	myeloid	-
J2MY2097	myeloid	-
HAFTL1	pro-B	-
BW 5147	mature T	-
EL4	mature T	-
M14T	pre-T	-
MEL	embryonal carcinoma	-
P19	embryonal carcinoma	-
NIH3T3	fibroblast	
54C12	A-MuLV transf. fibroblast	-
Tissues		
Brain		++ (4.0 and 6.0 kb)
Thymus		-
Spleen		-
Heart		-
Lung		-
Kidney		-
Testes		-
Liver		-

Most Murine Plasma Cell Tumors Express PCS but the Brain Appears to be its Normal Site of Expression

Northern blots of a variety of RNAs derived from cell lines and normal tissues were probed with a PCS restriction fragment specifying most of its encoded polypeptide without the 5' IAP sequences. About 30 out of 50 plasma cell tumors examined expressed variable levels of a 3.6 kb PCS RNA and occasionally other larger and smaller related transcripts (see 15 representative cases in Table I). PCS transcripts were not detected in a variety of other normal and transformed cell lines corresponding to pro-B, pre-B, mature B, T, myeloid, erythroid, fibroblast and embryonal carcinoma and stem cell lines (Table I and data not shown). PCS RNAs were also undetectable in normal thymus, heart, lung, kidney, testes, liver and spleen tissues, nor in LPS stimulated splenocytes (Table I and data not shown). However, two larger PCS RNAs of ~4.0 and ~6.0 kb were present in brain RNA.

Potential Significance of PCS Expression for Plasmacytomagenesis

PCS expression in murine plasma cell tumors maybe a consequence of ectopic gene activation. In the MPC-11 tumor, the PCS gene would appear to be activated by an IAP LTR which are known to become transcriptionally active in PCTs (Kuff and Lueders, 1988). Studies in progress will determine if this was a consequence of IAP proviral transposition into the PCS gene and if IAPs are involved in PCS expression in other PCTs. We surmise that the PCS gene is not likely expressed in normal plasma cell since LPS activated splenocytes were negative for PCS transcripts (data not shown) but this would also require direct confirmation. Given the larger sizes of the PCS transcripts in the brain and the consistent presence of a smaller 3.6 kb RNA in PCTs this would appear to be a case of abnormal if not ectopic gene expression in the PCTs. TAG-1, a PCS related glycoprotein, was found to be transiently expressed on the surface of a subset of neurons in the developing mammalian nervous system (Furley et al., 1990). TAG-1 is anchored to neurons via a glycosylphosphatidylinositol (GPI) linkage but also exists as a peripheral membrane protein which is released from neurons. TAG-1 facilitated the extension of neurites in vitro implying that it may play a role in axon growth and guidance in vivo (Furley et al., 1990). TAG-1 was suggested to promote the growth of commissural axons by homophilic interactions amongst its GPI-linked forms or by heterophilic binding to integrins or other axonal receptors. It will be interesting to determine whether the very related polypeptide encoded by the PCS gene also possesses similar properties. A function for PCS in plasmacytomagenesis remains unknown at present, but one can envision provocative roles in the progressive phases of this B cell malignancy. Ectopic PCS expression may contribute to abnormal B cell growth in vivo by enhancing their metastatic potential. Experiments designed to directly assess the effects of enforced PCS expression in normal B cells or in PCS negative B cell lines will hopefully speak to this important issue.

Acknowledgements:

We thank Joyce Schirmir for preparing figures 1 and 2 and Margarita Reyes for manuscript preparation. This research was supported by NCI grant CA36246 awarded to KBM.

References

Biczysko W, Pienkowski M, Solter D, Koprowski H. (1973) Virus particles in early mouse embryos. J. Nat. Canc. Inst. 51:1041-1050

Bieber AJ, Snow PM, Hortsch M, Patel NH, Jacobs JR, Traquina ZR, Schilling J, Goodman CS. (1989) Drosophila neuroglian: a member of the immunoglobulin super family with extensive homology to the vertebrate neural adhesion molecule L1. Cell 59:447-460

Brümmendorf T, Wolff JM, Frank R, Rathjen FG. (1989) Neural cell recognition molecule F11: homology with fibronectin type III and immunoglobulin type C domains. Neuron 2:1351-1361

Calarco PG, Szollosi D. (1973) Intracisternal A-particles in ova and preimplantation stages of the mouse. Nature New Bio. 243:91-93

Canaani E, Klar DA, Rechavi G, Ram D, Cohen JB, Givol D. (1983) Activation of the c-mos oncogene in a mouse plasmacytoma by insertion of an endogenous intracisternal A-particle genome. Pro. Natl. Acad. Sci. USA 80:7118-7122Cohen JB, Unger G, Rechavi E,

Canaani E, Givol D. (1983) Rearrangement of the oncogene c- mos in mouse myeloma NSI and hybridomas. Nature 306:797-799

Cole MD, Ono M, Huang RCC. (1982) Intracisternal A-particle genes: Structure of adjacent genes and mapping of the boundaries of the transcription unit. J. Virol. 42:123-130

Clynes R, Wax J, Stanton LW, Smith-Gill S, Potter M, Marcu KB. (1988) Rapid induction of IgM-secreting murine plasmacytomas by pristane and an immunoglobulin heavy-chain promoter/enhancer-driven c-myc/v-Ha-ras retrovirus. Proc. Natl. Acad. Sci. USA 85:6067-6071

Dalton AJ, Potter M, Merwin RM. (1961) Some ultrastructional characteristics of a series of primary and transplanted plasma cell tumors of the mouse. J. Nat. Cancer Inst. 26:1221-1267

Furley AJ, Morton SB, Manalo D, Karagogeos D, Dodd J, Jessell TM. (1990) The axonal glycoprotein TAG-1 is an immunoglobulin super family member with neurite outgrowth-promoting activity. Cell 61:157-170

Gennarini G, Cibelli G, Rougon G, Mattei M-G, Goridis C. (1989) The mouse neuronal cell surface protein F3: a phosphatidyl-inositol-anchored member of the immunoglobulin super family related to chicken contactin. J. Cell Biol. 109:775-788

Greenberg R, Hawley R, Marcu KB. (1985) Acquisition of an intracisternal A-particle element by a translocated c-myc gene in a murine plasma cell tumor. Mol. Cell. Biol. 5:3725-3728

Hawley RG, Shulman MJ, Murialdo H, Gibson DM, Hozumi N. (1982) Mutant immunoglobulin genes have repetitive DNA elements inserted into their intervening sequences. Proc. Natl. Acad. Sci. USA 79:7425-7429

Jessel TM. (1988) Adhesion molecules and the hierarchy of neural development. Neuron 1:3-13

Kuff EL, Wivel NA, Lueders KK. (1972) Some structural and antigenic properties of intracisternal A-particles occurring in mouse tumors. Proc. Natl. Acad. Sci. USA 69:218-222

Kuff EL, Lueders KK. (1988) The intracisternal A-particle gene family: structure and functional aspects. Adv. Cancer Res. 51:183-276

Lueders KK., Kuff EL. (1977) Sequences associated with intracisternal A -particles are reiterated in the mouse genome. Cell 12:963-972

Marcu KB, Bossone S, Patel AJ. (1992) MYC function and regulation. Ann. Rev. Biochem. 61, in press

Moos M, Tacke R, Scherer H, Teplow D, Fruh K, Schachner M. (1988) Neural adhesion molecule L1 as a member of the immunoglobulin super family with binding domains similar to fibronectin. Nature 334:701-703

Mushinski JF. (1988) C-myc oncogene activation and chromosomal translocation in BALB/c plasmacytomas In: Klein G (ed) Cellular oncogene activation. Dekker, New York, pp 181-222

Ohno S, Babonits M, Weiner F, Spira J, Klein G, Potter M. (1979) Nonrandom chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 18:1001-1007

Ohno S, Migita S, Weiner F, Babonits M, Klein G, Mushinski JF, Potter M. (1984) Chromosomal translocations activating myc sequences and transduction of v-abl are critical events in the rapid induction of plasmacytomas by pristane and Abelson virus.J. Exp. Med. 159:1762-1777

Ono M, Cole MD, White AT, Huang RCC. (1980) Sequence organization of cloned intracisternal A-particle genes. Cell 21:465-473

Potter M. (1972) Immunoglobulin-producing tumors and myeloma proteins in mice. Physiol. Rev. 52:631-719

Potter M, Sklar MD, Rowe WP. (1973) Rapid Viral induction of plasmacytomas in pristane-primed BALB/c mice. Science 182:592-594

Potter M, Mushinski JF, Mushinski EB, Brust S, Wax JS, Weiner F, Babonits M, Rapp UR, Morse HC. (1987) Avian v-myc replaces chromosomal translocation in murine plasmacytomagenesis. Science 235:787-789

Shen-Ong GLC, Cole MD. (1982) Differing populations of intracisternal A-particle genes in myeloma tumors and mouse subspecies. J. Virol. 42:411-421

Troppmair J, Potter M, Wax JS, Rapp UR. (1989) An altered v-raf is required in addition to v-myc in J3V1 virus for acceleration of murine plasmacytomagenesis. Proc. Natl. Acad. Sci. USA 86:9941-9945

Weissinger EM, Mischak H, Largaespada DA, Kaehler DA, Mitchell T, Smith-Gill SJ, Risser R, Mushinski JF. (1991) Induction of plasmacytomas secreting antigen-specific monoclonal antibodies with a retrovirus expressing v-abl and c-myc. Proc. Natl. Acad. Sci. USA 88:8735-8739

Inhibiting IL-6 in Human Multiple Myeloma

B. KLEIN, Z. Y. LU, J. P. GAILLARD, J. L. HAROUSSEAU, and R. BATAILLE

Laboratory for Immunohematological Oncogenesis, Institut de Biologie,
9 Quai Moncousu, 44035 Nantes Cedex 01, France

It has been well established by several groups that IL-6 is overproduced in patients with Multiple Myeloma (MM) and that this cytokine is an essential growth factor of myeloma cells both in vitro and in vivo (1-5). Most investigators also agree now that IL-6 is produced mainly by bone-marrow stromal cells (1,6). The aim of this report was to summarize our recent results concerning the mechanisms of IL-6 production control by the bone-marrow environment and the different means of neutralizing IL-6 activity in vitro and in vivo.

Myeloma Cells Produce IL-1β In Vivo

Several investigators have shown that populations enriched in myeloma cells produce IL-1β (7,8). However, as stromal cells adhere to myeloma cells, it is difficult to be absolutely certain that the latter really produce IL-1β in these studies. Moreover, purification of myeloma cells requires in-vitro manipulations which may lead to activation of IL-1β gene transcription in vitro. To determine whether myeloma cells produce IL-1β in vivo or not, we performed in situ hybridization on freshly explanted myeloma cells. Strong IL-1β gene expression was found in the bone-marrow plasma cells of the eight patients studied (unpublished observations). Interestingly, no IL-1β gene expression was noted in the circulating myeloma cells of patients with plasma-cell leukemia or in 10 human myeloma cell lines. This suggests that IL-1β production by myeloma cells is not due to constitutive activation of the IL-1β gene but to activation of the myeloma cells trapped in the bone-marrow environment.

Adhesion And Activation Antigens On Myeloma Cells

As shown in Figure 1, myeloma cells express a set of adhesion molecules enabling them to be trapped by the bone-marrow environment (unpublished observations). Myeloma cells express ICAM-1 antigen (Ag) but not its ligand the LFA-1 Ag. They bear the NCAM homotypic adhesion molecule, unlike normal plasma cells. Myeloma cells also express several adhesins such, particularly those adhering to fibronectin molecules (VLA-4 and VLA-5). Myeloma cells express TP44 Ag which binds to hyaluronidate and the epithelial membrane antigen (EMA). Concerning activation antigens, myeloma cells have a high density of CD28 Ag but also a low density of CD28-ligand (B7 Ag). Most myeloma cell lines fail to express

CD40 Ag but express CD30 Ag (Ki-1 Ag) which belongs to a large family of activation antigens including nerve growth factor receptor, TNF receptors, the CD40 Ag, Fas Ag, ...etc (9). The role of these various antigens in the reciprocal activation of myeloma cells and their bone-marrow environment is not presently known.

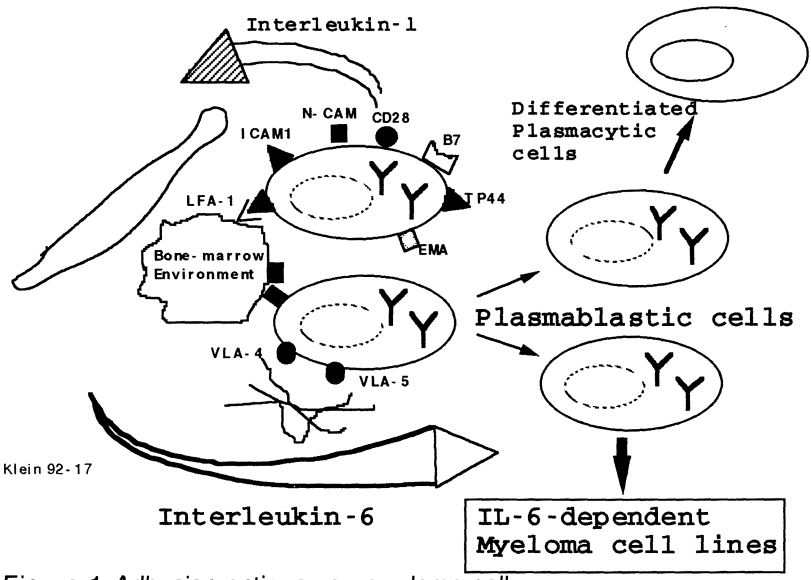


Figure 1. Adhesion antigens on myeloma cells

IL-1 Receptor Antagonist (IL-1RA) And Other Anti-Inflammatory Agents Inhibit IL-6 Production By The Myelomatous Tumoral Environment

IL-6 production was measured in 5-day cultures of bone-marrow cells of 14 patients with MM (unpublished observations). As shown in Figure 2, addition of 100 ng/ml of IL-1RA resulted in a 70% decrease of IL-6 production. The inhibitory effect was reversed by adding an excess of rIL-1β. Increasing IL-1RA concentrations up to 500 ng/ml resulted in almost complete inhibition of IL-6 production in these cultures. Recently, IL-1-induced production of IL-6 in monocytes was shown to be mediated by the PGE-2 synthesis (Lucien Aarden, oral communication). In agreement with these results, we found that the IL-6 production was inhibited 60% by indomethacin and that this inhibition could be reversed by adding synthetic PGE-2. Interleukin-4, a potent anti-inflammatory cytokine (10), also caused 60% inhibition of endogenous IL-6 production. Finally, dexamethasone inhibited IL-6 production by 80%, which is in agreement with the known repression of IL-6 promoter by the glucocorticoid receptor (11). The previously recognized strong efficacy of dexamethasone in treating patients with MM could be partly due to its strong ability to inhibit IL-6 production.

Thus, a variety of means of inhibiting IL-6 production in MM patients is now available. The use of these anti-IL-6 therapies should be of major interest since recent studies have shown that most newly diagnosed patients producing high levels of IL-6 in vivo failed to respond to conventional treatments (12-14). However, one critical question is whether IL-6 can be durably neutralized in vivo. IL-6 has many functions, some of which could be essential in vivo.

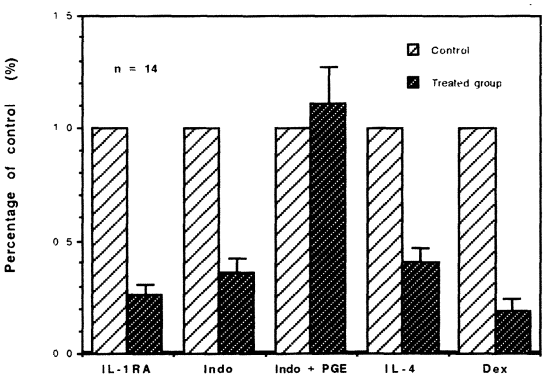


Figure 2. Inhibition of IL-6 production by various agents

Treatment Of MM Patients With Anti-IL-6 Monoclonal Antibodies

Nine patients were treated with anti-IL-6 monoclonal antibodies (mab), including one for two months (5). There was no occurrence of major adverse effects, except for partial thrombopenia; a reduction of several toxicities related to overproduction of IL-6 in vivo (fever, hypercalcemia, acute phase proteins, complement component); 3) and blockage of malignant plasmablastic proliferation in 5 out of the 9 patients. A critical point was whether anti-IL-6 mab had been injected in sufficient amounts to block IL-6 bioactivity in vivo.

Inhibition Of C Reactive Protein (CRP) Production By Anti-IL-6 Treatment

IL-6 is a major inducer of acute phase protein production in human hepatocytes. In particular, we previously reported complete inhibition of CRP production in vivo in a patient treated for 2 months with anti - IL-6 mab (5). Such complete inhibition of CRP production was found in 3 out of 6 patients treated with anti-IL-6 mabs for more than one week (Figure 3). However, in the other 3 patients, CRP inhibition was only partial and was associated with a lack of response to treatment (unpublished

observations). In these 3 patients, other cytokines may have stimulated CRP production and myeloma-cell growth or the anti-IL-6 mab may not have been injected in sufficient amounts to neutralize IL-6 in vivo. It is noteworthy that two other cytokines (leukemia inhibitory factor/HILDA and oncostatin M) are potent inducers of acute phase proteins which share the same transducer receptor chain with IL-6 (15).

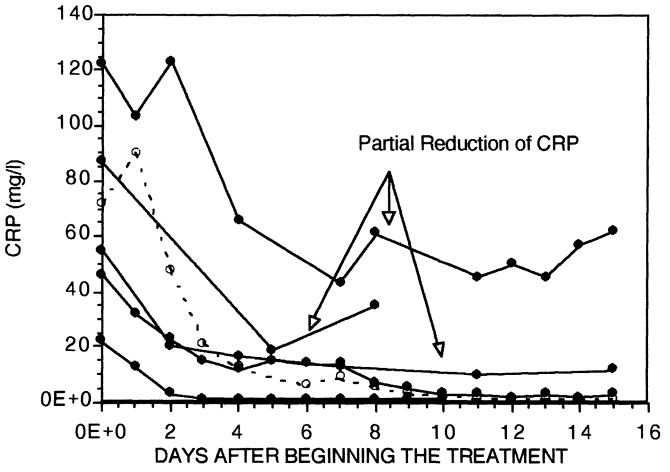


Figure 3. Inhibition of CRP production by anti-IL-6 treatments

Anti-IL-6 Treatment InducesHigh Amounts Of Circulating IL-6 In The Form Of Immune Complexes. Measurement Of Overall IL-6 Production In Vivo and Prediction Of Anti-IL-6 Treatment Efficacy

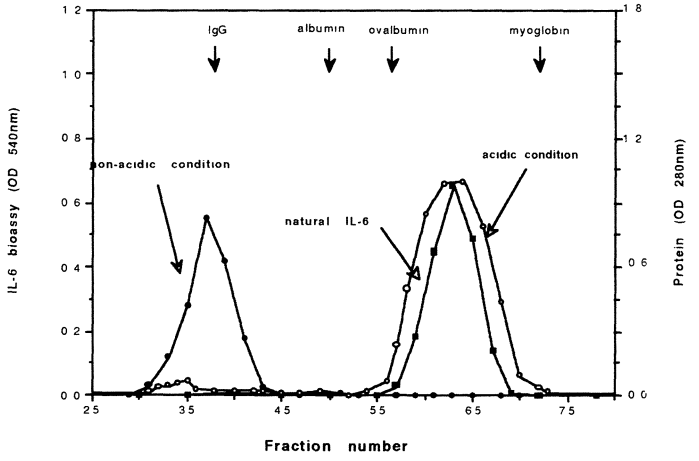


Figure 4. B9-stimulating activity after gel filtration of patient serum in acidic or non-acidic conditions

After discontinuation of anti-IL-6 mab injections, most patients treated with anti-IL-6 mab showed a marked rise in serum activity stimulating the growth of the B9 hybridoma (5). Chromatographies on protein A Sepharose and anti-murine Ig Sepharose demonstrated that this activity was bound to anti-IL-6 mab (unpublished results). As shown in Figure 4, this activity was recovered at a peak of 180 kDa MW after gel filtration. After treatment at acid pH, it eluted at a peak of 25 kDa MW and was then neutralized by different anti-IL-6 mab. Thus, anti-IL-6 mab, by preventing IL-6 binding to its high affinity receptor and renal elimination, induces high amounts of circulating IL-6 in the form of monomeric immune complexes. The accumulation kinetics of these IL-6/anti-IL-6 mab complexes and of free mab at the beginning of anti-IL-6 treatment demonstrated that these immune complexes have the same half-life as free mab (3,5 days), which is in agreement with previous observations about the elimination kinetics of multimeric immune complexes (16).

IL-6 Accumulation In The Form Of Circulating Monomeric Immune Complexes Enables Estimation Of Daily Overall IL-6 Production In Patients Treated With Anti-IL-6 mab In Vivo

As overall in vivo cytokine production has never been estimated in animals or humans, we developed a mathematical model to estimate this factor in our patients treated with anti-IL-6 mab (unpublished observations). The model assumes that (i) the half-life of monomeric immune complexes in vivo is similar to that of free mab (this is verified) and (ii) that there is no other IL-6 elimination route than by immune complexes. The 2 major routes of cytokine elimination are by cell consumption and renal elimination (17).

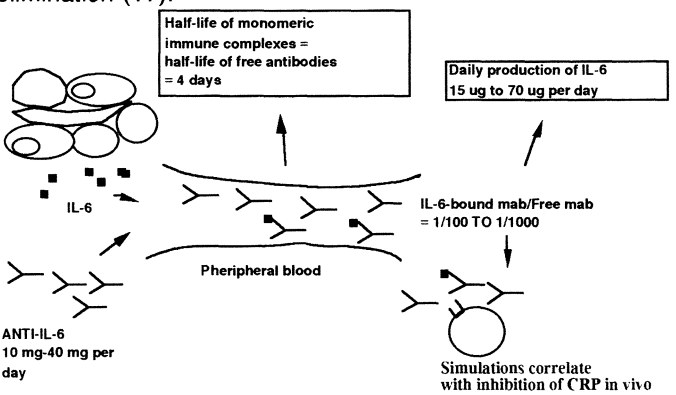


Figure 5. Accumulation of IL-6 in the form of circulating monomeric immune complexes during treatment with anti-IL-6 antibodies

The second assumption is likely in the case of complete CRP inhibition. In the case of partial inhibition, the mathematical model gave only a lower limit of overall in vivo overall IL-6 production. For the six patients studied, estimations of overall IL-6 production ranged from 5 μg/day to > 200 μg/day.

Determination Of The Amounts Of Circulating IL-6/anti-IL-6 Complexes Is Predictive Of Anti-IL-6 Treatment Efficacy

Determination of the ratio of IL-6-bound mab to free mab made it possible to simulate the ability of anti-IL-6 mab to inhibit IL-6 binding to its high affinity receptor. For the four patients studied, this ratio ranged from 1:100 to 1:1000 (Figure 5). Simulations of mab ability to inhibit IL-6 binding to its high affinity receptor indicated that, at a ratio of IL-6-bound mab to free mab of 1:1000, the mab concentration reached in vivo was just sufficient to inhibit IL-6 activity. At higher ratios (1:100), anti-IL-6 mab was unlikely to prevent IL-6 binding to cell surface receptors. Interestingly, these simulations fit well with the CRP inhibitions observed in vivo.
In patients with a measured ratio of IL-6-bound mab to free mab of 1:1000, CRP was undetectable and a significant anti-tumoral effect was found, whereas in patients with a ratio of 1:100, CRP production was only partially blocked.
Thus, the partial neutralization of CRP observed in three patients, in association together with a lack of clear anti-tumoral effect (Figure 3) was probably due to an excessively high production of IL-6 in vivo that could not be neutralized by anti-IL-6 mab.

How Should Be Carried Out Future Treatments With Anti-IL-6 Antibodies?

The circulation of high amounts of IL-6 in the form of monomeric and stable immune complexes is a major limitation of anti-IL-6 treatments using a single antibody. It is likely that circulation mobilizes high levels of IL-6 close to tumor subclones that are hypersensitive to IL-6. As indicated above, anti-IL-6 mab concentration, reached in vivo in the best cases, was just sufficient to neutralize IL-6 activity. This might explain why, in a patient, tumor cell proliferation was not more inhibited after 2 months of treatment whereas CRP production was still undetectable (5).
Improvement of these treatments with anti-IL-6 mab will be to reduce the half-life of IL-6/anti - IL-6 mab complexes. Previous studies in the literature using stabilized hapten/anti-hapten complexes indicated a considerable reduction in the half-life of polymeric as compared to monomeric immune complexes. Thus, it is essential to determine in an animal model whether this is also true for polymeric IL-6/anti-IL-6 complexes. The concept in this paper should be generalizable to a treatment design with anti-cytokine molecules whose target cytokine is known to circulate in a monomer form.

Conclusion

There are now many clinically available molecules that can be used to inhibit IL-6 in vivo in MM patients: IL-1 receptor antagonist, IL-4, non-steroid anti-inflammatory agents, corticosteroids, and anti-IL-6 antibodies. Other molecules could become clinically available such as mutated IL-6 or anti-IL-6 receptor antibodies. As MM patients with high in vivo IL-6 production have been shown to respond poorly to the usual treatments or to autologous bone-marrow transplantation, these anti-IL-6 therapies could be very useful either alone or in combination with chemotherapy.

References

1. Klein B, Zhang X G, Jourdan M, Content J, Houssiau F, Aarden L A, Piechaczyk M, and Bataille R (1989). Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73: 517.

2. Kawano M, Hirano T, Matsuda T, Taga T, Horii Y Iwato k, Asaoku H, Tang B, Tanabe O, Tanaka H Kuramato A, and Kishimoto T (1988). Autocrine generation and requirement of BSF-2/IL-6 for human Multiple Myelomas. Nature 332: 83.

3. Anderson K C, Jones R M, Morimoto C, Leavitt P, and Barut B A, (1989). Response patterns of purified myeloma cells to hematopoietic growth factors. Blood 73 ; 1915.

4. Nilsson K, Jernberg H, and Pettersson M (1990). IL-6 as a growth factor for human myeloma cells - a short overview. Current Topics of Microbiology and Immunology, vol. 166 F. Melchers, and M Potter (eds). Springer-Verlag, Berlin-Heidelberg, p 3.

5. Klein B, Wijdenes J, Zhang X G, Jourdan M, Boiron J M, Brochier J, Liautard J, Merlin M, Clement C, Morel-Fournier B, Lu Z Y, Mannoni B, Sany J, and Bataille R (1991). Murine Anti-Interleukin - 6 Monoclonal Antibody Therapy for a Patient With Plasma Cell Leukemia. Blood 5: 1198.

6. Portier M, Rajzbaum G, Zhang X G, Attal M, Rusalen C, Wijdenes J, Mannoni P, Maraninchi D, Piechaczyk M, Bataille R, and Klein B (1991). In vivo interleukin-6 gene expression in the tumoral environment in multiple myeloma. Eur J Immunol 21: 1759.

7. Carter A, Merchav S, Silvian-Draxler I, (1990) The role of interleukin-1 and tumor necrosis factor-α in human multiple myeloma. Br Haematol J 74: 424.

8. Cozzolino F, Torcia M, Aldinucci D, (1989). Production of interleukin-1 by bone-marrow myeloma cells. Blood 74: 380.

9. Dürkop Horst, Latza Ute, Hummel Michael, Eitelbach Florian, Seed Brian, and Stein Harald (1982). Molecular cloning and expression of a new member of the nerve growth factor receptor family that is characteristic for hodgkin's disease. Cell 68: 421.

10. Gibbons R, martinez O, Matli M, Heinzel F, Bernstein M, andWarren R (1990). Recombinant IL-4 inhibits IL-6 Synthesis by Adherent Peripheral Blood Cells In Vitro. Lymphokine Res. 93:283.

11. Waage A, Slupphaug G, and Shalaby R (1990). Glucocorticoids inhibit the production of IL-6 from monocytes, endothelial cells and fibroblasts. Eur J Immunol, 20: 2439-2443..

12. Bataille R, Jourdan M, Zhang X G, and Klein B (1989). Serum Levels of Interleukin-6, a Potent Myeloma Cell Growth Factor, as a Reflect of Disease Severity in Plasma Cell Dyscrasias. 84: 2008.

13. Bataille R, Boccadoro M, Klein B, Durie B, Pileri A. (1992). C-Reactive Protein and B-2 Microglobulin Produce a Simple and Powerful Myeloma Staging System. Blood, In press.

14. Reibnegger G, Krainer M, Herold M, Heinz L, Wachter H, and Heinz H (1991). Predictive Value of Interleukin-6 and Neopterin in Patients with Multiple Myeloma. Cancer Res 51: 6250.

15. Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasher KK, King JA, Gillis S, Mosley B, Ziegler SF, Cosman D. (1992) The IL-6 Signal Transducer, gp 130. An Oncostatin M Receptor and Affinity Converter for the LIF Receptor. Science 255:1434.

16. Plotz PH, Kimberly RP, Guyer RL, and Segal DM. (1979). Stable model immune complexes produced by bivalent affinity labeling haptens: in-vivo survival. Mol Immunol, 16: 721.

Pathophysiology of Human Multiple Myeloma – *Recent* Advances and Future Directions

B. BARLOGIE

Division of Hematology-Oncology and Arkansas Cancer Research Center,
University of Arkansas for Medical Sciences, Little Rock, Arkansas

Fundamental research in human myeloma (MM) has addressed the tumor cell hierarchy, [1] growth regulation by normal host cells [2] and a plethora of cytokines, [3] specific disease manifestations resulting directly from tumor growth or indirectly by way of tumor or normal cell secretory products, [4] and the area of genetic abnormalities [5] with the consequences of oncogene activation and tumor suppressor gene inactivation [6] (see Fig. **1**).

Tumor Cell Hierarchy

Consensus exists that the prevalent plasma cell phenotype is maintained by a more immature precursor compartment at the pre-B or even more primitive hemopoietic stem cell stage. [1,7,8] The origin of tumor stem cells from bone marrow, lymph nodes [9] or even spleen has become a subject of debate. [10] Circulating monoclonal tumor cells exist in the peripheral blood, [11,12,13,14] but conclusive evidence as to the pre-myeloma cell nature of cells expressing CD11b, CD45RA/RO and possibly MDR-1 is still lacking.

Future investigations should emphasize genetic and clonality studies of tumor cells with a discrete phenotype along with in vitro response to proliferative and differentiative stimuli.

Cytokine Network

Controversy continues regarding autocrine and paracrine growth loops especially involving the IL-6 molecule. [15,16,17] While several investigators were able to confirm proliferative responses in vitro upon IL-6 exposure of advanced disease with intrinsically high proliferative activity, [18] others were unable to observe tumor cell doubling or enhanced DNA synthesis upon exposure to IL-6. (S.M. Hsu, unpublished observations). Autologous bone marrow stromal cell layers, on the other hand, have proven effective for the growth and expansion of clonal B cells and terminal plasma cell differentiation from peripheral blood mononuclear cells. [2] Current research in several laboratories emphasizes the response of phenotypically discrete blood and bone marrow tumor cells to defined stromal cell populations and to recombinant cytokines alone or in combination. The demonstration of IL-6 transcripts and protein supports the original observation by the Kishimoto group of an IL-6 autocrine loop, [17] although its role in tumor cell self-renewal and/or differentiation has yet to be further investigated.

Apoptosis can be induced by dexamethasone in ARP-1 cells (a recently established human MM cell line), [19] probably as a result of down-regulation of endogenous IL-6 production,

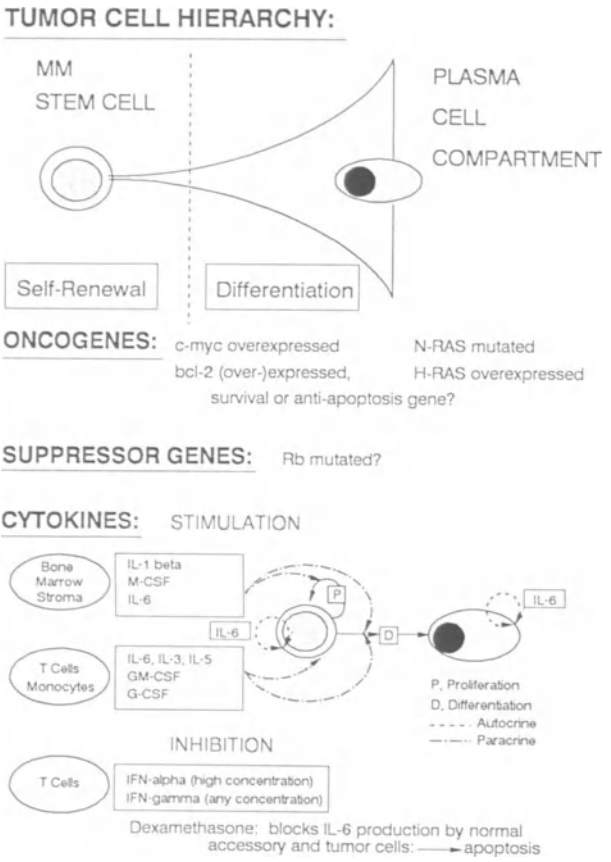


Fig. 1 Biology of Multiple Myeloma

a) Tumor cell hierarchy:
An exceedingly small tumor cell renewal compartment of undetermined phenotype gives rise to the prevalent myeloma plasma cell compartment at the terminal stage of B cell differentiation, exhibiting monoclonal cytoplasmic immunoglobulin and low proliferative activity (median plasma cell labelling index, 1%).

b) Oncogenes and tumor suppressor genes:
C-myc RNA is overexpressed in about 25% of myeloma cases, with high myc protein expression present in about 80%. N-ras is mutated in one-third of cases; p21 (H-ras protein) is strongly expressed in about 75% of patients. As in normal plasma cells, BCL-2 protein is expressed by the majority of myeloma cells of most patients. Monosomy 13 is frequently observed cytogenetically and some investigators have observed mutation of the retinoblastoma gene located on chromosome 13.

c) Cytokines in myeloma growth and differentiation:
IL-6 purportedly the major myeloma growth factor through autocrine and paracrine growth loops. It is not clear whether IL-6 may also induce tumor cell differentiation to the plasma cell stage. Bone marrow stromal cells, monocytes and T cells provide additional stimulatory molecules such as IL-1-ß, M-CSF, IL-3, IL-5, GM-CSF and G-CSF which exert growth stimulation directly and/or indirectly through an IL-6 mechanism. The major growth-inhibitory signal is provided by interferon-gamma. The therapeutic activity of dexamethasone may involve inhibition of IL-6 production by normal accessory cells and myeloma cells, leading to tumor cell apoptosis, which can be prevented in vitro by addition of IL-6. For further details, see text.

which can be prevented by co-incubation with IL-6 (J Epstein, unpublished observations). Thus, in the absence of enhanced tumor cell proliferation upon IL-6 exposure of ARP-1 cells, this cytokine may have an important role in maintaining myeloma cell survival.Several other cytokines exert, under special in vitro conditions, stimulation of tumor cell DNA synthesis (IL-3, IL-1, GM-CSF, G-CSF) partly mediated through IL-6; growth inhibition is observed with interferon (IFN), especially IFN-gamma and high doses of IFN-α. [3] Several of the clinical manifestations of MM can be traced to excessive elaboration of cytokines by tumor and host cells, the genetic basis of which however remains to be clarified. [4]

Genetic Alterations

Cytogenetic studes have been difficult to perform because of the low tumor mitotic yield associaed with low proportion of myeloma plasma cells engaged in DNA synthesis (median, 1%). [20,21] The use of fluorescently labelled chromosome-specific DNA probes permits the detection of both numeric and structural aberrations in interphase cells. [22]

Karyotypic abnormalities, present in about 30% of subjects evaluated, are very complex in marked contrast to often single structural or numeric aberrations in other malignancies of the hemopoietic system. [23,24,25] This is not surprising when one considers the long latency phase of 15 to 20 years observed among survivors of the atomic bomb who develop MM. [26] Myelomagenesis is probably different in the typically elderly population (>60 years) and in the rare younger patient under age 40.

Recent cytogenetic studies in newly diagnosed patients showed co-segregation of certain abnormalities such as monosomies 13 and 16, in addition to partial or complete deletions of chromosome 17p (the site of the p-53 tumor suppressor gene). [27] Lymphoma-like translocations have been observed in MM, e.g. t(8;14), t(14;18) and t(11;14). However, these account for only about 5% of cytogenetic aberrations and are infrequently associated with the expected oncogene rearrangements such as myc, bcl-2 and bcl-1, respectively. [6,28,29] High myc RNA message has been reported in one-quarter of MM patients, [28] and myc protein is detectable in about 90% using immunocytochemistry with monoclonal antibody 154. [30] Correlations with proliferative activity were not observed. The mechanism of myc activation in MM remains obscure, although more recent studies have demonstrated a higher incidence of rearrangements and abnormal RNA transcripts (R Hoover, unpublished observations). One-third of cases display mutated N-ras, [31] and about 80% have elevated levels of p21 (H-ras product). [32] The bcl-2 gene product is present in almost all cases examined and may, like in CLL, provide for tumor cell longevity. [33,34] The presence of monosomy 13 and of deletions of 17p [27] have stimulated research into abnormalities of Rb and p53 tumor suppressor genes, respectively.

At present, abnormalities in MM growth control and disease manifestations have not yet been traced to specific genetic events. Given the consistent involvement of c-myc and ras oncogenes as well as high levels of bcl-2, the consequences of these molecular abnormalities for the expression and production of cytokines and their receptors should prove fruitful. Interactions should be examined between the expression of c-myc, ras, and bcl-2 and the constitutive production of cytokines including IL-6, which in turn may modulate the expression and function of oncogenes and suppressor genes. The high frequency of DNA

aberrations detectable by flow cytometry, also among subjects with benign monoclonal gammopathy (BMG), suggests that cytogenetic studies in BMG should be especially informative for early genetic events. [35]

Lessons from the Clinic

Comparative research of B cell malignancies presenting with distinctly different tumor phenotypes should be rewarding. Thus, parallels and differences in tumor genetics and cytokine growth mechanisms have been reported for MM and CLL (Table). [26] Similar considerations apply to MM and immunoblastic lymphoma, a phenotype acquired frequently during the terminal stage of plasma cell myeloma. [36]

Different clinical presentations of plasma cell dyscrasias hold important clues to the understanding of the pathophysiology. [37] This applies especially to the syndrome of monoclonal gammopathy of undetermined significance (MGUS) or BMG with an annual progression to overt MM of about 1%, or to solitary plasmacytoma (SP) of bone or soft tissue which can be cured in 30 to 50%. Hence, lack of progression of MGUS or failure to develop systemic disease after SP should provide crucial insight into both tumor-intrinsic features and defects in immunosurveillance associated with the development of overt MM.

The secrets of tumor biology and pathophysiology can also be unveiled from therapeutic investigations. Thus, the potential for high doses of glucocorticoids to induce remissions in about one-half of subjects with overt MM [38] provides for unique opportunities in studying possible alterations of gene expression, especially concerning the production of cytokines and their receptors. [39,40] The prevention of DEX-induced tumor cell apoptosis by IL-6 in vitro may represent a novel mechanisms of resistance in vivo.[19] Furthermore, escape from glucocorticoid-inducible downregulation of tumor growth genes may unravel the molecular mechanisms associated with disease progression.

The potential of intensive alkylating agent and radiation therapy to induce durable complete remissions with reconstitution of normal T and B cell functions offers opportunities for investigating biological features permissive of cure. [41,42] Comparative studies of autologous bone marrow versus peripheral blood stem cells can contribute to an understanding of the relevance of circulating tumor cells in MM. Switches in immunoglobulin heavy and light chains have been observed in 15 to 20% of patients following intensive cytotoxic therapy both with autologous and allogeneic transplants; the underlying mechanism is yet to be elucidated. [43] Disease recurrence especially from complete remission, obtained in up to 50% of patients receiving intensive therapy with hemopoietic stem cell support, provides the unique opportunity for studying the molecular and biological events associated with disease progression.

References

1. Epstein J, Xiao H and He X Y (1990) Markers of multiple hematopoietic-cell lineages in multiple myeloma. N Engl J Med: 664-668.
2. Caligaris-Cappio F, Bergui L, Gregoretti MG, Gaidano G, Gaboli M, Schena M, Zallone AZ, Marchisio PC (1991) Role of bone marrow stromal cells in the growth of human multiple myeloma. Blood 77: 2688-93.
3. Klein B and Bataille R (1992) Cytokine network in human multiple myeloma. In: Hematology/Oncology Clinics of North America. Barlogie, B. (ed.), W.B. Saunders Company, Philadelphia, p. 273-284.
4. Bataille R, Chappard D and Klein B (1992) Mechanisms of bone lesions in multiple myeloma. In: Hematology/Oncology Clinics of North America. Barlogie, B. (ed.), W.B. Sanders Company, Philadelphia, p. 285-295.
5. Durie B (1992) Cellular and molecular genetic features of myeloma and related disorders. Barlogie, B. (ed.), W.B. Sanders Company, Philadelphia, p. 463-477.
6. Barlogie B, Epstein J, Selvanayagam P and Alexanian R (1989) Plasma cell myeloma - new biologic insights and advances in therapy. Blood 73:865-79.
7. Epstein J, Barlogie B, and Alexanian R (1988) Phenotypic heterogeneity in aneuploid multiple myeloma indicates pre-B cell involvement. Blood 71:861-65.
8. Grogan TM, Durie BG, Spier CM, Richter L, Vela E (1989) Myelomonocytic antigen positive multiple myeloma. Blood 73:371-84.
9. MacLennan ICM, Chan EYT (1991) The origin of bone marrow plasma cells. Epidemiology and biology of multiple myeloma. (Obrams GI, Potter M eds) Berlin, Springer-Verlag, p. 129.
10. Pilarski L and Jensen G (1992) Monoclonal circulating B cells in multiple myeloma: A continuously differentiating, possibly invasive, population as defined by expression of CD45 isoforms and adhesion molecules. In: Hematology/Oncology Clinics of North America. Barlogie, B. (ed.), W.B. Sanders Company, Philadelphia, p. 297-322.
11. Barlogie B and Alexanian R (1986) Cellular aspects of myeloma: Biologic and clinical implications. In: Multiple myeloma and other paraproteinemias. I.W. Delamore (ed), Churchill Livingstone, Edinburgh, pp. 154-168.
12. Bast E, Van Camp B, Reynaert P, Wirniga G, Ballinx R (1982) Idiotypic peripheral blood lymphocytes in monoclonal gammopathy. Clin Exp Immunol 47:677-82.
13. Berenson J, Wong R, Kim K, Brown N, Lichtenstein A (1987) Evidence of peripheral blood B lymphocyte but not T lymphocyte involvement. Blood 70:1550-1553.
14. Pilarski LM, Mant MJ, Reuther BA (1985) Pre-B cells in peripheral blood of multiple myeloma patients. Blood 66:416.
15. Kawano M, Hirano T, Matsuda T, et al (1988) Autocrine generation and requirement of BSF-2/KL-6 for human multiple myelomas. Nature 332:83-85.
16. Klein B, Zhang XG, Jourdan M, et al (1989) Paracrine but not autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73:517-26.
17. Epstein J: Myeloma Phenotype (1992) Clues to disease origin and manifestation. In: Hematology/Oncology Clinics of North America. Barlogie, B. (ed.), W.B. Saunders Company, Philapelphia, p. 249-256.
18. Bataille R, Jourdan M, Zhang XG, Klein B (1989) Serum levels of interleukin-6, a potent myeloma cell growth factor, as a reflection of disease severity in plasma cell dyscrasias. J Clin Invest 84:2008.
19. Hardin JW, MacLeod SL, Hsu SM, Epstein J and Barlogie B (1992) IL-6 prevents dexamethasone (DEX)-induced apoptosis in a human myeloma cell line (ARP-1). Proc 83rd Annual Mtg/AACR, 33:1674a.
20. Latreille J, Barlogie B, Dosik G, Johnston D, Drewinko B and Alexanian R (1980) Cellular DNA content as a marker of human multiple myeloma. Blood 55:403-08.
21. Durie BGM, Salmon SE, Moon TE (1980) Pretreatment tumor mass, cell kinetics and prognosis in multiple myeloma. Blood. 55:364-372.
22. Dao D, Epstein J, Barlogie B and Sawyer JR (1992) Interphase cytogenetics in multiple myeloma. Proc 83rd Annual Mtg/AACR, 33:1594a.

23. Dewald GE, Kyle RA, Hicks GA, Greipp PR (1985) The clinical significance of cytogenetic studies in 100 patients with multiple myeloma, plasma cell leukemia or amyloidosis. Blood 66:380-390.
24. Gould J, Alexanian R, Goodacre A, Pathak S, Hecht B, Barlogie B (1988) Plasma cell karyotype in multiple myeloma. Blood 71:453-56.
25. Lewis JP, MacKenzie MR (1984) Non-random chromosomal aberrations associated with multiple myeloma. Hematol Oncol 2:307.
26. Barlogie B and Gale RP (1992) Multiple myeloma and chronic lymphocytic leukemia: Parallels and contrasts. Am Jour Med, in press.
27. Sawyer JR, Thomas EL and Barlogie B (1990) Identification of new chromosomal breakpoints in multiple myeloma (MM) by high resolution (HR) cytogenetic (CG) analysis. Blood 74:1474a.
28. Selvanayagam P, Blick M, Narni F, et al (1988) Alteration and abnormal expression of the c-myc oncogene in human multiple myeloma. Blood 71:30-35.
29. Selvanayagam P, Goodacre A, Strong L, Saunders G and Barlogie B (1987) Alterations of bcl-1 oncogene in human multiple myeloma. Proc AACR 28:76a.
30. Greil R, Fasching B, Loidl P, Huber H (1991) Expression of the c-myc proto-oncogene in multiple myeloma and chronic lymphocytic leukemia: An in situ analysis. Blood 78:180-91.
31. Neri A, Murphy JP, Cro L, Ferrero D, Taraella C, Baldini L and Dalla-Favera R (1989) Ras oncogene mutation in multiple myeloma. J Exp Med 170:1715-25.
32. Tsuchiya H, Epstein J, Selvanayagam P, Dedman J, Gallick G, Alexanian R and Barlogie B (1988) Correlated flow cytometric analysis of H-ras p21 and nuclear DNA in multiple myeloma. Blood 72:796-800.
33. Durie BGM, Mason DY, Giles F, et al (1990) Expression of the bcl-2 oncogene protein in multiple myeloma. Blood 74:374a.
34. Hamilton MS, Barker HF, Ball J, Drew M, Abbot SD, Franklin IM (1991) Normal and neoplastic human plasma cells express bcl-2 antigen. Leukemia 5:768-771.
35. Barlogie B, Alexanian R, Pershouse M, Smallwood L, Smith L (1985) Cytoplasmic immunoglobulin content in multiple myeloma. J Clin Invest 76:765-69.
36. Barlogie B, Smallwood L, Smith T, Alexanian R (1989) High serum levels of lactic dehydrogenase identify a high-grade lymphoma-like myeloma. Ann Int Med 110:521-25.
37. Barlogie B, Alexanian R, Jagannath S (1992) Plasma cell dyscrasias. In: JAMA primer on allergic and immunologic diseases. DeShazo R (ed), in press.
38. Alexanian R, Dimopoulos M and Barlogie B (1991) Intermittent dexamethasone as initial chemotherapy for multiple myeloma. Blood 78:688a.
39. Kishimoto T. The biology of interleukin-6 (1989) Review article. Blood 74:1-10.
40. Ray A, LaForge S, Sehgal PB (1990) On the mechanism for efficient repression of the interleukin-6 promoter by glucocorticoids: Enhancer, TATA Box and RNA start site occlusion. Mol Cell Biol 10:5736-5746.
41. Jagannath S, Barlogie B, Dicke K, et al (1990) Autologous bone marrow transplantation in multiple myeloma: identification of prognostic factors. Blood 76:1860-1866.
42. Gahrton G, Tura S, Ljungman P. (1991) Allogeneic bone marrow transplantation in multiple myeloma. N Engl J Med 325:1267-73.
43. Barlogie B, Dicke KA and Alexanian R (1988) High dose melphalan for refractory myeloma-the M.D. Anderson Experience. Hematol Oncol 6:167-172.

An Exceptional Mouse Plasmacytoma with a New Kappa/N-myc [T(6; 12) (C1; B)] Translocation Expresses N-myc But Not c-myc

S. Silva, Y. Wang, M. Babonits, H. Axelson, F. Wiener and G. Klein

Department of Tumor Biology, Karolinska Institutet, Box 60400,
S-104 01 Stockholm, Sweden.

SUMMARY

Mouse plasmacytomas (MPC) carry one of three reciprocal translocations that juxtapose c-myc to one of the three immunoglobulin (Ig) loci. Here we describe an exceptional MPC, induced by pristane oil and Abelson (A-MuLV) virus. It does not carry any of the three c-myc/Ig translocations, but contains a previously unknown reciprocal T(6;12) translocation affecting the bands known to carry the IgK (6C/1) and N-myc (12B) loci, respectively. Northern blot analysis showed high N-myc but no c-myc expression. This is consistent with the constitutive activation of N-myc by a juxtaposition of the IgK and N-myc loci. Reciprocal translocation in B-cell derived tumors are believed to involve the Ig loci by the action of some enzyme that participates in the physiological rearrangement of the Ig loci. Only transcriptionally active chromatin regions are accessible to such recombinases (Alt et al. 1987). N-myc is not expressed in B-cells, but it is transcriptionally active during the early pro- and pre-B cell stage, whereafter it and the surrounding chromatin region becomes inactive (Smith et al. 1992). It is therefore most likely that the N-myc/Kappa translocation has arisen at an early stage of B-cell differentiation. This would imply that the myc/Ig translocations do not block B-cell differentiation. They also reaffirm the functional equivalence of N- and c-myc in relation to B-cell carcinogenesis, as shown by our previous work on tumor induction in N-myc transgenic mice (Wang et al. 1992).

INTRODUCTION

Intraabdominal injection of pristane oil can induce MPCs in BALB/c mice after latency periods of 180 to 360 days. The infection of pristane-primed BALB/c mice with Abelson virus can shorten the latency period to 35 to 70 days after the virus (Potter et al. 1973).

Current Topics in Microbiology and Immunology, Vol. 182

Similarly to rat immunocytomas (RIC) and human Burkitt lymphoma (BL) MPCs carry one of the three translocation that juxtapose the c-myc gene to one of the three Ig loci. This brings c-myc under the control of the linked Ig locus, leading to the constitutive activation of the gene.

More than 85 % of the pristane induced plasmacytomas (TEPCs) were found to contain the typical T(12;15) translocation, generated by the breakage of chromosome 15, 5' of the c-myc gene or within its first intron. As a result, the distal segment of chromosome 15 is juxtaposed to IgH sequences, in a head to head orientation. In the variant T(6;15) translocation, the kappa constant region is transposed to the tail end of the pvt-1 locus, located ca. 100-300 kb downstream of the c-myc, as a rule. Recently, we have identified the previously MPC-missing lambda/myc T(15;16) translocation that has a similar geometry.

Facsimile experiments with Emu-c-myc transgenic mice have proven that the IgH-enhancer-activated myc gene can generate B- and pre-B-cell lymphomas (Adams et al. 1985, Harris et al. 1988). We have found that Abelson virus or pristane + Abelson virus treatment of Emu-c-myc mice triggers the development of plasmacytomas in approximately 42 % of the mice. Another 58 % develop B and pre-B lymphomas (Sugiyama et al. 1990). In another type of facsimile experiment, retrovirally activated avian v-myc constructs were introduced into pristane treated BALB/c mice. This has led to the appearence of translocation-free plasmacytomas after a short latency period (Potter et al. 1987). The fact that the translocation related Ig/myc juxtaposition is obviated by the introduction of constitutively activated myc constructs is consistent with the tumorigenic role of the translocation.

All MPC, BL and RIC associated translocations so far investigated were found to have displaced c-myc, to the exclusion of all other oncogenes, including other members of the c-myc family. Since the c-myc carrying chromosome can break at many different sites (upstreams or downstreams of the gene, outside the gene or within the first intron), and since no homologous recombination is involved, it was assumed that chromosome breaks at random. Tumorigenic translocations are generated, according to this hypothesis, by the accidental joining of intact myc coding exons and constitutively active Ig sequences (Calame et al. 1982, Klein & Klein. 1985). Our recent studies on Emu-N-myc transgenic mice (Wang et al. 1992) have shown that activated c- and N-myc can perform the same carcinogenic function within the B-cell series. The lack of N-myc involvement in the Ig/myc translocations was therefore probably due to the inactivity of N-myc and the surrounding chromatin region in B-cells (Smith et al. 1992). This should be in line with Alt's postulate that only transcriptionally active genes are available to normal and, by inference, pathological recombinase action.

In this paper, we describe an exceptional pristane + Abelson virus induced MPC that did not contain any of the three c-myc/Ig translocations. Instead, it carried a novel T(6;12)(C1;B) translocation. The breakpoints corresponded to the localization of the kappa and N-myc genes, respectively (Swan et al. 1979, Klett et al. 1991). The tumor expressed high levels of N-, but no c-myc, suggesting that it has arisen by N-myc/kappa juxtaposition.

MATERIAL AND METHODS

MICE. MPCs were induced in (C4.12xCB-20)F_1 hybrids, derived from the crossing of BALB/c4.12 and CB-20, a BALB/c congenic stocks. The BALB/c4.12 subline was established in our department by introducing the Robertsonian 4.12 (Rb4.12) chromosome from the Wild 'Alp' Rb(4.12)9Bnr after repeated backcrosses (N:10) into the BALB/cAnPt strain. The mice were bred and housed in our department under conventional conditions.

PLASMACYTOMA INDUCTION. One of the experimental groups was treated twice with 0.5 ml of pristane at 2 months intervals, followed by infection with helper free A-MuLV 2 - 3 weeks after the second injection of pristane, administered in the form of 0.5 ml (2 x 10^5 ffu/ml) of filtered supernatant obtained from the psi-2pAB4 cell line as described by Sugiyama et al. (1989).

PLASMACYTOMA DIAGNOSIS. One of the 21 mice that have developed MPC in a total group of 54 as signaled by the appearence of ascites, was punctured with a 25-gauge needle attached to a 2-ml syringe. Several drops of fluid were collected and diluted with buffer salt solution (BSS) containing 5% fetal calf serum (FCS). Cytosmears were stained with May-Grünwald-Giemsa. MPC was diagnosed according to the criteria described by Silva et al. (1991). It was further confirmed by transplanting the cells into pristane treated syngeneic mice, followed by the confirmation of the histopathological diagnosis. Metaphase plates were prepared from the ascites of the tumor bearing mice. G-banded chromosomes were identified by the criteria of the Committee on Standardized Genetic Nomenclature for mice (1972). The supernatants of short term (24 hrs) cultured cells were assayed for immunoglobulins by the Outcherlony method.

SOUTHERN AND NORTHERN BLOTTING. Ascites cells and tumor tissue from primary tumors, were used to prepare high molecular weight DNA and total RNA according to methods described by Sambrook et al. (1989). Southern blotting was performed as described by Pear et al. (1988). Liver and spleen of an untreated BALB/c mouse, TEPC-HI-24, a cell line established from a pristane induced plasmacytoma, Eu-mycKBC-3, an Eu-myc spontaneous lymphoma that developed in a (Eu-c-myc X AKR) mouse (BC-3) were used as control. Samples of 10 ug of total RNA were separated on 1.2% agarose-formaldehyde gels and transferred to Hybond-N membranes (Amersham). Hybridization was performed by using the following ^{32}p labelled probes: -) Pmc-myc 54 contains a 2.2 kb c-myc cDNA fragment (Stanton et al. 1983), -) the N-myc cDNA probe kindly provided by Dr C Cepko (Harvard University), contains part of murine N-myc exon 1, exon 2 and exon 3, -) PMN-7 is a SmaI/BamHI genomic fragment begining upstream of exon I and extending 150 bp into this exon (DePinho et al. 1986), -) K2abl, a BglII fragment spanning nucleotides 2546-3991 of the Abelson proviral genome which was a kind gift from Dr A. Srinivasan (Reddy et al. 1983), -) the murine alpha-actin probe is a 0,9 kb PstI cDNA fragment (Minty et al. 1981).

RESULTS

MPC-DEVELOPMENT. One of the tumors, ABPC(C4.12xCB20)132 (hereafter referred as ABPC-132) originated in a male 110 days after the first pristane injection and 29 days after A-MuLV infection.

CYTOGENETIC ANALYSIS. ABPC-132 did not contain any of the known translocations. It carried a novel translocation instead, generated by the reciprocal exchange of one chromosome 6 with the short arm of the Rb4.12. The segment of chromosome 6 between 6C2 and 6D was deleted and the segment distal to 6D was attached to chr 12. Chromosome 12 was severed at band B, leading to the juxtaposition of the distal segment to 6C1 (Fig 1). In addition to the translocation, all metaphase plates contained an extra copy of chromosome 11. There were no other regular chromosome anomalies and no noticeable changes at the usual c-myc/Ig translocation breakpoints.

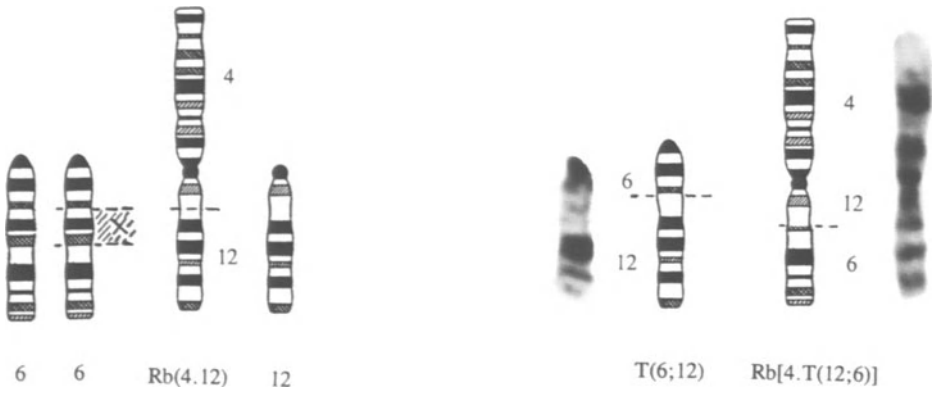


Fig. 1. Selected G-Banded chromosomes involved in the reciprocal T(6;12)(C1;B) translocation.

IMMUNOGLOBULIN SECRETION. ABPC-132 did not secrete any detectable immunoglobulins.

MOLECULAR ANALYSIS. The total RNA of ABPC-132 and controls were Northern blotted onto nitrocellulose filters that were hybridized with a c-myc probe. The filters were autoradiographed. ABPC-132 showed no c-myc expression (Fig. 2a). The filter was stripped and rehybridized with the N-myc probe. ABPC-132 showed a high level of N-myc expression (Fig 2b), while liver, spleen, TEPC-Hi-24 and Eu-mycKBC-3 were negative. Hybridization with an alpha-Actin probe confirmed that similar amounts of RNA were loaded in the blots (Fig 2c). Southern blot analysis (DNA) showed that ABPC-132 but not TEPC-Hi-24 contained integrated v-abl, detected by the K2abl probe (data not shown).

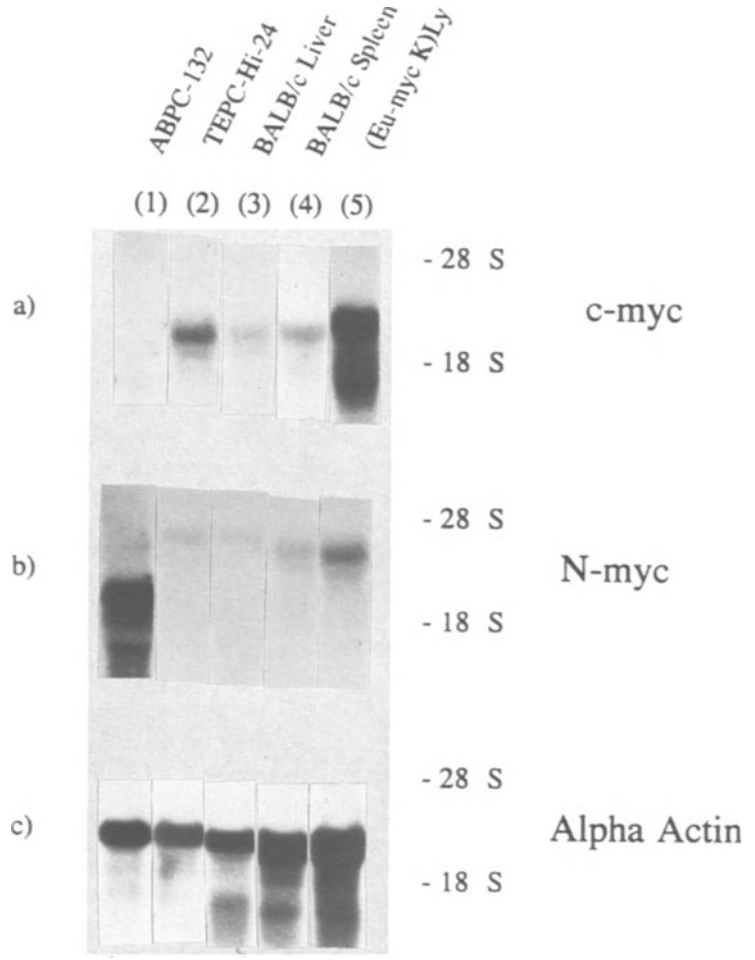


Fig. 2. Northern blot hybridization of RNA from ABPC-132 and control samples. (For details see the text).

DISCUSSION

The 6C1 and 12B breakpoints in ABPC-132 correspond to the localization of Igkappa and N-myc, respectively. This, together with the fact that the tumor expresses N-myc at a high level, but no c-myc, indicates that the translocation has brought the N-myc gene under the influence of the constitutively active light chain gene.

Activation of c-myc by chromosomal translocation or gene amplification has been found in numerous human and animals tumors. N- and L-myc, the two other best known members of the myc family (Reviewed by Zimmerman and Alt 1990) were less frequently activated in tumors, and then always by amplification, not by translocation. N-myc activation has been largely restricted to tumors of neuroendocrine and embryonic origin like primarily neuroblastoma and Wilms' tumour (Kohl et al. 1983, Lee et al. 1984). N-, L- and c-myc were alternatingly amplified in the highly invasive variant forms of small cell lung carcinoma (Nau et al. 1985).

Translocations in B- and T-cell derived tumors preferentially affect the physiologically rearranging Ig and TCR loci, respectively. The breakpoint corresponds to the target sites of the normal recombinases. Only transcriptionally active genes are accessible to recombinase action during physiological immunoglobulin rearrangement (Alt et al. 1987). The c-myc/Ig translocations appear in MPC, BL and RIC, after a long preneoplastic history that involves chronically stimulated cell division in the target cell at risk. This, together with the lack of clustered breakpoints or fragile sites on the myc chromosome suggests that the chromosome breaks accidentally, at random locations. Conceivably, the recombinases involved in an Ig-gene rearrangement that take place in parallel with the chronically stimulated cell division may join the wrong chromosome pieces together. The fact that only c-myc has been involved in the several hundred translocations so far studied in MPC, RIC and BL, respectively, suggests that constitutive activation of c-myc is particularly suitable to drive the continuous division of B-cells. Other oncogenes that may become involved in similar translocations may be less suitable to transform B-cells or, alternatively, may require additional specific changes for their tumorigenic effect. Two rare events are not likely to coincide in the same cell. The exclusive involvement of c-myc, to the exclusion of other members of the myc family, may either mean that N-myc is less competent to stimulate continuous cell division of B-cells or, alternatively, that the c- but not the N-myc chromatin is open at the appropiate time. Available evidence supports the second possibility. Emu-N-myc mice are equally prone to develop B-cell tumors as their c-myc transgenic counterparts (Dildrop et al. 1989, Rosenbaum et al. 1989). Constitutively activated N-myc is thus equally competent to trigger the development of B-cell neoplasia as c-myc .

Both the N- and the c-myc containing chromatin regions, are active in early pre-B-cells, but the N-myc chromatin becomes inactive in mature B-cells (Zimmerman et al. 1986, Smith et al. 1992). This suggests that the N-myc/Kappa juxtaposition in ABPC-132 has taken place at the pre-B stage, at a time when both genes were open for transcription. If so, the constitutive activation of N-myc has not interfered with plasma cell differentiation. This is in line with our earlier findings (Altiok et al. 1989), showing that the IgH switch -mu region is particularly translocation prone in pro-B cells but that such translocations do not prevent the cell from turning into sterile "activated immunoblast".

The fact that plasmacytomas can be induced in both Emu-c-myc and Emu-N-myc transgenic mice were the c- or the N-myc gene is turned on constitutively in all pro- and pre-B cells also shows that activated myc does not interfere with plasmacytic differentiation.

In typical IgH/myc translocation carrying MPCs, a switch region, most frequently S_{alpha}, serves as the most common myc receptor site. This has led to the assumption that a class switching enzyme may be responsible for the translocations (Calame et al. 1982, Klein & Klein 1985). Class switching occurs in mature B, not in pre-B cells. Therefore, it may be reasonable to assume that the typical translocations that involve a switch region, occur in B- and not in pre-B cells.

Rearrangement of heavy and light chains is believed to take place in Abelson virus transformed pre-B cells in a sequential, ordered fashion. Pre-B cells must express a complex of surface mu and certain pre-B associated light chain precursors, in order to develop further. Using targeted gene deletion, Rajewsky's group has shown (Kitamura et al. 1991, Gu et al. 1991) that pre-B cells that cannot express surface mu, are eliminated, probably by a scavenging mechanism. It has been suggested that surface -mu must be expressed on pre-B cells in order to trigger light chain assembly. At least one third of all kappa rearrangements are non-functional (Coleclough et al. 1981). Non-functionally rearranged pre-B cells are presumably eliminated by scavenging and/or as a consequence of positive clonal selection (Osmond et al. 1991). This may include light chain/myc translocation carrying pre-B cells that produce no functional immunoglobulin. Conceivably, v-abl, known to immortalize pre-B cells, may protect them from elimination. This is consistent with our earlier finding that the relative frequency of variant (light chain/myc) to typical (heavy chain/myc) translocations shifts from 1:9 in pristane oil induced, to 1:1 in pristane + Abelson virus induced plasmacytomas. The "risk" of an early N-myc/kappa translocation could be readily accomodated in the same scenario.

REFERENCES

Adams JM, Harris AW, Pinkert CA, Corcoran L M, Alexander WS, Cory S, Palmiter RD & Brinster RL (1985) Nature, **318**, 533-538

Alt FW, Blackwell RK and Yancopoulus GD (1987) Science **238**:1079-1087

Altiok E, Klein G, Zech L, Uno M, Ehlin Henriksson B, Batta S, Ono T and Ernberg I (1989) Proc Natl Acad Sci USA **86**:6333-6337

Calame K, Kim S, Lalley P, Hill R, Davis M and Hood L (1982) Proc Natl Acad Sci USA **79**:6994-6998

Coleclough C, Perry RP, Karjalainen K and Weigert M (1981) Nature (London) **290**: 372-377

Committee on Standard Genetic Nomenclature for mice, Standard karyotype of the mouse, Mus musculus (1972) J Hered **63**: 69-72

DePinho R, Legouy E, Feldman L, Kohl NE, Yancopoulos GD and Alt FW (1986) Proc Natl Sci USA **83**:1827-1831

Dildrop R, Ma A, Zimmerman K, Hsu E, Tesfaye A, DePinho R and Alt FW (1989) The EMBO Journal **8** (4) pp 1121-1128

Gu H, Kitamura D and Rajewsky K (1991) Cell **65**:47-54

Harris AW, Pinkert CA, Crawford M, Langdon W, Brinster RL and Adams JM (1988) J Exp Med **167**: 353-371

Kitamura D, Roes J, Kühn R and Rajewsky K (1991) Nature **350**:423-426

Klein G and Klein E (1985) Immunology Today **6**:208-215

Klett Ch, Adolph S and Hirning U (1991) Mouse Genome **89** (1) 275

Kohl N, Gee C and Alt FW (1984) Science **226**: 1335-1337

Lee WH, Murphree AL and Benedict WF (1984) Nature **309**:458-460

Minty AJ, Caravatti M, Robert B, Cohen A, Daubas P, Weydert A, Gros F and Buckingham M (1981) J Biol Chem **256**: 1008-1014

Nau M, Brooks B, Battey J, Sausville E, Gazdar A, Kirsch I, McBride W, Bertness V, Hollis G and Minna J (1985) Nature **318**:69-73

Osmond D, (1991) Mechanisms of B-cell Neoplasia pp 1-19 Eds Roche, Basel, Switzerland.

Pear WS, Wahlström G, Nelson SF, Axelson H, Szeles A, Wiener F, Bazin H and Klein G (1988) Mol Cell Biol **8**:441-445

Potter M, Sklar MD and Rowe WP (1973) Science **182**:592-594

Potter M, Mushinski JF, Mushinski EB, Brust S, Wax JS, Wiener F, Babonits M, Rapp UR and Morse III, HC (1987) Science **235**:787-789

Reddy EP, Smith MJ and Srinivasan A (1983) Proc nat Acad Sci (Wash) **80**, 3623-3627

Rosenbaum H, Webb E, Adams JM Cory S and Harris AW (1989) The EMBO Journal **8** (3) pp 749-755

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory manual. Cold Spring Harbor Laboratory Press

Silva S, Sugiyama H, Babonits M, Wiener F and Klein G (1991) Int J Cancer **49**:224-228

Smith RK, Zimmerman K, Yancopoulos GD, Ma A and Alt FW (1992) Mol Cell Biol **12**:1578-1584

Stanton LW, Watt R and Marcu KB (1983) Nature **303**:401-406

Sugiyama H, Weber G, Silva S, Babonits M, Wiener F and Klein G (1989) Int J Cancer **44**:348-352

Sugiyama H, Silva S, Wang Y, Weber G, Babonits M, Rosén A, Wiener F and Klein G (1990) Int. J. Cancer, **46**:842-852

Swan D, DÉustachio P, Leinwand L, Siedman J, Keithley D and Ruddle FH (1979) Proc Nat Acad Sci USA **76**:2735-2739

Wang Y, Sugiyama H, Axelson H, Panda Ch, Babonits M, Ma A, Steinberg JM, Alt FW, Klein G and Wiener F (1992) Oncogene (In press)

Zimmerman KA, Yancopoulos GD, Collum RG, Smith RK, Kohl NE, Denis KA, Nau MM, Witte ON, Toran-Allerand D, Gee CE, Minna JD and Alt FW (1986) Nature **319**:780-783

Zimmerman KA and Alt FW (1990) Crit Rev Oncogenes **2**:75-95

ACKNOWLEDGEMENTS

This investigation was supported by PHS grant 5 R01 CA 14054-15 from the US National Cancer Institute and by the Swedish Cancer Society. S.S. is a recipient of fellowships from the Cancer Research Institute, New York and the Concern Foundation, Los Angeles, CA. Y.W was supported by fellowships from the Swedish Cancer Society.

Experimental Model of Human Light-Chain-Associated Disease

A. SOLOMON, D. T. WEISS and T. K. WILLIAMS

Department of Medicine, Human Immunology & Cancer Program,
University of Tennessee Medical Center/Graduate School of Medicine

Introduction

The three types of human light-chain-associated disease—"myeloma kidney" disease (myeloma [cast] nephropathy), light-chain deposition disease, and AL amyloidosis—are characterized by renal and systemic deposition of monoclonal light chains, i.e., Bence Jones proteins, as casts, linear precipitates, or fibrils, respectively [1]. These light-chain deposits ultimately result in the impairment of kidney (and other organ) function and account for much of the morbidity and mortality in patients with these disorders [2]. The fact that, clinically and experimentally [1], tubular casts, basement membrane deposits, amyloid formation, and other physiological aberrations are *not* an invariant accompaniment of Bence Jones proteinuria or are *not* necessarily related to the amount of monoclonal light chain synthesized or excreted implies that, in addition to host or exogenous factors (e.g., dehydration, hypercalcemia, sepsis, nephrotoxic antibiotics, etc.), certain light chains are "malignant" while others are "benign" [3].

Despite our increased understanding of the structural properties of light chains, as well as the genes and regulating elements that control light-chain synthesis, we have as yet limited knowledge concerning the molecular properties of light chains or of the host factors that facilitate the pathological deposition of these proteins. Such information will provide for the clinician new insight into the pathogenesis of the light-chain-associated renal and systemic diseases and the ability to design more effective methods to diagnose, treat, and, ultimately, prevent these disorders. Several types of *in vitro* and *in vivo* models have been developed that have provided a means to assess the potential nephrotoxicity of Bence Jones proteins [4-8]. We have used one such *in vivo* model, first described in 1976 by Koss, Pirani, and Osserman [4], in which mice were injected intraperitoneally (i.p.) with Bence Jones proteins and the presence or absence of human light-chain deposits in mouse organs determined by conventional microscopic and immunohistochemical analyses. Our studies have demonstrated that such proteins can be deposited in the form of intraluminal casts, crystals, basement membrane precipitates, or fibrils [8,9]. The clinical relevance of this *in vivo* model was evidenced by the demonstration that a) the lesions induced experimentally were comparable to those found in the patients from whom the injected Bence Jones proteins were obtained and b) through the use of the experimental mouse model, it was possible to differentiate "nephrotoxic" from "non-nephrotoxic" Bence Jones proteins [8].

The current report updates our recent experience with this model that has provided new information on the pathogenesis and treatment of the human light-chain-associated diseases.

Results

Using previously described experimental conditions [8], 6-week-old, 15- to 20-g C3H/HeJ mice were injected with up to 300 mg of purified human Bence Jones protein and sacrificed 48 hours later. Tissue was examined by light microscopy after routine histochemical staining; immunohistochemical analyses were performed using anti-human κ- or λ-chain-specific antisera. Among the 43 different proteins studied to date (23 κ, 20 λ), 27 were deposited predominately as tubular casts, basement membrane precipitates, or crystals in the mouse kidneys; no light-chain deposits were detected in the other 16. In 18 cases where human tissue was available for analysis, the clinical and experimental findings were comparable in 14. Further, 22 of 27 proteins obtained from patients with abnormally high serum creatinine concentrations (≥168 μmol/liter) were deposited in the mouse kidneys, in contrast to the deposition of only 6 of 16 proteins from patients with a serum creatinine of <168 (Table 1).

Table 1. Nephrotoxicity of urinary Bence Jones proteins from 43 patients with multiple myeloma or AL amyloidosis

Serum creatinine level (μmol/liter)	Number of patients	Light-chain deposits[a]	
		Absent	Present
<168	16	10	6[b]
≥168	27	5	22[c]

[a]Denotes deposits detected experimentally in mice.
[b]Basement membrane deposits (4); casts (2).
[c]Basement membrane deposits (6); casts (13); crystals (3).

Two of the three types of human light-chain-associated disease—light-chain deposition disease and AL amyloidosis—are characterized by systemic as well as renal light-chain deposits [1]. However, in the third and most common form of this disease, myeloma (cast) nephropathy, it has not as yet been established if the precipitation of Bence Jones protein is limited to the renal tubules or also occurs systemically. To address this question, we have begun to analyze, using immunohistochemical methods, liver, spleen, and heart tissue from mice injected with human cast-forming Bence Jones proteins. Our preliminary results indicate that one such protein was not only deposited in renal tubular lumens but was also found in the membranes of myocardial cells (Fig. 1). In contrast, no systemic human light-chain deposits were found in a mouse injected with a Bence Jones protein that failed to form renal casts.

It is not as yet known if the entire light polypeptide chain is required for light-chain deposition. Because certain properties of intact Bence Jones proteins (i.e., their characteristic behavior upon heating) are expressed by the isolated variable domain (V_L) but not by the constant domain (C_L) [10], we tested if V_L or C_L fragments prepared from a nephrotoxic light chain could also induce tubular casts. A mouse was injected with a cast-producing Bence Jones protein, while two other animals received equimolar amounts of either V_L or C_L fragments prepared by pepsin digestion of the same protein [10]. Similar results were obtained when the V_L fragment as well as the intact light chain were injected; i.e., there was extensive formation of tubular casts (Fig. 2A). In contrast, injection of the C_L fragment or of comparable amounts of the V_L or

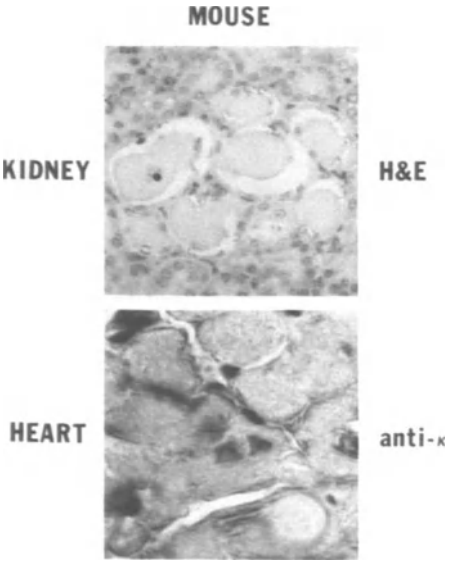


Fig. 1. Systemic deposition of a "cast-forming" Bence Jones protein. Mice were injected with a human κ Bence Jones protein and sacrificed after 48 hours. *(Upper)* H&E-stained section of mouse kidney revealed the presence of renal tubular casts that were shown immunohistochemically to be composed of the injected human light chain. *(Lower)* Immunohistochemical demonstration of human light-chain deposition in myocardial cell membranes; primary antiserum, anti-human κ-chain (X250).

C_L components prepared from a "non-nephrotoxic" Bence Jones protein did not result in tubular casts.

The mouse model was also utilized to study various host factors that have been attributed to potentiate or ameliorate renal dysfunction in patients with Bence Jones proteinuria—e.g., dehydration and hydration, respectively. For the dehydration experiments, two animals were water deprived for a 48-hour period after injection of 50 or 100 mg of Bence Jones protein. Another mouse was dehydrated for 48 hours but was not injected with protein, and a fourth mouse was permitted water ad libitum and received 150 mg of protein. Under these conditions, tubular casts were induced only in the dehydrated, protein-injected mice (Fig. 2B). The extent of cast formation in the dehydrated animal that received as little as 50 mg of the Bence Jones protein was comparable to that found in other experiments when non-dehydrated mice were injected with 300 mg of the protein.

For the hydration experiments, pairs of mice were permitted water ad libitum and injected with 300 mg of the same Bence Jones protein used in the dehydration study. One member of each pair received additional fluids administered parenterally every 8 hours via 2-ml i.p. injections of a physiological buffer solution beginning 12 hours before the protein injection and continuing twice daily until the time of sacrifice. As a control, the second member received no supplemental fluids. In contrast to the control mice, no tubular casts occurred in animals that received the parenteral hydration (Fig. 2C).

To determine if experimentally-induced cast nephropathy persisted during the life span of the mouse or eventually disappeared, we injected the nephrotoxic Bence Jones

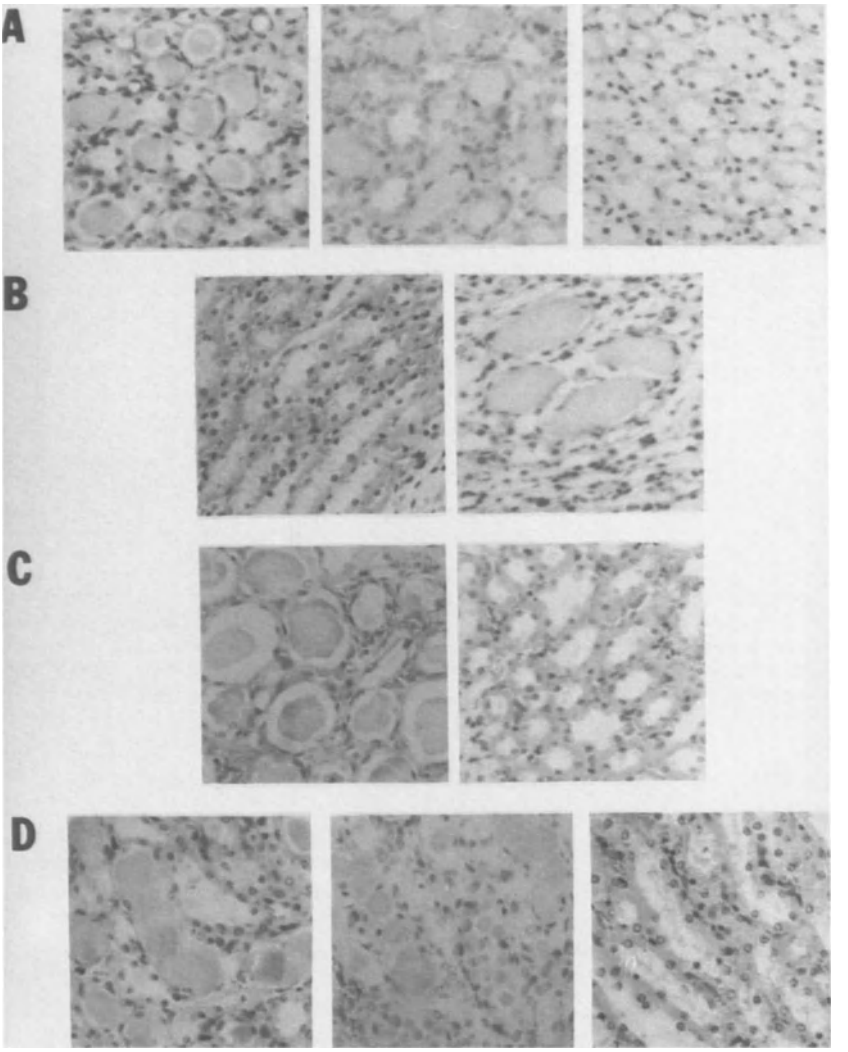


Fig. 2. *(A)* Molecular localization to the V_L of Bence Jones protein nephrotoxicity. Tubular casts occurred in a mouse injected with *(left)* 200 mg of a "nephrotoxic" Bence Jones protein, *(middle)* 100 mg of its pepsin-derived V_L or *(right)* C_L fragment. *(B)* Potentiation of cast formation by dehydration. *(Left)* No tubular casts occurred in a mouse permitted water ad libitum and injected with 150 mg of a "nephrotoxic" Bence Jones protein. *(Right)* Extensive cast deposition in a mouse dehydrated and injected with 50 mg of the same protein. *(C)* Reversal of cast formation by hydration. *(Left)* Tubular casts occurred in a mouse injected with 300 mg of a "nephrotoxic" Bence Jones protein and sacrificed 1 week later. *(Right)* No casts were evident when a mouse received supplemental parenteral hydration beginning 72 hours after protein injection. *(D)* Resolution of cast nephropathy. *(Left)* Cast deposition in renal tubules of a mouse injected with a "nephrotoxic" Bence Jones protein and sacrificed 72 hours later. *(Middle)* Fragmentation of casts in a mouse injected with the same protein and sacrificed 2 weeks later. *(Right)* Resolution of cast nephropathy after 6 weeks (X250).

protein into each of five mice and sacrificed one animal 72 hours and 1, 2, 3, and 6 weeks later. Extensive tubular casts were present at 72 hours and were undiminished after 1 week. By 2 weeks, the casts appeared smaller and fragmented, and by 3 weeks, they were even less evident. By 6 weeks, virtually no casts were present (Fig. 2D).

The intraluminal proteinaceous casts characteristic of myeloma "kidney" disease contain, in addition to Bence Jones protein, the Tamm-Horsfall glycoprotein [11]. Experimentally, it has been shown that Bence Jones proteins that form casts *in vivo* co-aggregate *in vitro* with human Tamm-Horsfall glycoprotein and that, under appropriate physiological conditions, this interaction is responsible for pathologic cast formation [11]. Further, suppression of Tamm-Horsfall protein synthesis and/or alteration of its carbohydrate moiety by colchicine can prevent cast formation *in vitro* [12]. To determine if colchicine administration would also inhibit the *in vivo* development of casts, a mouse was given 0.2 μg/mg body weight doses of the drug i.p. twice daily for 2 days prior to Bence Jones protein injection, the day of injection, and for an additional 2 days. A second mouse received protein but no colchicine. In contrast to the control animal, where tubular casts were readily evident, no intratubular pathology was apparent in the colchicine-treated mouse (Fig. 3).

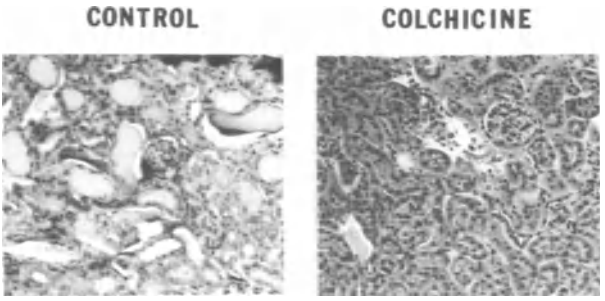


Fig. 3. Effect of colchicine on experimental induction of myeloma (cast) nephropathy. *(Left)* Renal tubular casts in the kidney of a mouse (control) injected with a "nephrotoxic" Bence Jones protein. *(Right)* No tubular casts occurred in a mouse injected with the same protein and treated with colchicine (0.2 μg/mg body weight) twice daily for 5 days (X100).

Another presumably host-related factor was the observation that, in certain cases, the injection of human Bence Jones protein into the mouse resulted in unilateral renal tubular cast formation. This phenomenon, as noted previously in murine Bence Jones protein-producing plasma cell neoplasms [13], was not limited to the left or right kidney.

Discussion

We have shown that under defined conditions, the injection of human Bence Jones proteins into mice can result in the pathological deposition of these proteins in a manner comparable to that found in the human light-chain-associated diseases, myeloma (cast) nephropathy, light-chain deposition disease, or AL amyloidosis. Further, we have

found the experimental mouse model useful in the differentiation of "nephrotoxic" from "non-nephrotoxic" light chains.

That the deposition of Bence Jones proteins as tubular casts is not limited to the kidney was evidenced in the mouse model. The extent and pathophysiological significance of systemic light-chain deposits by cast-forming Bence Jones proteins remain to be established.

The molecular factors responsible for deposition of monoclonal light chains as casts, crystals, precipitates, or fibrils (or the total lack of deposition of these proteins) are as yet unknown. However, our comparative studies with the intact light chain *vs.* V_L and C_L fragments indicate that, at least for cast formation, the V_L portion of the molecule is responsible for such pathology.

Host factors that contribute to the renal disease found in patients with multiple myeloma and Bence Jones proteinuria [1] were also studied in the mouse model. In particular, the effect of fluid restriction or supplementation on myeloma (cast) nephropathy was evaluated. When mice were dehydrated, there was a marked increase in cast formation, even when "sub-toxic" doses of protein were injected. Conversely, vigorous hydration prevented or lessened the deposition of Bence Jones protein. The fact that light-chain-induced pathology was found to be reversible over time has therapeutic implications; namely, improvement in renal function may occur in patients whose Bence Jones protein synthesis is suppressed or abolished by chemotherapy. Additionally, our experiments showing that colchicine treatment inhibited the pathologic deposition of Bence Jones proteins as casts provided further evidence to support the role of another host factor—the Tamm-Horsfall protein—in myeloma (cast) nephropathy and offers a new therapeutic modality.

With regard to exogenous factors, it has been presumed that patients with Bence Jones proteinuria develop acute renal failure after intravenous pyelography (IVP) as a result of sudden precipitation of protein within the renal tubules [1]. We have utilized the mouse model to determine if this event is a consequence of the injected radiocontrast medium or is due to the dehydration that results from the preparation required for this diagnostic procedure—e.g., the mandated fluid restriction or the fluid loss that results from laxative-induced diarrhea. Our preliminary studies in the mouse indicate that dehydration and not the injected contrast medium is responsible for the nephropathy.

In summary, the *in vivo* mouse model has provided us a means to identify monoclonal light chains of pathological import and to investigate protein and host factors that contribute to the pathogenesis of these diseases. This information has prognostic and therapeutic importance; the knowledge gained will ultimately reduce the morbidity and mortality of patients with light-chain-associated diseases.

Acknowledgements

We thank Julie Ottinger for preparation of the manuscript. This research was supported, in part, by USPHS grant CA10056 from the National Cancer Institute, an I.M.A. Barger Memorial Grant for Cancer Research (IM-430) from the American Cancer Society, and the Stein Cancer Research Fund.

References

1. Solomon A (1986) Clinical implications of monoclonal light chains. Semin Oncol 13:341-349.
2. Alexanian R, Barlogie B, Dixon D (1990) Renal failure in multiple myeloma: pathogenesis and prognostic implications. Arch Intern Med 150:1693-1695.
3. Solomon A (1982) Bence Jones proteins: malignant or benign? N Engl J Med 306:605-607.
4. Koss MN, Pirani CL, Osserman EF (1976) Experimental Bence Jones cast nephropathy. Lab Invest 34:579-591.
5. Clyne DH, Pollak VE (1981) Renal handling and pathophysiology of Bence Jones proteins. Contrib Nephrol 24:78-87.
6. Smolens P, Barnes JL, Stein JH (1986) Effect of chronic administration of different Bence Jones proteins on rat kidney. Kidney Int 30:874-882.
7. Sanders PW, Herrera GA, Galla JH (1987) Human Bence Jones protein toxicity in rat proximal tubule epithelium in vivo. Kidney Int 32:851-861.
8. Solomon A, Weiss DT, Kattine AA (1991) Nephrotoxic potential of Bence Jones proteins. N Engl J Med 324:1845-1851.
9. Solomon A, Weiss DT, Pepys MB (1992) Induction in mice of human light-chain-associated amyloidosis. Am J Pathol 140:629-637.
10. Solomon A, McLaughlin CL (1969) Bence-Jones proteins and light chains of immunoglobulins. I. Formation and characterization of amino-terminal (variant) and carboxyl-terminal (constant) halves. J Biol Chem 244:3393-3404.
11. Sanders PW, Booker BB, Bishop JB, Cheung HC (1990) Mechanisms of intranephronal proteinaceous cast formation by low molecular weight proteins. J Clin Invest 85:570-576.
12. Sanders PW, Booker BB (1992) Pathobiology of cast nephropathy from human Bence Jones proteins. J Clin Invest 89:630-639.
13. McIntire KR, Potter M (1964) Studies of thirty different Bence Jones protein-producing plasma cell neoplasms in an inbred strain of mouse. JNCI 33:631-648.

Lymphomas:
B-CLL, Follicular
Lymphomas BCL-2, BCL-1

On the Role of Endogenously Produced TNF-α and IL-6 as Regulators of Growth and Differentiation of B-Type Chronic Lymphocytic Leukemia Cells In Vitro

K. Nilsson[1], L.-G. Larsson[1], O. Söderberg[1], M. Schena[2], D. Gottardi[2], F. Caligaris-Cappio[2], and M. Carlsson[1]

[1]Laboratory of Tumor Biology, Department of Pathology, Uppsala University, Uppsala, Sweden and
[2]Dipartimento di Scienze Biomediche e Oncologia Umana, Sezione Clinica, Università di Torino, Torino, Italy.

Introduction

Chronic lymphocytic leukemia of CD5+ B cells is clinically a heterogeneous disease [1]. This heterogeneity is also easily demonstrable in vitro [2]. In the vast majority of the cases B-CLL is a malignancy of CD5+ B cells with a very low capacity for proliferation in peripheral blood and with a phenotype resembling that of virgin [2] and mantle zone memory [3] B cells. In some cases the stage of differentiation is more advanced, as reflected by the capacity of the leukemic cells to secrete immunoglobulin [Ig]. In other rare cases Ig production may be very low and undetectable by immunofluorescence. The possibility that B-CLL might be a disease of differentiating leukemic B cells has not been extensively studied, but our own unpublished observations suggest that the stage of differentiation of the B-CLL cells may be different in peripheral lymphoid organs and in the bone marrow of the patient than in the peripheral blood, and the capacity for proliferation seems often to be about a 10-fold higher in these tissues.

B-CLL cells were for long assumed to to be irreversibly blocked at a particular stage of the B cell differentiation lineage. In 1974 Fu and collaborators [4] succeeded in inducing DNA synthesis and plasmacytoid differentiation in B-CLL cells from patients with a monoclonal serum IgM in vitro using pokeweed mitogen and allogeneic T cells as stimulatory agents. It was not, however, until phorbol myristic acetate (PMA) was used as a stimulus that a large fraction (50-60%) of "common" B-CLL clones could be induced to lymphoblastoid-plasmacytoid differentiation in vitro [5].

Studies in several laboratories during the last decade, employing PMA, Staphylococcus Aureus Cowan I (SAC) or anti-μ antibodies to deliver pre-activation signal(s) and thioredoxin (Trx) and some cytokines as co-stimulatory agents, have now clearly demonstrated that a large fraction of B-CLL clones are inducible to DNA synthesis and differentiation in vitro [reviewed in 2]. The dissection of the regulation of growth and differentiation of B-CLL cells have generated many, sometimes contradictory, results. One may, however, conclude that IL-2 is a powerful growth and differentiation-inducing cytokine for most B-CLL clones provided that they respond to any of the pre-activating agents. IL-4, on the other hand, is stimulatory or inhibitory depending on the type of pre-activating agent and the type of co-stimulatory signals. Interferon-α and interferon-γ, like PMA, may activate B-CLL cells to become Ig secretory in the absence of concomitant proliferation. In other cases various degrees of proliferation are simultaneously induced. It is thus clear that B-CLL cells are by no means autonomous but can be regulated by several members of the cytokine network, particularly by T cell derived lymphokines and by cell-cell contacts.

The complexity of the regulation of proliferation/differentiation in B-CLL cells has further been underlined by the recent findings that B-CLL cells may produce several cytokines and express cytokine receptors spontaneously or after induction to proliferation and/or differentiation in vitro. Due to the problem of biological heterogeneity among the studied B-CLL clones, their

Current Topics in Microbiology and Immunology, Vol. 182

contamination by various amounts of non-B-CLL cells and some variations in the experimental setups the published data on the profile of cytokine production and cytokine receptor expression in B-CLL may not always be reliable. However, B-CLL cells seem to be able to produce IL-1-α, IL1-β, IL-6, TGF-β, TNF–α and TNF-β and release the soluble form of CD23 and TNF-αR. Only TNF–α, TGF-β and IL-1β were consistently found [6-14]. Receptors for IL-2 [15-16], TNF–α [17-18] and IL-6 [19-20] have also been reported. Taken together these findings suggest that B-CLL cells may interact with other cells in their microenvironment by paracrine cytokine interaction, and that some cytokines may function as constitutively produced, or inducible autocrine growth-and differentiation factors.

In this study we have used a well characterized clone of primary B-CLL cells, designated I-83 [2, 16, 21], to examine two cytokines, TNF–α and IL-6, which seem to be controversial with respect to their role in the proliferation/differentiation control of B-CLL cells. The expression of these cytokines and their receptors in I-83 cells, resting or stimulated by PMA, SAC, IL-2, IL-4 and Trx and combinations thereof, were analyzed at the mRNA and protein level. Furthermore the effect of exogeneous cytokines were examined, as was the effect of antibodies against the cytokines and their corresponding receptors. The results suggest that TNF–α may function as a paracrine and perhaps also as an autocrine regulator of proliferation/differentiation, while IL-6, which also can be produced by I-83 cells, appears not to affect growth or differentiation of this B-CLL clone [22, Carlsson M, unpublished results].

TNF–α is a growth and differentiation factor for I-83 B-CLL cells

The stock of I-83 cells, isolated by leukapheresis, was kept in liquid nitrogen [16]. The I-83 clone has been extensively characterized with respect to phenotype and has been found to contain 2.4% contaminating T cells and monocytes [2, 21]. The use of this clone allows repeated experiments with minimal inter-experimental variation. The various protocols that can be used to stimulate the I-83 cells to proliferation with minimal differentiation, to proliferation with concomitant differentiation and to differentiation only are depicted in Fig. 1.

Spontaneous and IL-2 enhanced synthesis and secretion of TNF–α

As studied by ELISA in supernatants from control cells and from cells stimulated for 6 days according to some of the different protocols, no TNF-α was found in untreated or SAC activated cells. The combination of PMA or SAC and IL-2+Trx, however, induced TNF-α secretion (0.11-0.19 ng/ml). Small amounts were found in cultures stimulated with PMA or IL-2 only (0.03-0.04 ng/ml). TNF-α secretion thus occurred in I-83 cells stimulated to proliferation and differentiation but IL-4, which strongly enhanced both growth and Ig secretion [22], did not further increase TNF-α secretion in the IL-2 stimulated PMA pre-activated cells.

Immunocytochemistry performed to detect TNF-α production at the single cell level essentially confirmed the ELISA results. In untreated cells the staining with the anti-TNF-α antibodies was very weak but specific in approximately 90% of the cells, demonstrating that resting I-83 cells indeed may constitutively produce TNF-α, although not secreting the factor in amounts detectable with the ELISA. Increased amounts of TNF-α was found after pre-activation of the cells by PMA or SAC followed by co-stimulation by IL-2+Trx.

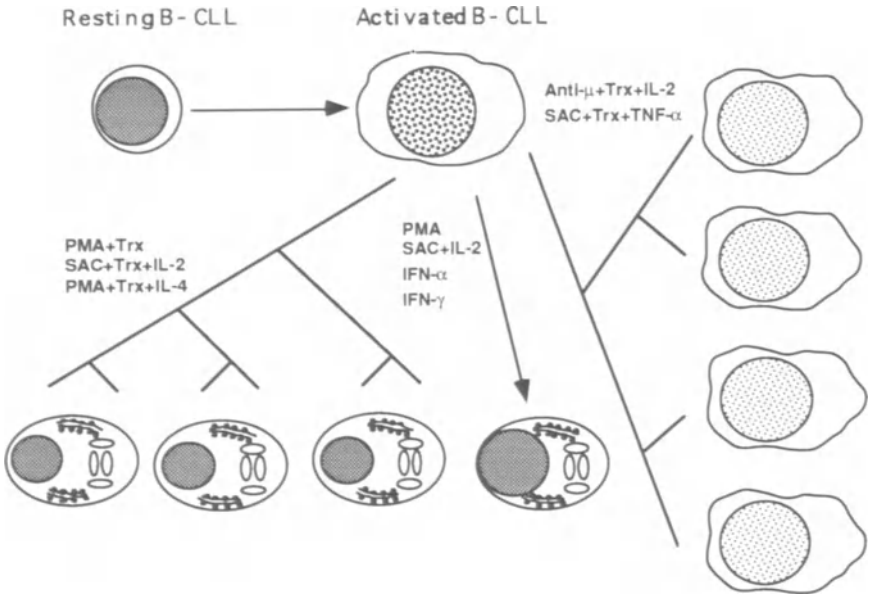


Figure 1. Protocols for the induction of activation followed by differentiation, or proliferation with and without concomitant differentiation

Northern blot analyses of total mRNA prepared from untreated and induced cells demonstrated a low level of TNF-α mRNA in the non-stimulated cells, confirming the immunocytochemical findings, and a 10-fold increase at day 6 after induction by SAC+IL-2+Trx. This increase was inhibited by IL-4, thus in agreement with the ELISA data.

TNF−α receptor (TNF−αR) expression and turn-over in resting, activated and proliferating/differentiating I-83 cells

The expression of receptors for TNF-α was assayed by a ^{125}I-labelled TNF-α binding assay [22]. No receptors could be demonstrated on untreated cells. After stimulation with the SAC+Trx+IL-2 and the PMA+Trx+IL-4 protocols protocol TNF-α receptors were detectable. As calculated from Scatchard plots the receptor density in SAC stimulated cells was 330 receptors/cells with a dissociation constant of 71 pM. The corresponding values for cells stimulated according to the PMA protocol were 880 and 84, respectively.

An assay for TNF-α binding protein was used to study the turn-over of TNF-αR. Receptor turn-over was found after pre-activation by PMA alone, PMA+Trx and PMA+IL-2. SAC activation, although stimulating the expression of TNF-αR, did not increase TNF-αR turn-over.

TNF−α is a potent inducer of growth and differentiation of pre-activated I-83 cells

TNF-α was found to increase the ^{3}H-incorporation of PMA+Trx stimulated cells in a dose-dependent manner (maximal stimulation at concentrations >50 ng/ml). The stimulation of PMA+Trx induced cells was of the same magnitude as that induced by IL-2. TNF-α alone did not affect proliferation but minimally, and Ig secretion was not induced.

When TNF-α was added to I-83 cells, pre-activated by PMA in combination with Trx and IL-2 or IL-4 it was found to slightly enhance the proliferation/differentiation induced by these stimulation protocols.

TNF-α also stimulated DNA synthesis of SAC pre-activated cells, alone or in combination with Trx and IL-2. The enhancement of the IL-2 stimulation was restricted to an increase of DNA synthesis while the Ig secretion was unaffected.

Antibodies against TNF–α and TNF–αR do not inhibit growth and differentiation of I-83 cells

I-83 cells were stimulated by the various induction protocols, together with antibodies against TNF-α and/or TNF-αR, to examine the presence of a possible autocrine TNF-α loop. Such an autocrine TNF-α stimulatory mechanism for B cells has been suggested previously [7] and by the present findings that the induced growth and differentiation of the I-83 cells is enhanced by exogeneous TNF-α, that TNF-α secretion is detectable during the induced proliferation/differentiation and that TNF-αR expression and turn-over increase during this process.

Neutralizing rabbit anti-human TNF-α was obtained from Genzyme and as a kind gift from Dr. I. Olsson, Lund, Sweden. Mouse monoclonal anti-human TNF-α antibodies were kindly provided by Dr G. Adolf, Boeringer-Ingelheim, Vienna. A rabbit antiserum against the TNF-αR was a gift from Dr. Olsson.

Cells were induced by the stimulation protocols leading to maximal DNA synthesis in the presence of various concentrations of anti-TNF-α antibodies. In no case did the antibodies block the DNA synthesis induced by PMA+Trx+IL-4, PMA+Trx and SAC+Trx+IL-2. In control experiments the enhancing effect on the DNA synthesis in PMA+Trx stimulated cells by exogeneous TNF-α could be abrogated by neutralizing antibodies. Attempts to block the induced proliferation/differentiation by the anti-TNF-αR antibodies also failed.

IL-6 is produced by I-83 B-CLL cells but seems not to be a growth and differentiation factor for these cells

I-83 cells were examined for their capacity to produce IL-6 and to express IL-6R in vitro. Furthermore, as an additional test for the possible involvement of IL-6 in the regulation of growth and differentiation of I-83 cells, attempts were made to block the induced activation and proliferation/differentiation by anti-IL-6 and anti-IL-6R antibodies.

Spontaneous and induced synthesis and secretion of IL-6 in the I-83 B-CLL clone

I-83 cells, stimulated to growth and/or differentiation by the various protocols, were examined for IL-6 production by an ELISA and immunocytochemistry. Uninduced cells were essentially negative by immunocytochemistry, and no IL-6 was detectable in the supernatant. In the majority of the PMA activated cells, and in cells activated by SAC and co-stimulated by Trx+IL-2, a faint staining was found. However, stimulation by PMA+Trx+IL-4 led to a strong staining of the I-83 cells. That IL-4 is a strong inducer of IL-6 in the I-83 cells was supported by the finding that IL-6 was demonstrable by ELISA in the supernatant in cultures stimulated by PMA+Trx+IL-4 and PMA+ IL-4 but not by the other protocols of induction tested.

IL-6 does not induce growth/differentiation in resting or pre-activated I-83 cells alone or in combination with IL-2 and IL-4

The effect of IL-6 alone on unstimulated I-83 cells and on cells induced to proliferation and/differentiation according to Fig. 1 was tested in short and long term assays with negative results. Neither the ^{3}H-thymidine uptake and Ig secretion, nor the morphology, were altered in IL-6 stimulated cultures.

I-83 cells do not express IL-6R spontaneously or after induction to proliferation/differentiation, and anti-IL-6 and anti-IL-6R antibodies do not affect induced proliferation/differentiation

To examine whether the insensitivity to IL-6 might be the result of an autocrine IL-6 loop as has been suggested for the human U-266-1970 cell line [23] the I-83 cells were examined for IL-6R expression, and the effect of anti-IL-6 and anti-IL-6R antibodies was analyzed.

The expression of IL-6R on I-83 cells, uninduced or stimulated according to the protocol most efficiently inducing IL-6 production (PMA+Trx+IL-4), was examined by an ^{125}I-labelled IL-6 binding assay as described [23]. No receptors were detectable, neither in the control nor in the cultures induced to proliferation/differentiation.

The polyclonal anti-IL-6 and monoclonal anti-IL-6R antibodies (PM1), kindly donated by Dr. T. Kishimoto, Osaka, Japan, did not affect the proliferation and/or differentiation induced by the various protocols of induction.

Discussion and conclusions

TNF-α has previously been shown to be produced by normal B cells [24, 25] and has been proposed to function as an autocrine growth-and differentiation factor for such cells [26]. In some B-cell malignancies, e.g. B-CLL and hairy cell leukemia, production of TNF-α and expression of TNF-αR have been described [7-9, 13, 27 -30], and it has been suggested that TNF-α may represent an autocrine growth factor in these malignancies [7]. However, the results from studies on B-CLL in vitro concerning the growth promoting effects of exogeneously added TNF-α are somewhat conflicting [7, 8, 27], as are the published data on the effect on anti-TNF-α and anti-TNF-αR antibodies in blocking experiments [8, 29].

This study clearly shows that resting B-CLL cells, as exemplified by the well characterized I-83 clone, indeed may synthesize TNF-α and express receptors for this cytokine. Secretion of TNF-α occurs, however, only after activation of the cells. The most powerful inducers of TNF-α secretion in vitro were PMA or SAC+ Trx+IL-2. That IL-2 is a potent inducer of TNF-α has recently been shown for, activated normal B cells [26]. Exogeneous TNF-α also seemed to induce moderate TNF-α secretion, suggesting the excistance of an autoregulatory mechanism in the I-83 cells as has been reported for other B-CLL clones [7].

We also show that TNF-αRs are present on I-83 B-CLL cells and that they are inducible by the stimulation protocols using PMA and SAC as pre-activating agents and Trx+IL-2, or Trx+IL-4 as co-stimulatory factors.The number of receptors and the dissociation constant are in good agreement with what has been published for B-CLL cells [27, 28]

Exogeneous TNF-α induced strong proliferation of PMA and SAC activated I-83 cells. Previous studies have shown extensive heterogeneity among B-CLL clones with respect to a mitogenic response to TNF-α. In the studies by Cordingly et al [7] and Digel et al [27] the vast majority of the studied cases responded while in the study by Foa et al [8] only rare B-CLL clones were stimulated by TNF-α.

The facts that the I-83 cells inducibly expressed secretory TNF-α and TNF-αR and responded to TNF-α by proliferation suggest that TNF-α may function as an autocrine growth factor for activated B-CLL cells. In vivo such a stimulation of TNF-α and TNF-αR production may take place as a result of an activation by the immune system, e.g by bacterial infections. However, we have not been able to prove that TNF-α is autostimulatory in I-83 cells since all attempts to against the TNF-αR have failed.

Future experiments will therefore have to be performed to prove or disprove the existance of an autocrine TNF-α loop in B-CLL.

The findings, a) that IL-6 is inducible to secretion in I-83 cells by the protocol most efficiently stimulating growth and Ig secretion, b) that IL-6R seems not to be expressed and c) that anti-IL-6 and anti-IL-6R antibodies do not affect induced growth/differentiation, suggest that IL-6 in this B-CLL clone is produced as a paracrine cytokine, which in vivo may function in the communication between the B-CLL cells and accessory cells, e.g. T cells in the bone marrow and peripheral lymphoid organs. This is in contrast to what has been shown for normal B cells in vitro. For such cells IL-6 has been found to increase Ig secretion but not proliferation [31, 32] perhaps by an autocrine mechanism, at least in B cells actiated by SAC+IL-2 to Ig secretion [31]. Our results also contrast to the finding of both a growth and differentiation stimulatory effect by IL-6 in Epstein-Barr virus immortalized B cells [33]. Similar observations of no or minimal effects of exogeneous IL-6 on B-CLL clones have previously been reported [34-36], However, regarding IL-6 production and IL-6R expression in B-CLL cells the data are conflicting [6, 10, 14, 20, 26]. It is therefore possible that B-CLL clones in fact are heterogeneous with respect to the biological effects of IL-6, perhaps depending on the stage of B cell differentiation they represent or on possible differences in the suggested, malignancy-associated defect in the signal transduction [34].

Acknowledgements

This work was supported by grants from the Swedish Cancer Society and the Associazone Italiana Ricerca Cancero, Milano, Italy.

References

1. Freedman AS (1990) Immunobiology of chronic lymphocytic leukemia. Hematol Oncol Clin North Am 4:405-430
2. Nilsson K (1992) The control of growth and differentiation in chronic lymphocytic leukemia (B-CLL) cells. In: Cheson BD (ed) Chronic lymphocytic leukemia. Marcel Dekker, New York, pp 33-61
3. Schena M, Larsson L-G, Gottardi D Gaidano G, Carlsson M, Nilsson K, caligaris-Cappio (1992) Growth and differentiation-associated expression of BCL-2 in B-chronic lymphocytic leukemia ells. Blood, in press
4. Fu SM, Winchester, RJ, Feizi T, Walzer PD, Kunkel HG (1974) Idiotypic specificity of surface immunoglobulin and the maturation of leukemic bone marrow-derived lymphocytes. Proc Natll Acad Sci USA 71: 4487-4490
5. Tötterman TH, Nilsson K, Sundström C (1980) Phorbol ester-induced differentiation of chronic lymphocytic leukaemia cells. Nature 288:176-178
6. Aguilar-Santelises M, Magnusson R, Svenson SB, Loftenius A, Andersson B, Mellstedt H, Jondal M (1991) Expression of interleukin-1α, interleukin-1β and interleukin-6 in chronic B lymphocytic leukaemia (B-CLL) cells from patients at different stages of disease progression. Clin exp Immunol 84:422-428
7. Cordingley FT, Hoffbrand AV, Heslop HE, Turner M, Bianchi A, Reittie JE, Vykarnam A, Brenner MK (1988). Tumor necrosis factor as an autocrine tumour growth factor for chronic B-cell malignancies. Lancet 969-971
8. Foa R, Massaia M, Cardona S, Tos AG, Bianchi A, Attisano C, Guarini A, Di Celle PF, Fierro MT (1990) Production of tumor necrosis factor-alpha by B-cell chronic lymphocytic leukemia cells: A possible regulatory role of TNF in the progression of the disease. Blood 76:393-400
9. Schena M, Gaidano G, Gottardi D, Malavasi F, Larsson L-G, Nilsson K, Caligaris-Cappio F (1992) Molecular investigation of the cytokines produced by normal and malignant B lymphocytes. Leukemia 6:120-125
10. Biondi,A. Rossi V, Bassan,R. Barbui,T. Bettoni,S. Sirono,M. Mantovani,A. Rambaldi A (1989) Constitutive expression of the interleukin-6 gene in chronic lymphocytic leukemia. Blood 73:1279-1284
11. Safarti M, Fournier S, Christoffersen M, Biron G (1990) Expression of CD23 antigen and its regulation by IL-4 in chronic lymphocytic leukemia. Leuk Res 14:47-55

12. Foa R, Massaia M, Cardona S, Tos AG, Bianchi A, Attisano C, Guarini A, di Celle PF, Fierro MT (1990) Production of tumor necrosis factor-alpha by B-cell chronic lymphocytic leukemia cells: A possible regulatory role of TNF in the progression of the disease. Blood 76:393-400
13. Hahn T, Kusminsky G, Bassous L, Berrebi A (1991) Tumor necrosis factor production in B cell chronic lymphocytic leukemia. Leuk Lymphoma 4:117-121
14. Lahat N, Aghai E, Maroun B, Kinarty A, Quitt M, Froom P (1991) Increased spontaneous secretion of IL-6 from B cells of patients with B chronic lymphatic leukaemia (B-CLL) and autoimmunity. Clin exp Immunol 85:302-306
15 Lantz O, Grillot-Courvalin C, Schmitt C, Fermand J-P, Brouet J-C (1985) Interleukin-2 induced proliferation of leukemic B cells. J. Exp. Med. 161: 1225-1230
16. Carlsson M, Tötterman TH, Matsson P, Nilsson K (1988) Cell cycle progression of B-chronic lymphocytic leukemia cells induced to differentiation by TPA. Blood 71: 415-421
17. Digel W, Schöniger W, Stefanic M, Janssen H, Buck C, Schmid M, Raghavachar A, Porzsolt F (1990) Receptors for tumor necrosis factor on neoplastic B cells from chronic lymphocytic leukemia are expressed in vitro but not in vivo. Blood 76:1607-1613.
18. Heilig B, Mapara M, Brockhaus M, Krauth K, Dörken B (1991) Two types of TNF receptors are expressed on human normal and malignant B lymphocytes. Clin Immunol Immunopathol 61:260-267
19. Hirata Y, Taga T, Hibi M, Nakano N, Hirano T,Kishimoto T (1989) Characterization of IL-6 receptor expression by monoclonal and polyclonal antibodies. J Immunol 143:2900-2906
20. Brown RD, Gorenc B, Gibson J, Warburton P, Joshua D (1992) Interleukin 4 and 6 receptor expression on B cell lines and the lymphocytes of patients with B cell malignancies. Leuk Lymphoma 6: 377-383
21. Larsson L-G, Schena M, Carlsson M, Sällström J, Nilsson K (1991) Expression of the c-myc protein is down-regulated at the terminal stages during in vitro differentiation of B-type chronic lymphocytic leukemia cells. Blood 77:1025-1032
22. Larsson L-G, Carlsson M, Schena M, Lantz M, Caligaris-Cappio F, Nilsson K (1992) Interleukin-2 enhances the production of tumor necrosis factor-α in activated B-type chronic lymphocytic leukemia (B-CLL) cells. Leukemia in press.
23. Jernberg-Wiklund H, Pettersson M, Carlsson M, Nilsson K (1992) Increase in interleukin 6 (IL-6) and IL-6 receptor expression in a human multiple myeloma cell line, U-266, during long term in vitro culture and the development of a possible autocrine IL-6 loop. Leukemia 6:310-318
24. Sung S-SJ, Jung LKL, Walters JA, Chen W, Wang CY, Fu SM (1988) Production of tumor necrosis factor/cachectin by human B cell lines and tonsillar B cells. J Exp Med 168:1539-1551
25. Kehrl JH, Miller A, Fauci AS (1987) Effect of tumor necrosis factor alpha on mitogen-activated human B cells. J Exp Med 166:786-791
26. Rieckmann P,D Alessandro F, Nordan RP, Fauci AS, Kehrl JH (1991) IL-6 and tumor necrosis factor-alpha. Autocrine and paracrine cytokines involved in B cell function. J Immunol 146:3462-3468
27. Digel W, Stefanic M, Schöniger W, Buck C, Raghavachar A, Frickhofen N, Porzsolt F (1989) Tumor necrosis factor induces proliferation of neoplastic B cells from chronic lymphocytic leukemia. Blood 73:1242-1246
28. Digel W, Schöniger W, Stefanic M, Janssen H, Buck C, Schmid M, Raghavachar A, Porzsolt F (1990) Receptors for tumor necrosis factor on neoplastic B cells from chronic lymphocytic leukemia are expressed in vitro but not in vivo. Blood 76:1607-1613
29. Heilig M Mapara M, Brockhaus M, Krauth K, Dörken B (1991) Two types of TNF receptors are expressed on human normal and malignant B lymphocytes. Clin Immunol Immunopathol 61:260-267
30. Lindemann A, Ludwig W-D, Oster W, Mertelsmann R, Herrmann F (1989) High-level secretion of tumor necrosis factor-alpha contributes to hematopoietic failure in hairy cell leukemia. Blood 73:880-884
31. Hirano T, Taga T, Nakano N, Yasukawa K, Kashiwamura S, Shimizu K, Nakajima K, Pyun KH, Kishimoto T (1985) Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSFp-2). Proc Natl Acad Sci USA 82:5490-5494
32. Splawski B, McAnally,LM, Lipsky PE (1990) IL-2 dependence of the promotion of human B cell differentiation by IL-6 (BSF-2). J.Immunol 144:562-569
33. Tosato G, Gerrard TL, Goldman NG, Pike S (1988) Stimulation of EBV-activated human B cells by monocytes and monocyte products. Role of IFN-β2/B cell stimulatory factor/IL-6. J Immunol 140:4329-4336
34. Drexler HG, Brenner MK, Coustan-Smith E., Gignac SM, Hoffbrand AV,. (1988) Analysis of signal transduction in chronic lymphocytic leukemia cells. Blood 71:1461-1469
35. Ghaderi AA, Richardson P, Cardona C, Millsum MJ, Ling N, Gillis S, Ledbetter J, Gordon J. (1988) Stimulation of B-chronic lymphocytic leukemia populations by recombinant interleukin-4 and other defined growth-promoting agents. Leukemia 2:165-170
36. Defrance T, Fluckiger A-C, Rossi J-F, Rousset F, Bancgereau J (1991) In vitro activation of B-CLL cells. Leuk Lymphoma , Supplement, pp. 13-19

BCL-2 in B-Chronic Lymphocytic Leukemia

F. CALIGARIS-CAPPIO^, P. GHIA^, D. GOTTARDI^, G. PARVIS^, M. G. GREGORETTI^, K. NILSSON* and M. SCHENA^

^Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Torino, Torino, Italy and
*Department of Pathology, University of Uppsala, Uppsala, Sweden.

B-chronic lymphocytic leukemia (B-CLL) is a human B-cell malignancy characterized by the relentless accumulation of long-lived mature B cells that have two distinctive features. First, more than 99% of the circulating malignant lymphocytes are in the Go phase of the cell cycle (Andreeff et al. 1980; Carlsson et al. 1988). Second, B-CLL cells express the CD5 surface molecule (Caligaris-Cappio and Janossy 1985). Crucial to our understanding of the development and natural history of the disease is to define which is the cellular origin of B-CLL and which mechanisms favour the progressive accumulation of malignant resting CD5+ B cells. The phenotype of B-CLL cells (Caligaris-Cappio and Janossy 1985, Freedman and Nadler 1990; Schena et al. 1992) suggests a similarity with mature B lymphocytes that are found in the mantle zone of secondary follicles and lends credit to the hypothesis that the normal counterpart of B-CLL may be a long-lived, recirculating subpopulation of mantle zone B cells (Galton and MacLennan 1982). As, in adult lymphoid tissues, CD5+ B lymphocytes are located within the mantle zone of secondary follicles (Kipps et al. 1991) it is not unreasonable, though still unproven, to suggest that the CD5+ B cell population might be the actual normal counterpart of B-CLL.
In recent years, several functional features of CD5+ malignant, CD5+ normal and B mantle cells have been comparatively studied (the data are summarized in Table 1). In the present work we have investigated the expression and the significance of BCL-2 in B-CLL cells.

Table 1: comparative features of B-CLL, CD5+ normal B and B mantle zone cells.

	B-CLL	CD5+ normal B	B mantle zone
CD5 capping [a]	yes	no	--
sIg capping [b]	yes	yes	yes
CD5 modulation[a]	no	yes	--
Adhesive properties[c]	yes	no	no
F-actin organization[c]	podosomes	submembranous	submembranous
Na^+/H^+ antiporter activity d	low	high	intermediate

a: Bergui et al. 1989; b: Antin et al. 1986; c: Marchisio et al. 1988; d: Ghigo et al. 1991.

Current Topics in Microbiology and Immunology, Vol. 182

The rationale for studying BCL-2 in B-CLL

The BCL-2 gene codes for a 25 kD protein (Tsujimoto and Croce 1986, Chen-Levy et al. 1989) that is located mainly at the inner mitochondrial membrane and blocks programmed cell death (apoptosis: Hockenbery et al. 1990). The gene is translocated into the heavy chain immunoglobulin (Ig) locus in a high proportion of human follicular lymphomas (Tsujimoto et al. 1984, Bakhshi et al. 1985, Cleary et al. 1985) resulting in a high constitutive expression of BCL-2 mRNA and protein (Chen-Levy et al. 1989, Graninger et al. 1987, Seto et al. 1988).
The rationale for studying BCL-2 in B-CLL cells is at least threefold. First, transgenic mice that possess the BCL-2-Ig fusion product observed in the chromosomal breakpoint of human follicular lymphomas develop an expanded compartment of mature B cells that are 99% in the G0 phase of the cell cycle (McDonnell et al. 1989). Also, in the transgenic mouse model, BCL-2 influence the generation and maintenance of G0 B memory cells (Nunez et al. 1991). These observations document that the BCL-2-Ig transgene is able to confer a survival advantage to mature resting B cells and lead to their selective expansion. Finally, the gene is translocated into the light chain Ig loci in few (< 5 %) cases of B-CLL (Adachi et al. 1990; Raghobier et al. 1991).

Expression of Bcl-2 in B-CLL Cells

In a series of 18 consecutive B-CLL cases studied, we have observed, in accordance with literature data (Pezzella et al. 1990, Zutter et al. 1991), a high expression of BCL-2 mRNA and protein, irrespective of the clinical stage of the patients. Further, we have recently (Schena et al. 1992) shown that the levels of BCL-2 mRNA and protein expressed by malignant CD5+ B-CLL cells are comparable to those observed in cells of the Karpas 422 cell line (Dyer et al. 1990) that contains a translocated BCL-2 gene. On the contrary, BCL-2 mRNA levels are undetectable by conventional Northern Blotting in normal CD5+ B cells from cord blood.
One possible interpretation of these data is to suggest that malignant B-CLL cells may be unable to switch off the BCL-2 expression and hypothesize that BCL-2 protein may confer to B-CLL cells the survival advantage that favours their relentless accumulation. As the overexpression of BCL-2 blocks apoptosis in murine FL5.12 pro-B-lymphocytes (Hockenbery et al. 1990), it is tempting to speculate that a similar effect may be operating in B-CLL cells as well. Still, we have shown that, in two selected clones of B-CLL, the expression of BCL-2 is not constitutive, but can be consistently downregulated by mitogenic stimulation (Schena et al. 1992). Thus, the expression of BCL-2 in B-CLL cells appears to mirror the modifications that occur in normal B cells (Schena et al. 1992), where resting mantle zone B cells are BCL-2 negative and activated proliferating germinal center B cells are BCL-2 positive. Therefore, the expression of BCL-2 in resting and activated B-CLL cells may simply reflect the normal B cell population from which B-CLL has arisen. Recent data (Schena et al. 1992) link BCL-2 expression with mantle zone normal B cells and show that normal CD5+ B cells are BCL-2 negative. However, the CD5+ B cells studied (Schena et al.

1992) have been obtained from cord blood and have an activated phenotype: B cells activated within germinal centers downregulate BCL-2 (Pezzella et al 1990; Zutter et al. 1991; Schena et al. 1992). Thus, CD5+ normal B cells from adult lymphoid organs are also to be studied in order to evaluate whether the BCL-2 negativity merely reflects the activation of B cells or whether instead it is a feature consistently associated with the CD5+ normal B cell phenotype. If the latter hypothesis holds true, the BCL-2 positivity of B-CLL cells might be a malignancy-associated property.

The Mechanisms Controlling the Overexpression of Bcl-2 in B-CLL Cells

We have asked which mechanisms control the overexpression of BCL-2 in B-CLL cells. To this end, DNA was extracted from Ficoll-Hypaque separated cells obtained from 16 B-CLL cases in different stages of their diseases (Rai et al. 1975) and analyzed in Southern Blotting. Two different BCL-2 probes were used: a 2.8 Kb EcoRI-HindIII genomic fragment corresponding to the major breakpoint region (MBR) in the untranslated 3' end of the BCL-2 gene and a 1.6 kb EcoRI-EcoRI fragment corresponding to the 5' end breakpoint region (kind gifts of Dr. C. Croce, Jefferson University, Philadelphia, PA). None of the cases showed BCL-2 rearrangement within the MBR and the 5' end breakpoint region. Thus, in agreement with recently reported data (Raghobier et al. 1991) and in contrast with follicular lymphomas (Tsujimoto et al. 1984, Bakhshi et al. 1985, Cleary et al. 1985), the level of BCL-2 expression in B-CLL does not usually reflect a rearrangement of BCL-2 gene, which appears to be a rare event in CLL cells.

We have then explored the possibility that the overexpression of BCL-2 in B-CLL cells might depend upon a higher stability of BCL-2 mRNA leading to its prolonged half-life. B-CLL cells from 4 different cases as well as Nalm-6 cells which overexpress BCL-2 without having the t(14;18) translocation and normal B cells purified from the mantle zone of tonsil follicles (Schena et al. 1992) were cultured in presence of 10 ug/ml actinomycin D (Seto et al. 1988). The cells were harvested at 0, 2, 3, 4, 6, 8 h, RNA was extracted and 10 ug total RNA were hybridized with the MBR probe. The half life of BCL-2 mRNA was 2 and half h in B-CLL, in Nalm-6 cells (Fig. 1) as well as in normal B cells (data not shown).

These data rule out two classical reasons underlying the overexpression of a gene in malignant cells. The possibility remains that point mutations (Tanaka et al., 1992) in the promoter region may explain the overexpression. To this end, we are now screening PCR amplified fragments with Denaturing Gradient Gel Electrophoresis and Single Strand Conformation Polimorphism.

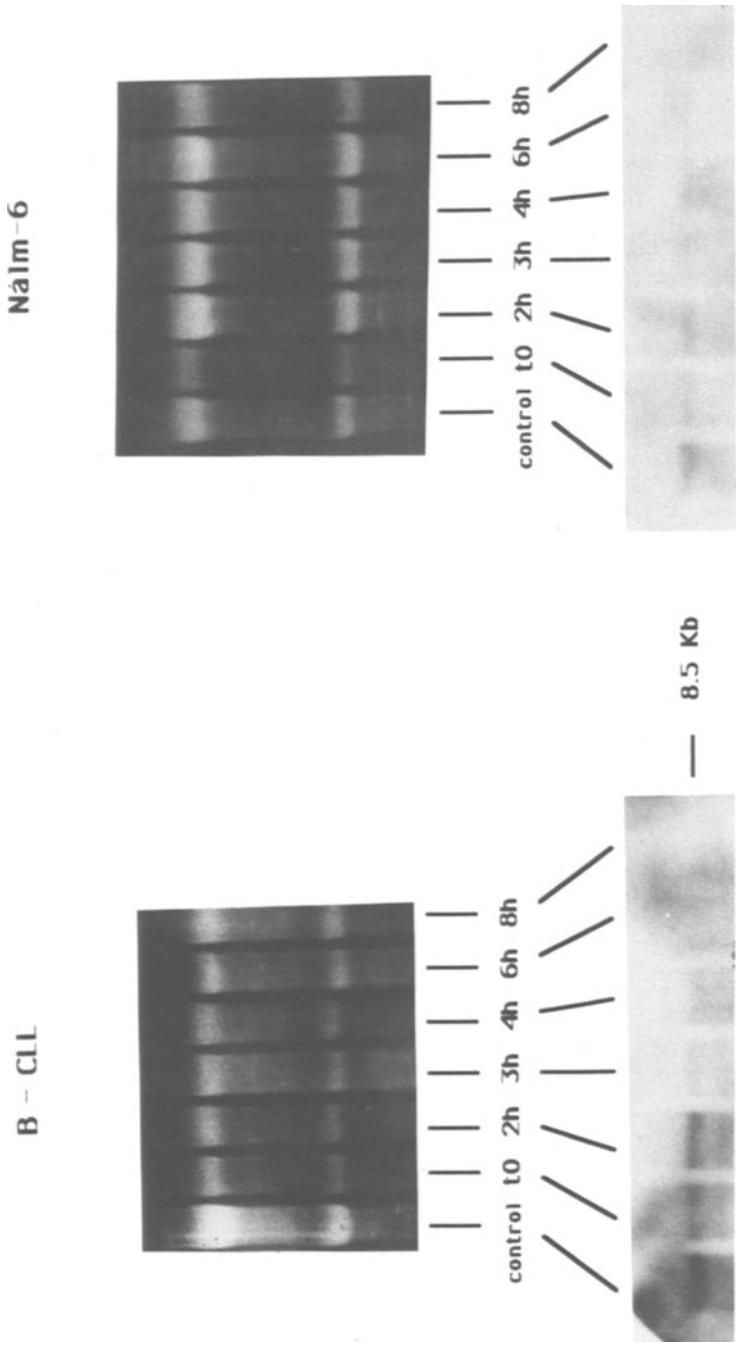


Fig. 1: BCL-2 mRNA half life in B-CLL and Nalm-6 cells.

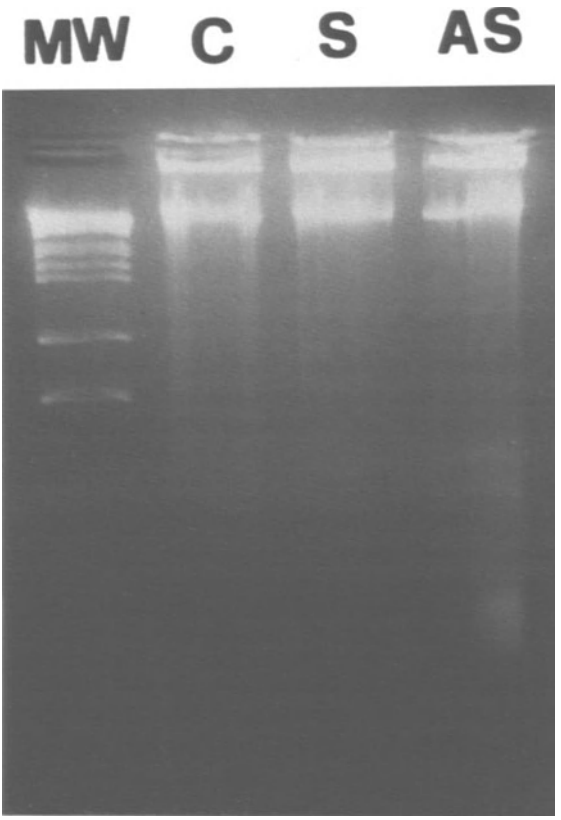


Fig. 2: B-CLL cells cultured for 4 days in presence of 12.5 uM sense (S) and antisense (AS) BCL-2 oligodeoxynucleotides. AS, but not S, nor cells grown in medium only (control: C) show a partial internucleosomal DNA cleavage.

Has BCL-2 overexpression a role in the natural history of B-CLL ?

As discussed above, it is by no means clear whether BCL-2 overexpression in B-CLL cells has any specific role in the progressive accumulation of the G0 cells or whether it merely reflects a feature of B-CLL normal counterpart. The role of BCL-2 as a survival oncogene (Hockenbery et al. 1990) and the observation that BCL-2 negative germinal center B cells, rescued from apoptosis, upregulate BCL-2 expression (Liu et al. 1992) lead to suggest that BCL-2 expression might save B-CLL cells from undergoing apoptosis. BCL-2 positive B-CLL cells would thus become long-lived elements "immortalized" in the G0 phase of the cell cycle and progressively accumulate.
In order to understand whether BCL-2 is actually involved in preventing apoptosis of B-CLL cells, malignant peripheral blood B lymphocytes from 4 different patients were separated on Ficoll-Hypaque gradient and cultured in serum-free RPMI-1640 at the concentration of 2 x 10^6 cells/ml for 6 days. Eighteen nucleotide phosphorotioate BCL-2 sense and antisense oligodeoxynucleotides complementary to the translation-initiation site of human BCL-2 were added at the concentration of 12.5 and 25 uM with the aim of downregulating BCL-2 expression. Cells were harvested at 24, 48, 72, 96 h and after 6 days of culture. DNA was extracted and electrophoresed on 2% agarose gel: the gels were photographed under UV light to evaluate the internucleosomal DNA cleavage as an indicator of apoptosis. The concentration of 25 uM oligonucleotydies was toxic for > 50% cells after 4 days of culture. On the contrary, with the 12.5 uM antisense BCL-2 oligodeoxynucleotide concentration, a proportion of cells underwent apoptosis (Fig. 2) in one case: the percentage of cells undergoing apoptosis was about 30% of the total cell population exposed to the antisense oligo. This observation suggests that indeed BCL-2 may be involved in preventing apoptosis in malignant B-CLL cells. However, they also indicate that the regulation of cell death and cell survival in B-CLL cells is a complex phaenomenon which may not be uniquely due the overexpression of BCL-2.

Acknowledgements: this work was supported by grants from Associazione Italiana Ricerca Cancro to F.C.C. and from Swedish Cancer Society to K.N. D.G. and M.G.G. are recipients of a fellowship from G. Ghirotti Foundation.

References

Adachi M, Tefferi A, Greipp PR, Kipps TJ, Tsujimoto Y (1990) Preferential linkage of BCL-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. J Exp Med 171:559-564.

Andreeff M, Darzynkiewicz Z, Sharpless TK, Clarkson BD, Melamed MR (1980) Discrimination of human leukemia subtypes by flow cytometric analysis of cellular DNA and RNA. Blood 55:282-289.

Antin JH, Emerson SG, Martin P, Gadol N, Ault K (1986) Leu-1+ (CD5+) B cells. A major lymphoid subpopulation in human fetal spleen: phenotypic and functional studies. J Immunol 136:505-510.

Bakhshi A A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: Clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41:899-906.

Bergui L, Tesio L, Schena M, Riva M, Malavasi F, Schulz T, Marchisio PC, Caligaris-Cappio F (1988) CD5 and CD21 molecules are a functional unit in the cell/substrate adhesion of B-CLL. Eur J Immunol 18: 89-96.

Caligaris-Cappio F, Janossy G (1985) Surface markers in chronic lymphoid leukemias of B cell type. Seminars in Hematol 22:1-12.

Carlsson M, Totterman TH, Matsson P, Nilsson K (1988) Cell cycle progression of B-chronic lymphocytic leukemia cells induced to differentiate by TPA. Blood 71:415-421.

Chen-Levy Z, Nourse J, Cleary ML (1989) The *bcl*-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. Mol Cell Biol 9:701-710.

Cleary ML, Sklar J (1985) Nucleotide sequence of a t(14:18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA 82:7439-7443.

Cleary ML, Smith SD, Sklar J (1986) Cloning and structural analysis of cDNAs for *bcl*-2 and a hybrid *bcl*-2/Immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19-28.

Dyer MJS, Fischer P, Nacheva E, Labastide W, Karpas A (1990) A new human B-cell non-Hodgkin's lymphoma cell line (Karpas 422) exhibiting both t(14;18) and t(4;11) chromosomal translocations. Blood 75:709-714.

Freedman AS, Nadler LM (1990) The relationship of chronic lymphocytic leukemia to normal activated B cells. Leuk Lymph 1:293-298.

Galton DAG, MacLennan ICM (1982) Clinical patterns of B cell malignancy Clin Hematol 11:561-587.

Ghigo D, Gaidano GL, Treves S, Bussolino F, Pescarmona G, Caligaris-Cappio F, Bosia A (1991) Na+/H+ antiporter has different properties in human B lymphocytes according to CD5 expression and malignant phenotype. Eur J Immunol 21:583-588.

Graninger WB, Seto M, Boutain B, Goldman P, Korsmeyer SJ (1987) Expression of *bcl*-2 and *bcl*-2-Ig fusion transcripts in normal and neoplastic cells. J Clin Invest 80:1512-1515.

Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334-336.

Kipps TJ, Duffy SF (1991) Relationship of the CD5 B cell to human tonsillar B lymphocytes that express autoantibody-associated cross-reactive idiotypes. J Clin Invest 87:2087-2095.

Korsmeyer SJ, McDonnell TJ, Nunez G, Hockenbery D, Young R (1990) Bcl-2: B cell life, death and neoplasia. Current Topics in Microbiol Immunol 166:203-211.

Liu YJ, Mason DY, Johnson GD, Abbot S, Gregory CD, Hardie DL, Gordon J, MacLennan ICM (1991) Germinal center cells express bcl-2-protein after activation by signals which prevent their entry to apoptosis. Eur J Immunol 21:1905-1910.

Marchisio PC, Bergui L, Corbascio G, D'Urso N, Tesio L, Schena M, Caligaris-Cappio F (1988) LFA1, vinculin and talin co-localization at specific adhesion sites in the malignant B lymphocytes of B-CLL. Blood 72,:830-833.

McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP, Korsmeyer SJ (1989) *bcl*-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57:79-88.

Nunez G, London L, Hockenbery D, Alexander M, McKearn JP, Korsmeyer SJ (1990) Deregulated *bcl*-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. J Immunol 144:3602-3610.

Nunez G, Hockenberry D, McDonnell TJ, Sorensen CM, Korsmeyer SJ (1991) Bcl-2 maintains B cell memory. Nature 353: 71-73.

Pezzella F, Tse AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY (1990) Expression of the *bcl*-2 oncogene protein is not specific for the 14;18 chromosomal translocation. Am J Pathol 137:225-232.

Raghobier S, van Krieken JHJM, Kluin-Nelemans JC, Gillis A, van Ommen GJB, Ginsberg AM, Raffeld M, Kluin PM (1991) Oncogene rearrangements in chronic B-cell leukemia. Blood 77:1560-1564.

Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS (1975) Clinical staging of chronic lymphocytic leukemia. Blood 46:219-234.

Reed JC, Tsujimoto Y, Alpers JD, Croce CM, Nowell PC (1987) Regulation of *bcl*-2 proto-oncogene expression during normal human lymphocyte proliferation. Science 236:1295-1299.

Schena M, Gaidano G, Gottardi D, Malavasi F, Larsson LG, Nilsson K, Caligaris-Cappio F (1992) Molecular investigation of the cytokines produced by normal and malignant B lymphocytes. Leukemia 2:120-125.

Schena M, Larsson LG, Gottardi G, Gaidano G, Carlsson M, Nilsson K, Caligaris-Cappio F (1992) Growth and differentiation-associated expression of BCL-2 in B-chronic lymphocytic leukemia cells. Blood, in press.

Seto M, Jaeger U, Hocket RD, Graninger W, Bennett S, Goldman P, Korsmeyer SJ (1988) Alternative promoters and exons, somatic mutation and deregulation of the *bcl*-2-Ig fusion gene in lymphoma. EMBO J 7:123-131.

Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226: 1097-1099.

Tsujimoto Y, Croce CM (1986) Analysis of the structure, transcripts, and protein products of *bcl*-2, the gene involved in human follicular lymphoma. Proc Natl Acad Sci USA 83:5214-5418.

Tsujimoto Y, Bashir MM, Givol I, Cossman J, Jaffe E, Croce CM (1987) DNA rearrangements in human follicular lymphoma can involve the 5' or the 3' region of the *bcl*-2 gene. Proc Natl Acad Sci USA 84:1329-1331.

Tanaka S., Louie D.C., Kant J.A. and Reed J.C. (1992) Frequent incidence of somatic mutations in the translocated BCL2 oncogenes of non-Hodgkin's lymhomas. Bllod 79:229-237.

Vaux DL, Cory S, Adams JM (1988) *bcl*-2 gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. Nature 335:440-442.

Zutter M, Hockenberry D, Silverman GA, Korsmeyer SA (1991) Immunolocalization of the Bcl-2 protein within hematopoietic neoplasms. Blood 78:1062-1068.

Subcellular Localization of *bcl-2* Protein

D. DE JONG, F. PRINS, H. H. J. M. VAN KRIEKEN, D. Y. MASON[1], G. B. VAN OMMEN[2], P. M. KLUIN

Laboratory of Pathology, University of Leiden, Leiden, The Netherlands.
[1]Department of Haematology, John Radcliffe Hospital, Headington, Oxford, United Kingdom.
[2]Department of Human Genetics, University of Leiden, Leiden, The Netherlands.

The bcl-2 oncogene is normally transiently expressed both in lymphoid and myeloid cells as well as in various epithelia (Hockenbery et al. 1991). It may have a function in the transition of dividing (precursor) cells to mitotically inactive and relatively longliving daughter cells (Vaux et al., 1988, Nunez et al. 1991). The protein acts through protection against programmed cell death (apoptosis). Deregulation by the t(14;18) translocation in lymphoid malignancies induces inappropriate cell survival and serves as one of the steps towards a fully malignant behaviour.

The bcl-2 gene encodes two proteins, bcl-2α and bcl-2ß. The bcl-2α protein consists of 239 aminoacids (26kD) and is most abundantly present in lymphoid cells. The bcl-2ß protein consists of 205 aminoacids (22 kD). The two forms are identical except for their carboxy terminus (Tsujimoto and Croce, 1986). Protein structure analysis suggests that the bcl-2α protein has a hydrophobic carboxy-terminal membrane-spanning motif and a hydrophilic domain extending only at the cytoplasmic surface (Chen-Levy et al. 1989; Tsujimoto et al. 1987).

Membrane separation techniques and immuno-precipitation have been used by several groups to study the detailed localization of the protein within the cell. A relation to mitochondrial inner membranes was found by Hockenbery et al., while others demonstrated bcl-2α protein in the separated membrane fractions containing nuclear envelope, plasma membrane and endoplasmic reticulum of t(14;18) translocated cells (Tsujimoto et al. 1987; Chen-Levy et al. 1989; Hockenbery et al. 1990).

We used both pre- and postembedding immuno-electronmicroscopy on normal blood peripheral lymphocytes, CLL-cells and on a lymphoid cell-line with a t(14;18) translocation (DoHH2, Kluin-Nelemans, 1991) to study subcellular distribution of the protein.

pre-embedding

- 2% paraformaldehyde-lysine-periodate (PLP), 1 h, 4°C
- pelleted, 10% gelatin, post-fixation in PLP (ON)
- snap-freezing, 7µm frozen sections
- 2-step immunoperoxidase: anti-bcl-2, peroxidase conjugate, diaminobenzidine (positive control CD45)
- postfixation in OsO4
- embedding in in EPON 812, ultrathin sectioning
- analysis of serial utrathin sections by reflection-contrast light microscopy and EM

post-embedding

- 2% paraformaldehyde-lysise-periodate (PLP), 1 h, 4°C
- pelleted, 10% gelatin, post-fixation in PLP (ON)
- 2.3M sucrose/PBS
- snap-freezing, 60 nm frozen sections
- 3-step immunogold labeling: anti-bcl-2, rabbit-anti-mouse, goat-anti-rabbit, 15nm gold
- methylcellulose and uranyl acetate
- EM analysis and counting of particles

With reflection-contrast light microscopy on the same preparations as used for pre-embedding EM-studies, we could confirm the patchy intracytoplasmic immunoreactivity in all tree types of lymphoid cells studied. Immuno-electron-microscopy both with pre- and post-embedding labelling techniques showed immunoreactivity on the circumference of the mitochondria, on the nuclear envelope and also on the cell membrane. There was no significant staining of the mitochondrial cristae and matrix. There was no essential difference in staining pattern between normal peripheral blood lymphocytes on one hand and CLL-cells and DoHH2-cells with a known high expression of bcl-2 on the other hand. In CLL, additional staining of endoplasmic reticulum and intra-cytoplasmic vesicles was seen, which was interpreted as intracellular processing of the protein and reflecting the high protein expression.

Using pre-embedding staining, bcl-2 showed a non-homogeneous, patchy, staining of the mitochondrial outer structures, suggestive of association with contact zones. These are highly stable areas in which the mitochondrial outer and inner membrane closely approximate and are involved in the transport of precursors from the cytosol into the mitochondrial matrix (Schleyer and Neupert 1985; Pain et al. 1988).

Membrane spanning proteins, containing receptor-like extra-mitochondrial domains and membrane-embedded domains have been identified in yeast and Neurospora crassa (Hawlitschek et al. 1988; Murakami et al. 1990; Pfanner et al. 1991). Sequence comparison of bcl-2α aminoacid sequences and a putative Yeast mitochondrial import receptor and phosphate carrier (mir-1) (Pain et al. 1988, Murakami et al. 1990)showed an overall homology. In contrast to mir-1, a phosphate carrier motif was lacking.

Table 1 Quantitation of immunogold-staining in 17 peripheral blood lymphocytes

	relative surface	absolute number of particles	relative staining/ relative surface area
mitochondria	4.2%	196	3.13 (32.0%)
nucleus	50.6%	663	0.88 (9.0%)
nuclear envelope	3.9%	206	3.56 (36.4%)
cytoplasm	35.9%	295	0.55 (5.6%)
cell membrane	5.4%	132	1.65 (16.8%)

In 17 lymphocytes, relative surfaces of the cell membrane, cytoplasm, mitochondria, nuclear membrane and nucleus were assessed by point-hit counting. Gold particles were attributed to these areas with the same hit-margin as the cross sections in point-hit counting.

These data suggest that bcl-2 may belong to a group of proteins involved in import of precursors across membranes. Association of bcl-2 to mitochondrial contact sites could also very well explain the association of bcl-2α immunoreactivity with mitochondrial inner membrane fractions in membrane separation experiments (Hockenbery et al. 1990), since these sites are highly stable and remain associated with inner membrane after shearing off of the outer membranes (Schleyer and Neupert, 1985).

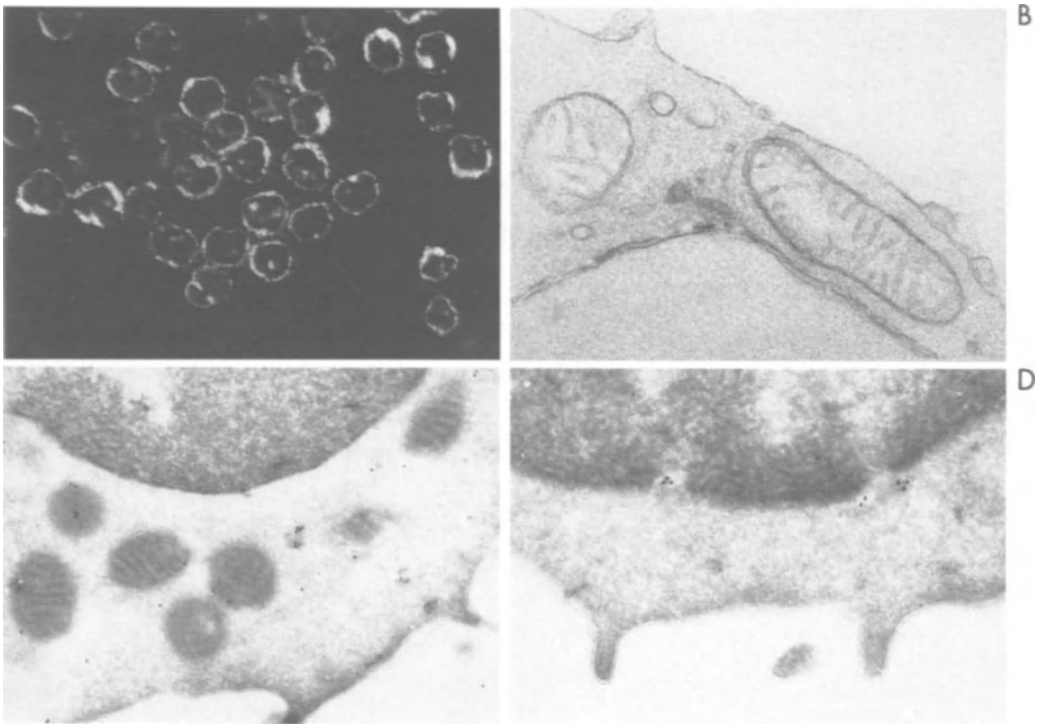


Fig.1.
pre-embedding DAB/bcl-2-staining of mitochondria and nuclear membranes of blood peripheral lymphocytes.

A: reflection-contrast light microscopy of an ultrathin section of a cryostat-preparation of peripheral blood lymphocytes shows bright, intra-cytoplasmic, non-homogeneous bcl-2 staining as visualized by DAB-complexes. No intra-nuclear staining is seen.
B: immuno-electron-microscopy on a sequential section of the same sample as A showing patchy localization of bcl-2 along the nuclear envelope as well as on the outer circumference of mitichondria (original magnification 26000x)

post-embedding immunogold/bcl-2-staining of mitochondria and nuclear membranes of blood peripheral lymphocytes.

C: bcl-2 distribution as visualized by immuno-gold labeling shows essentially the same distribution as seen with the DAB-technique. Preferential staining along the mitochondrial outer circumference is seen as well as staining along the nuclear envelope. (original magnification 22000x).
D: immuno-electron-microscopy as in C: immuno-gold-complexes may be associated with nuclear pores. (original magnification 26000x)

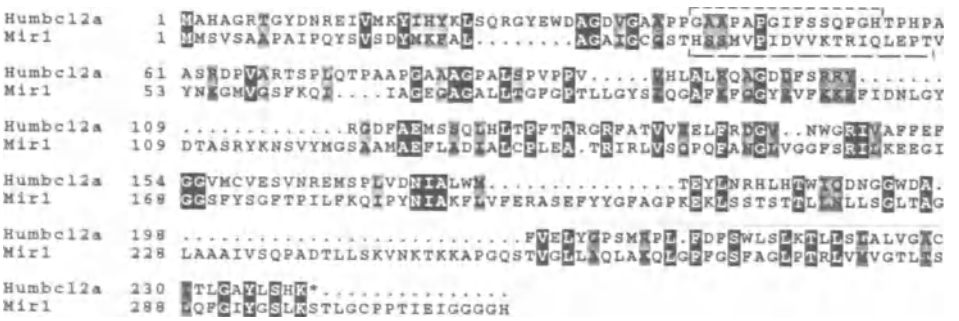


Fig.2. AALIGN-protein analyis of human bcl-2 (upper panel) and the putative Yeast mitochondrial import receptor and phosphate carrier mir-1 (lower panel).

-------- sequence against which the bcl-2 antibody used was raised.
— — — phosphate carrier motif in mir-1
——— hydrophobic membrane embedded domain in bcl-2
■ full aminoacid homology
conserved/similar aminoacid residues

References

Chen-Levy, Z., Nourse, J., Cleary, M.L., (1989) The bcl-2 canditate proto-oncogene product is a 24 kD integral membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. Mol.Cell.Biol. **9**, 701-710

Hawlitschek G., Schneider H., Schmidt B., Tropschug M., Hartl F-U., Neupert, N., Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. (1988) **53**, 795-80.

Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D., Korsmeyer, S.J., Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. (1990) Nature, **348**, 334-336

Hockenbery, D., Zutter, M., Hickey, W., Nahm, M., Korsmeyer, S.J., Bcl-2 protein is topograffically restricted in tissues characterized by apoptotic cell death. (1991) Proc.Natl.M. Acad.Sci. **88**, 6961-6965

Kluin-Nelemans, H.C., Limpens, J., Meerabux J., Beverstock, G.C., Jansen, J.H., de Jong, D., Kluin, Ph.M., a new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18)(q32;q21) (1991) Leukemia **5**, 221-224.

Murakami, D., Blobel G., Pain, D., Isolation and characterization of the gene for a yeast mitochondrial import receptor. (1990) Nature, **347**, 488-491

Nunez, G., Hockenbery, D., McDonnell, T.J., Sorensen, C.M., Korsmeyer, S.J., Bcl-2 maintains B-cell memory (1991) Nature, **353**, 71-73

Pain, D., Yashpal, Y.S., Blobel G., Identification of a receptor for protein import into chloroplasts and its localization to envelope contact zones. (1988) Nature, **331**, 232-237.

Pfanner, N., Sollner, T., Neupert, W. Mitochondrial import receptors for precursor proteins. (1991) TIBS **16**, 63-67.

Schleyer, Neuport W., Transport of proteins into mitochondria: translocation intermediates spanning contact sites between outer and inner membranes (1985) Cell, **43**, 339-350

Tsujimoto, Y., Croce, C.M., Analysis of the structure, transcripts and protein products of bcl-2, the gene involved in human follicular lymphoma. (1986) Proc.Natl.Acad.Sci., **83**, 5214-5218

Tsujimoto, Y., Ikegaki, N., Croce, C.M., Characterization of bcl-2, the gene invoved in human follicular lymphoma. (1987) Oncogene, **2**, 3-7

Vaux, D.L., Cory, S., Adams, J.M., Bcl-2 promotes haematopoietic cell survival and cooperates with c-myc to immortalize pre-B-cells. (1988) Nature, **335**, 440-442

Collaboration of PIM-1 and BCL-2 in Lymphomagenesis

D. ACTON[1], J. DOMEN[1], H. JACOBS[1], M. VLAAR[1], S. KORSMEYER[2] and A. BERNS[1]

[1]Division of Molecular Genetics of the Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, The Netherlands;
[2]The Howard Hughes Medical Institute and the Department of Medicine and Molecular Microbiology, Washington University School of Medicine, 660 South Euclid, St. Louis, Missouri 63110, USA.

Introduction

Previously, we have shown that pim-1 transgenic mice are predisposed to lymphomagenesis. Although the spontaneous tumor incidence in pim-1 transgenic mice is extremely low, tumor induction by carcinogens (Breuer et al., 1989; 1991) or murine leukemia virus infection (van Lohuizen et al., 1989) showed a dramatic acceleration of disease in the transgenic mice as compared to nontransgenic littermates. Either the proto-oncogenes c-myc or N-myc were found to be activated in the MuLV-induced T-cell lymphomas in pim-1 transgenic mice, whereas activation of myc by proviral insertion was only found in a fraction of the lymphomas induced in nontransgenic littermates. This suggested that pim-1 and myc were efficient collaborators in MuLV-induced T-cell lymphomas. Support for the strong synergistic interaction between pim-1 and myc came also from MuLV-induced tumor acceleration studies in $E\mu$-myc transgenic mice. Here activation of the pim-1 proto-oncogene was frequently found in pre-B cell lymphomas (van Lohuizen et al., 1991). This was further substantiated in crossbreeding experiments without the use of any tumor-accelerating agent: double transgenic mice carrying both an activated pim-1 and c-myc oncogene died in utero from lymphoblastic leukemia (Verbeek et al., 1991). The phenotype of crosses between pim-1 and either N-myc or L-myc was somewhat less severe, in agreement with the reduced oncogenicity of the myc oncogenes in the order c-myc > N-myc > L-myc as was observed in cell culture (Möröy et al., 1991). Transplantation of leukemic cells from double transgenic embryos carrying the pim-1 and c-myc oncogenes indicated that additional events were involved in the progression of these tumors to full malignancy. MuLV infection of $E\mu$-myc and $E\mu$-pim-1 transgenic mice has uncovered a number of new genes that collaborate with myc and pim-1 in transformation. These encompass bmi-1, pal-1 (van Lohuizen et al., 1991) and others which are currently being identified (Jonkers & Berns, unpublished results). However, other oncogenes that can contribute to

these tumor types, such as myb, abl, and bcl-2 appeared not to be involved. We were particularly intrigued by the lack of involvement of bcl-2, which was previously shown to collaborate with c-myc in vitro (Vaux et al., 1988) and in vivo. Evidence for collaboration between c-myc and bcl-2 in vivo came from the presence of c-myc rearrangements in immunoblastic lymphomas that spontaneously developed in bcl-2-Ig mice (McDonnell et al., 1991). Additional support came from crossbreeding of Eμ-myc and Eμ-bcl-2: A high incidence of primitive lymphoid tumors was found in double transgenic mice (Strasser et al., 1990). Here we report on the interaction of pim-1 and bcl-2 in crosses between pim-1 and bcl-2 transgenic mice and on the MuLV-induced tumorigenesis in these mice.

Crossbreeding of Eμ-pim-1 and bcl-2-Ig Transgenic Mice.

Both Eμ-pim-1 and bcl-2-Ig transgenic mice have been shown to exhibit a very low tumor incidence. Mice overexpressing these oncogenes show only minor (pim-1) or moderate (bcl-2) changes in their hematopoietic compartment. Whereas Eμ-pim-1 transgenic mice exhibit a somewhat enlarged spleen with normal ratios of T- and B-cells, bcl-2-Ig transgenic mice show an increased number of B-cells, probably as the result of the increased lifespan of cells overexpressing the bcl-2 oncogene and contributing to the prolonged memory seen in these mice (McDonnell et al., 1989; Nuñez et al., 1991; Strasser et al., 1991). Eμ-pim-1 transgenic mice show a low incidence of T-cell lymphomas; bcl-2-Ig transgenic mice tend to develop B-cell malignancies. The differences in the target cell population for transformation in Eμ-pim-1 and bcl-2-Ig transgenic mice is not a reflection of the expression patterns of these transgenes. The Eμ-pim-1 transgene is expressed in both the T-cell, the B-cell, and the myeloid lineage, whereas the Eμ-bcl-2 transgene is expressed in the B-cell compartment and, to a somewhat lesser extent, in T-cells.

The tumor incidences of Eμ-pim-1 and bcl-2-Ig single and double transgenic mice are depicted in figure 1. Co-expression of pim-1 and bcl-2 strongly accelerated tumorigenesis in these mice. However, the long and variable latency period (average 30 weeks) indicated that additional events were required for tumorigenesis. To determine what tumor types were induced FACS analyses were performed on fresh tumor cells with a number of T- and B-cell-specific cell surface markers. In addition, we determined the rearrangement status of the T-cell receptor β-chain gene, the μ immunoglobulin heavy chain gene and the κ immunoglobulin light chain gene. The results of this analysis are depicted in table 1.

In double transgenic mice a variety of tumors were observed, ranging from pro-B cell types (B220+;CD4+) to mature sIg-positive immunoblasts. A

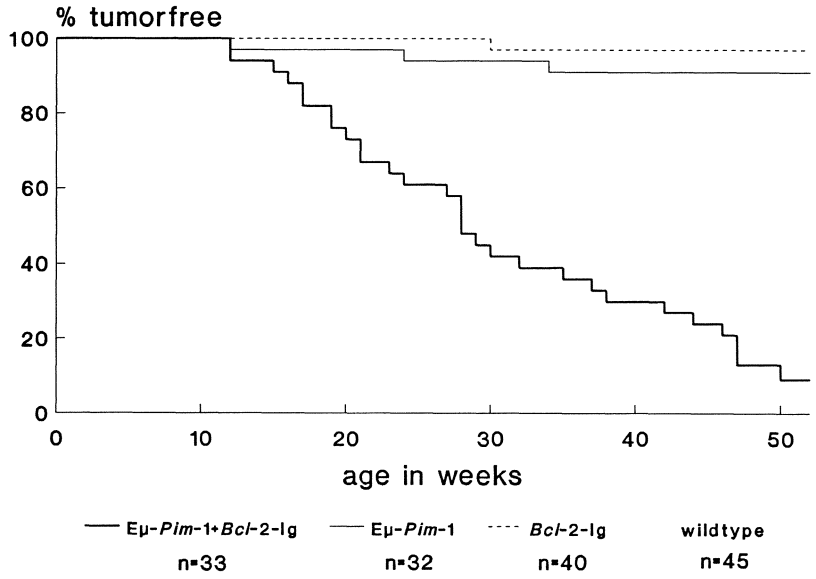


Fig. 1. Lymphoma incidence in offspring from matings between heterozygous Eμ-pim-1 and bcl-2-Ig transgenic mice. n indicates the number of mice monitored for each group.

significant fraction of the tumors constituted T-cell lymphomas expressing the T-cell receptor complex at their cell surface. Apparently, the combination of bcl-2 and pim-1 can affect many different cell types. In view of the specific immortalizing properties assigned to bcl-2 this indicates that pim-1 can contribute to the transformation of cells of very different stages of lymphoid differentiation. The occurrence of tumors with early lymphoid markers is in agreement with the notion that pim-1 knock-out mice appear to have undergone a size reduction in this compartment as measured by colony assays in vitro using IL-7 or IL-7 + SCF (Domen, in preparation), whereas pim-1 transgenic mice show an expansion of that same early compartment.

MuLV infection of Eμ-pim-1 and Bcl-2-Ig Transgenic Mice

Offspring from matings between heterozygous Eμ-pim-1 and bcl-2-Ig transgenic mice were infected with MuLV and monitored for their tumor incidence and tumor type. The results of infection of Eμ-pim-1 transgenic mice have been reported previously. The tumors in these mice, which are found 7-8 weeks after infection with MuLV, constitute CD4+CD8+ double

Table 1. Lymphomas in Eμ-*pim*-1 and *bcl*-2-Ig double transgenic mice

Mice No.	Mice %	Surface marker expression	Gene configuration[a] TCRβ;	μIg;	κIg	Cell type
11	55	B220; sIg; Ia	G;	R;	R	B-cell
5	25	TCR$\alpha\beta$;CD3;CD4/CD8	R;	G/R[b];	G	T-cell
2	10	Mixed[c]	R;	R;	R	B-and T-cell
2	10	B220; CD4	G;	G;	G	non-B, non-T-cell

[a]G, germline configuration; R, rearranged.
[b]In 3 mice μIg rearrangements were observed.
[c]Mice developed mixed lymphomas: 30-50% of cells expressed B220; sIg and 30-50% of cells expressed CD3; TCR$\alpha\beta$.

or CD4+ or CD8+ single positive cells, in which either c-myc or N-myc is activated by proviral insertion. Remarkably, MuLV-induced tumorigenesis in bcl-2-Ig transgenic mice did not significantly differ from the tumor incidence observed in control nontransgenic littermates (see figure 2), suggesting that the overexpression of bcl-2 is not an important predisposing factor for MuLV-induced disease. At present, it is unclear why we see a rather strong synergism between bcl-2 and pim-1 in crossbreeding experiments, whereas such synergism is not apparent in MuLV-induced disease. This was corroborated by the coincidence of the tumor latency curves of MuLV-infected Eμ-pim-1 and Eμ-pim-1/bcl-2-Ig transgenic mice. Tumors were exclusively of T-cell origin as is the case for pim-1 transgenic mice. The vast majority showed expression of CD3 and CD4, whereas the remainder showed expression of CD3 and CD4 + CD8. This was surprising in view of earlier observations in Eμ-pim-1 trangenic mice in which a preponderance for immature T cell tumors was found. At present, it is unclear whether this is caused by the co-expression of bcl-2 and pim-1 or due to the mixed genetic background of the transgenic mice (C3H, C57Bl/6, CBA). One intriguing possibility is that pim-1/bcl-2 positive T-cells in which myc is activated by proviral insertion (see below) become refractory to negative selection in the thymus resulting in tumors of predominantly T-helper origin. Superantigen stimulation of the CD4+ cells could add to their selective outgrowth. This mechanism of action would predict that the Vβ repertoire in the tumors is skewed to a subset which is normally deleted by negative selection. Therefore 13 tumors were screened for the expression of Vβ chain genes. A variety of different TCR Vβ chain genes were found to be expressed, indicating that selective outgrowth of cells carrying only a small subset of Vβ chain genes had not occurred.

Common proviral insertion sites that were found to be occupied in tumors of pim-1/bcl-2 double transgenic mice were N-myc, c-myc, and pal-1, the

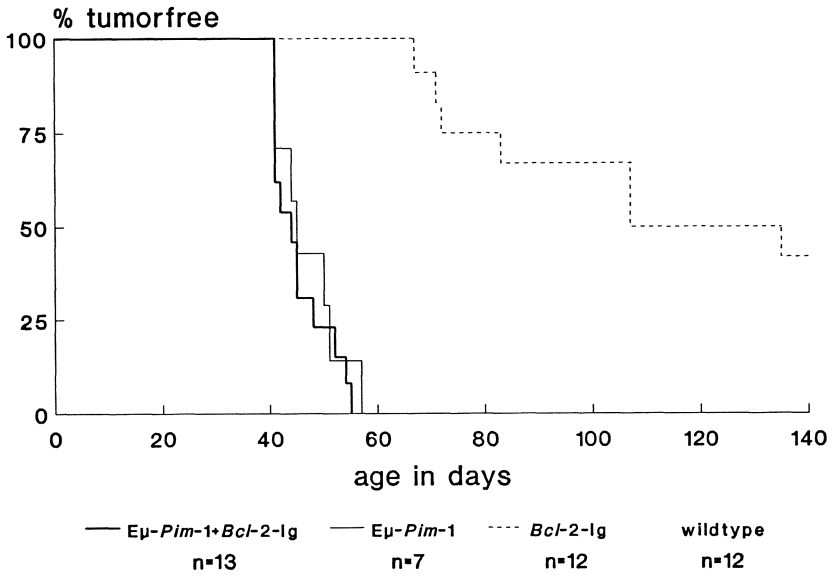


Fig. 2. Lymphoma incidence after MuLV infection of offspring from heterozygous Eμ-pim-1 and bcl-2-Ig matings. n indicates the number of mice monitored for each group.

same loci that were preferentially found in MuLV-induced T-cell lymphomas in Eμ-pim-1 transgenic mice. Remarkably, the majority of tumors showed the activation of N-myc rather than c-myc. Whether this relates to the subtype of tumors found in these mice or to other factors is at present unknown.

With respect to tumorigenesis in mice overexpressing the pim-1 and bcl-2 oncogene it is worth noting that in addition to the earlier reported synergism between pim-1 and myc we here observe collaboration between pim-1 and bcl-2 as well. This indicates that pim-1 overexpression can enhance or complement both the effects of oncogenes promoting cell proliferation (myc) and cell survival (bcl-2). This places pim-1 in an intriguing "intermediate" position with respect to the pathways in which myc and bcl-2 act. We are currently determining the effects of co-expression of pim-1 and bcl-2 on the longevity of lymphoid cells under various conditions in vivo and in vitro.

Acknowledgements

This work was supported by the Dutch Cancer Society

References

Breuer M, Slebos R, Verbeek S, van Lohuizen M, Wientjens E, Berns A (1989) Very high frequency of lymphoma induction by a chemical carcinogen in pim-1 transgenic mice. Nature 340:61-63

Breuer M, Wientjens E, Verbeek S, Slebos R, Berns A (1991) Carcinogen-induced lymphomagenesis in pim-1 transgenic mice: dose dependence and involvement of myc and ras. Cancer Res. 51:958-963

McDonnell TJ, Deane N, Platt FM, Nuñez G, Jaeger U, McKearn JP, Korsmeyer SJ (1989) Bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57:79-88

McDonnell TJ, Korsmeyer SJ (1991) Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14;18). Nature 349:254-256

Möröy T, Verbeek S, Ma A, Achacoso P, Berns A, Alt F (1991) Eμ N- and Eμ L-myc cooperate with Eμ pim-1 to generate lymphoid tumors at high frequency in double transgenic mice. Oncogene 6:1941-1948

Nuñez G, Hockenbery D, McDonnell TJ, Sorensen GM, Korsmeyer SJ (1991) Bcl-2 maintains B cell memory. Nature 355:71-73

Strasser A, Harris AW, Bath ML, Cory S (1990) Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 348:331-333

Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S, Harris AW (1991) Enforced Bcl-2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc Natl Acad Sci USA 88:8661-8665

van Lohuizen M, Verbeek S, Krimpenfort P, Domen J, Saris C, Radaszkiewicz T, Berns A (1989) Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. Cell 56:673-682

van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A (1991) Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. Cell 65:737-752

Vaux DL, Cory S, Adams JM (1988) Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335:440-442

Verbeek S, van Lohuizen M, van der Valk M, Domen J, Kraal G, Berns A (1991) Mice bearing the Eμ-myc and Eμ-pim-1 transgenes develop pre-B-cell leukemia prenatally. Mol Cell Biol, 11:1176-1179

The Role of *bcl*-2 in Lymphoid Differentiation and Neoplastic Transformation

A. STRASSER, A. W. HARRIS and S. CORY

The Walter and Eliza Hall Institute of Medical Research,
P.O. Royal Melbourne Hospital, Australia, 3050.

Introduction

Follicular lymphoma is one of the most common hematopoietic malignancies in the western world. Most (70-80%) of these tumors exhibit a characteristic t(14;18) chromosome translocation caused by recombination of the *bcl*-2 gene into the immunoglobulin (Ig) heavy chain locus [1-3]. The coding portion of *bcl*-2 remains unchanged but its regulation is presumably perturbed because of the influence of immunoglobulin regulatory sequences. The *bcl*-2 gene encodes a 26 kD non-glycosylated cytoplasmic protein [4, 5] which associates with membranes via its hydrophobic C-terminus [6]. It has been variously reported to be associated with the plasma membrane and perinuclear endoplasmic reticulum [5] and with the inner membrane of the mitochondrion [7]. The first clue to the unusual function of *bcl*-2 was the discovery that enforcing its expression in factor dependent cell lines enabled them to survive in the absence of growth factor [8]. This observation raised the possibility that the normal role of *bcl*-2 is to govern the life and death of lymphocytes during the generation and function of the immune system. Transgenic mice have been developed to test this hypothesis and to evaluate the oncogenic potential of *bcl*-2 [9-13].

bcl-2 Transgene Promotes Survival of B and T Lymphoid Cells *in vitro*

Lymphoid cells expressing a *bcl*-2 transgene exhibited a prolonged lifespan *in vitro* in simple tissue culture medium [9-13]. All differentiation stages analyzed have displayed this property: pre-B, virgin B, mature B, activated B and plasma cells; all four major types of thymocytes ($CD4^-8^-$, $CD4^+8^+$, $CD4^+8^-$, $CD4^-8^+$); and resting and activated peripheral T cells ($CD4^+8^-$ and $CD4^-8^+$). Significantly, the surviving cells were all small, having retreated to the G_0 cell cycle state, but they could be stimulated to proliferate again by treatment with mitogens or antigens [12]. As well as enduring factor-deprived medium, thymocytes from *bcl*-2 transgenic mice were more resistant *in vitro* to a wide range of cytotoxic agents: glucocorticoids, γ-radiation, phorbol ester, ionomycin, anti-CD3 antibody and sodium azide [12]. Most of these treatments are known to induce apoptoptic cell death, but sodium azide induces necrosis. *bcl*-2 is not the antidote to all forms of cell death, however, since the T cells remained sensitive to killing by antibody plus complement and by cytotoxic T cells [12].

Because they are so robust, *bcl*-2-expressing lymphoid cells should prove ideal for *in vitro* studies of lymphoid differentiation and signaling, a property which we are currently exploiting. The resistance of immature Eμ-*bcl*-2 thymocytes to death induced by engagement of the T cell receptor (TCR) complex, for example, has enabled us to purify the minor subset of $CD4^+8^+$ thymocytes which express high levels of CD3/TCR and to demonstrate that these cells give rise to both $CD4^+8^-$ and $CD4^-8^+$ mature cells upon intrathymic transplantation, but only to $CD4^-8^+$ cells in culture (Petrie et al., submitted).

Current Topics in Microbiology and Immunology, Vol. 182

bcl-2 Transgene Perturbs B but not T Lymphoid Homeostasis *in vivo*

Expression of the *bcl*-2 transgene in B lymphoid cells *in vivo* markedly perturbed homeostasis in this cellular compartment [9-11, 14]. The mice accumulated a 3- to 5-fold excess of small non-cycling conventional (IgM^+, IgD^+, class II MHC^+) B cells in all organs where B cells are normally found (peripheral blood, spleen, bone marrow and lymph nodes). After 6 to 8 weeks, however, there was essentially no further expansion, suggesting that the rate of B cell production might decrease with age. The number of Ig-secreting cells was also markedly elevated in *bcl*-2 mice and there was an associated increase in the level of polyclonal Ig in the serum. Injection of antigen provoked an amplified and greatly prolonged humoral immune response, presumably because of the increased longevity of Ig-secreting cells [11, 15].

Expression of the *bcl*-2 transgene in the T lymphoid compartment conferred significant resistance *in vivo* to treatment with γ-radiation, dexamethasone and anti-CD3 antibody [12, 13]. Furthermore, the enhanced and sustained response of peripheral T cells to superantigen [12] suggested that activated T cells had an enhanced lifespan. Surprisingly, however, homeostasis was not perturbed [12, 13]. Both the number and relative proportions of the major thymic and peripheral T cell subsets remained normal through at least the first year of life of the transgenic animals. Furthermore, self-reactive T cells were still eliminated, even though they appeared to persist somewhat longer than usual in the thymus [12]. These observations indicate that negative selection in the thymus is not induced merely by premature activation of the TCR complex but probably involves an additional apoptosis-inducing pathway for which *bcl*-2 expression is an insufficient antidote.

bcl-2 Transgene Induces a Strain-dependent Autoimmune Disease

Our transgenic mice often developed a fatal autoimmune disease resembling human systemic lupus erythematosus. It was characterised by immune complex glomerulonephritis and high levels of anti-nuclear antibodies [11]. The autoimmunity occurred in all lines whose B cells expressed the *bcl*-2 transgene but not in those expressing the transgene only in T cells (Table 1).

Table 1. Autoimmune disease in Eμ-*bcl*-2 mice

Transgenic strain	Transgene expression	Autoimmune disease[a]	
		Frequency	Latency
bcl-2-25	T	0/98 (0%)	-
bcl-2-36	T & B	4/33 (12%)	44 wk (25 to 45 wk)
bcl-2-15	T & B	24/111 (22%)	40 wk (16 to 52 wk)
bcl-2-22	B	51/108 (47%)	29 wk (11 to 50 wk)
bcl-2-22 x C57BL/6 (N1)		0/10[b]	
bcl-2-22 x C57BL/6 (N2)		0/13	

[a]Mice were monitored up to 12 months of age. Autoimmune disease was diagnosed in sick mice by the grossly abnormal appearance of the kidneys at autopsy and by histological detection of severe glomerulonephritis as previously described [11].

[b]One mouse in this group was found dead at 47 wk and could not be autopsied informatively.

The long-lived B cells and plasma cells presumably included those with anti-self reactivity, causing autoreactive antibodies to accumulate to pathologic concentrations. Perturbation of deletion mechanisms that normally operate in germinal centres may have increased the frequency of self-reactive clones. In view of these results, it was puzzling that the *bcl*-2 mice generated by Korsmeyer and his colleagues [9] were not reported to exhibit this phenotype. Although the structure of the transgenes used by the two laboratories differed significantly, it was also possible that the discrepancy was due to differences in genetic background; our mice were C57BL/6 x SJL whereas those generated by McDonnell et al. were C57BL/6 x C3H. To test this possibility, strain 22, which has an incidence of terminal kidney failure of about 50% by 12 months of age on the C57BL/6 x SJL background, was serially bred with C57BL/6 mice. Already, in the first two generations of the backcross, a dramatic reduction in the incidence of the autoimmune disease has become apparent (Table 1). Of 23 mice followed for 11-12 months, only one died of suspected glomerulonephritis. Thus the SJL background apparently provides a genetic environment that fosters the onset of the autoimmune disease.

Lymphomagenic Potential of *bcl*-2

Studies of large cohorts of several independent lines of *bcl*-2 transgenic mice have revealed that *bcl*-2 can facilitate the spontaneous transformation of B lymphoid cells but displays very little (if any) lymphomagenic potential for T cells (Strasser et al., manuscript submitted). The principal types of B lineage tumors observed were plasmacytomas secreting clonal immunoglobulin and novel lymphomas exhibiting a phenotype consistent with an origin early in B lymphoid differentiation. Both types of tumors were of low incidence and developed only after long latent periods, indicating that *bcl*-2 expression was not sufficient for transformation and that somatic mutation must have played an important role. Rearrangement of the *myc* gene was common in the plasmacytomas, implying a synergistic role for *myc* and *bcl*-2 in their etiology, but was not detected in the lymphomas. These results argue that translocation of *bcl-2* is insufficient for the development of malignancy, a conclusion consistent with the finding that a low level of *bcl*-2 translocation can be detected even in non-lymphomatous, immunologically stimulated germinal follicles [16].

The synergistic potential of *myc* and *bcl*-2 was confirmed by crossing *bcl*-2 transgenic mice with Eμ-*myc* transgenic mice. Mice expressing both transgenes rapidly succumbed to tumors with a cell surface phenotype and gene expression pattern which suggested that they derived from a primitive stem or progenitor cell [17]. These tumor cells were readily transplantable but did not survive *in vitro* in medium supplemented with any of a wide variety of hematopoietic growth factors. Like very immature hematopoietic cells, however, they were able to grow for a limited period as 'cobblestone' colonies in layers of certain stromal cells. Since the cloned tumor cells can be forced to differentiate down both lymphoid and macrophage pathways, they resemble the B/myeloid progenitor cells recently identified in normal mice [18].

In conclusion, *bcl*-2 is a novel oncogene which promotes cell survival rather than proliferation. Its role in follicular lymphoma is apparently to enable an affected clone to resist apoptosis in the germinal center. The extended lifespan of the cells increases their probability of acquiring other more frankly oncogenic mutations. Significantly, germinal center cells are uniquely exposed to mutagenic processes. They accumulate multiple point mutations within the variable region of the assembled immunoglobulin heavy chain gene and undergo DNA deletion to switch expression to different heavy chain isotypes. We hypothesise that expression of the enzymes involved in these processes also promotes oncogenic mutations. It is pertinent in this regard that progression of certain follicular lymphomas to a highly aggressive phenotype has been correlated with acquisition of a *myc*/IgH (8;14) translocation [19, 20], a recombination event believed to represent aberrant Ig gene recombination.

Acknowledgments

This work was supported by a Special Fellowship of the Leukemia Society of America to A.S. and by the National Health and Medical Research Council of Australia and the US National Cancer Institute (CA43540).

References

1. Tsujimoto Y, Finger LR, Yunis J, Nowell PC and Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226:1097-1099.
2. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL and Korsmeyer SJ (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 41:899-906.
3. Cleary ML, Smith SD and Sklar J (1986) Cloning and structural analysis of cDNAs for *bcl*-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell 47:19-28.
4. Tsujimoto Y, Ikegaki N and Croce CM (1987) Characterization of the protein product of *bcl*-2, the gene involved in human follicular lymphoma. Oncogene 2:3-7.
5. Chen-Levy Z, Nourse J and Cleary ML (1989) The *bcl*-2 candidate proto-oncogene product is a 24-kilodalton integral-membrane protein highly expressed in lymphoid cell lines and lymphomas carrying the t(14;18) translocation. Mol Cell Biol 9:701-710.
6. Chen-Levy Z and Cleary ML (1990) Membrane topology of the *Bcl*-2 proto-oncogenic protein demonstrated *in vitro*. J Biol Chem 265:4929-4933.
7. Hockenbery D, Nunez G, Milliman C, Schreiber RD and Korsmeyer SJ (1990) *Bcl*-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348:334-336.
8. Vaux DL, Cory S and Adams JM (1988) *Bcl*-2 gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. Nature 335:440-442.
9. McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP and Korsmeyer SJ (1989) *bcl*-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57:79-88.
10. Strasser A, Harris AW, Vaux DL, Webb E, Bath ML, Adams JM and Cory S (1990) Abnormalities of the immune system induced by dysregulated *bcl*-2 expression in transgenic mice. Curr Top Microbiol Immunol 166:175-181.
11. Strasser A, Whittingham S, Vaux DL, Bath ML, Adams JM, Cory S and Harris AW (1991) Enforced *BCL*-2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc Natl Acad Sci U S A 88:8661-8665.
12. Strasser A, Harris AW and Cory S (1991) *Bcl*-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell 67:889-899.
13. Sentman CL, Shutter JR, Hockenbery D, Kanagawa O and Korsmeyer SJ (1991) *bcl*-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67:879-888.
14. McDonnell TJ, Nunez G, Platt FM, Hockenberry D, London L, McKearn JP and Korsmeyer SJ (1990) Deregulated *Bcl*-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. Mol Cell Biol 10:1901-1907.
15. Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM and Korsmeyer SJ (1991) *Bcl*-2 maintains B cell memory. Nature 353:71-73.
16. Limpens J, de Jong D, van Krieken JHJM, Price GA, Young BD, van Ommen G-JB and Kluin PM (1991) *Bcl*-2/J_H rearrrangements in benign lymphoid tissues with follicular hyperplasia. Oncogene 6:2271-2276.
17. Strasser A, Harris AW, Bath ML and Cory S (1990) Novel primitive lymphoid tumours induced in transgenic mice by cooperation between *myc* and *bcl*-2. Nature 348:331-333.
18. Cumano A, Paige CJ, Iscove NN and Brady G (1992) Bipotential precursors of B cells and macrophages in murine fetal liver. Nature 356:612-615.
19. de Jong D, Voetdijk MH, Beverstock GC, van Ommen GJB, Willemze R and Kluin PM (1988) Activation of the c-*myc* oncogene in a precursor-B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. New Eng J. Med. 318:1373-1378.
20. Lee JT, Innes DJ and Williams ME (1989) Sequential *bcl*-2 and c-*myc* oncogene rearrangements associated with the clinical transformation of non-Hodgkin's lymphoma. J Clin Invest 84:1454-1459.

Evolution of Leukemic Heterogeneity of Human B-CLL Lymphocytes Between and Within Patients

G. E. MARTI[1], G. B. FAGUET[2], C. STEWART[3], P. BRANHAM[1], P. H. CARTER[1], G. C. WASHINGTON[1], P. BERTIN[1], J. MULLER[1], V. ZENGER[1], N. CAPRAOSA[4], J. WHITEHOUSE[4], C. I. AMOS[4], T. A. FLEISHER[5], and R. VOGT[6]

[1]LCMB, DBB, CBER, FDA, NIH Bdg 29, Rm 502, Bethesda, MD 20892 (301-4960178);
[2]Heme-Oncology, VAMC, Depts Med and Biochem and Molec Biol, Medical College of Georgia, (111N), Augusta, GA 30910;
[3]Laboratory of Flow Cytometry, Roswell Park Cancer Institute, 666 Elm Street, Buffalo, New York 14263;
[4]Family Studies Section, Environmental Epidemiology Branch, NCI, NIH;
[5]Immunology Service, CPD, CC, NIH;
[6]Division of Environmental Health Laboratory Sciences, Centers for Disease Control, Atlanta, Georgia 30333

Introduction

B-chronic lymphocytic leukemia (B-CLL) is a disease manifested by the proliferation and accumulation of a clone(s) of medium-sized lymphocytes with mature-appearing chromatin and a characteristic surface immunophenotype. Monoclonality is usually evidenced by the presence of a single heavy and light chain immunoglobulin and or a rearranged Ig gene(s). The surface fluorescence intensity (FI) of membrane Ig is markedly diminished and ineffective rearrangements are common. Typically patients fifty years of age or older present with a stable, asymtomatic blood and marrow monoclonal B lymphocytosis. Blood lymphocytes may show considerable cytological or morphological variation between patients and within patients.

During the past several years we have conducted experiments on B-CLL lymphocytes directed at defining the B-CLL lymphocyte surface immunophenotype (1-8). We now present additional flow cytometric data based on three (1 color), four (2 color) and five (3 color) parameter studies as well as cell cycle evaluations. Cluster analysis of individual clones and image cytometry (ICM) has also been applied in selected cases. Clinical histories, blood films, marrow studies and cytogenetics were reviewed when available. Normal whole blood (WB), hypaque ficoll (HF) peripheral blood lymphocytes (PBL) and E rosette depleted (ENEG) preparations were used as controls. These experiments were carried out with the intent to further our understanding of the molecular lesion(s) underlying B-CLL.

Materials and Methods

WB, HF, and ENEG preparations from normal donors and WB and HF preparations from patients with B-CLL were prepared using established methods. Commercially available reagents were used for the majority of experiments. An EPICS V flow cytometer was used initially and a FACScan has been used for all subsequent work. Data display and analysis of forward scatter (FSC), side scatter (SSC) and log

Current Topics in Microbiology and Immunology, Vol. 182

fluorescence (FL1, FL2, FL3) were carried out using software provided by the manufacturers and some locally developed software (FDAPLOT, CAP). Data exchange was accomplished using Internet and some samples were shipped via overnight mail. A Macintosh IIcx and the software program Image 1.43 (W. Rasband, NIH) was used for image cytometry (ICM).

Results and Discussion

Initially, a panel of reagents (data not shown) was used to immunophenotype 40 normal individuals and 21 patients with B-CLL. In these studies, FSC was approximately twice as large for B-CLL lymphocytes when compared to normal PBLs while there was no difference in SCC. Single parameter histograms for H and L chain Ig expression from several B-CLL patients demonstrated low FI for IgM and IgD. K-S analysis for clonal excess was best demonstrated with heterologous antisera rather than murine monoclonal antibodies. Class II expression is markedly increased, CD20 is characteristically reduced and CD19 expression is similar to normal PBLs. The significance of the various patterns seen for CD5, CD19-21, CD25, and TQ1/Leu 8 expression is unknown; however, it does suggest considerable heterogeneity.

Two color studies were initiated with approximately 30 B-CLL patients using a panel of paired, directly conjugated reagents. Gating reagents (CD45, CD14) were evaluated on WB and HF B-CLL preparations. Our data demonstrate that WB lysis was equivalent or superior to HF. In addition, the FI of B-CLL CD45 expression appears to be lower than normal PBLs in the majority of examples examined and may be bimodal when mixed with normal cells. Figure 1 is a selected summary of CD20 CD5 two parameter contour plots of B-CLL patients. The presence of the clone, any remaining normal CD5 T cells, and the double negatives cells are demonstrated as well as the low FI of CD20. The composite display clearly shows that each B-CLL clone has a characteristic and unique coexpression of FI for CD20 and CD5. This can be visualized in Figure 2 which presents a synopsis of several experiments evaluating the presence of the CD5, CD20 B cell subpopulation. The figure demonstrates a normal child, a normal adult and a B cell enriched (ENEG) fraction from a normal adult contrasted with two patients with B-CLL. One of the patients was restudied at a six month interval. The initial stage may be the development of a B cell lymphocytosis followed by a CD5 B cell expansion and the final step being the clonal expansion of a lymphocyte characterized by low CD20 FI. The ENEG equivalent stage may occur early and is generally not seen. And there appears to be an intermediate stage between the ENEG equivalent stage and the fully developed clone as evidenced by epidemiological studies (Marti, personal observation). Both treatment and terminal transformation appear to modify this pattern and CD5 negative B-CLL would follow a different pattern.

A combination of CD19, CD5 and CD10 expression were then evaluated (Figure 3) as part of a panel to differentiate acute leukemia (ALL) from B-CLL. An unexpected finding of these experiments was the finding that some clones express low density CD10 . In this three color analysis, the larger cells tended to be CD10 positive. Our previous experience suggested that an occasional patient's lymphocytes expressed this antigen in low density which is now confirmed by these more extensive studies In a related experiment, we have determined cell cycle parameters in several patients and confirmed the well known observation of "deep" G0/G1 cells in B-CLL. In an additional experiment, cell cycle analysis was carried out in a single patient, the proband of three generations of familial B-CLL. CD20 was used to mark the clone and CD20 negative cells (remaining normal T cells) served as an internal

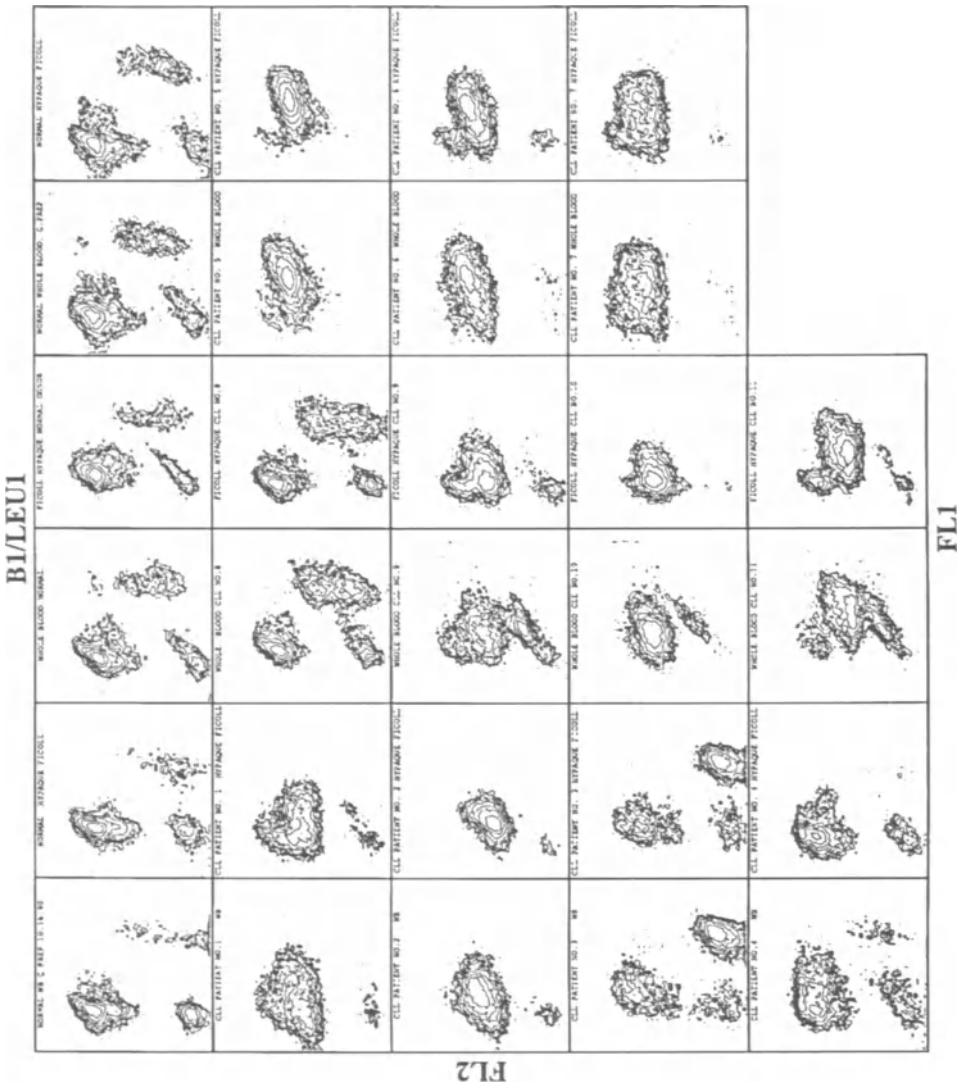


Figure 1. Selected two parameter fluorescent contour plots of CD20 (FL1, B1) and CD5 (FL2, Leu 1) expression. The upper row (under B1/Leu1 label) contains normal donors while all of the remaining panels are of B-CLL patients. There is also a comparison of whole blood (WB) and hypaque ficoll (HF).

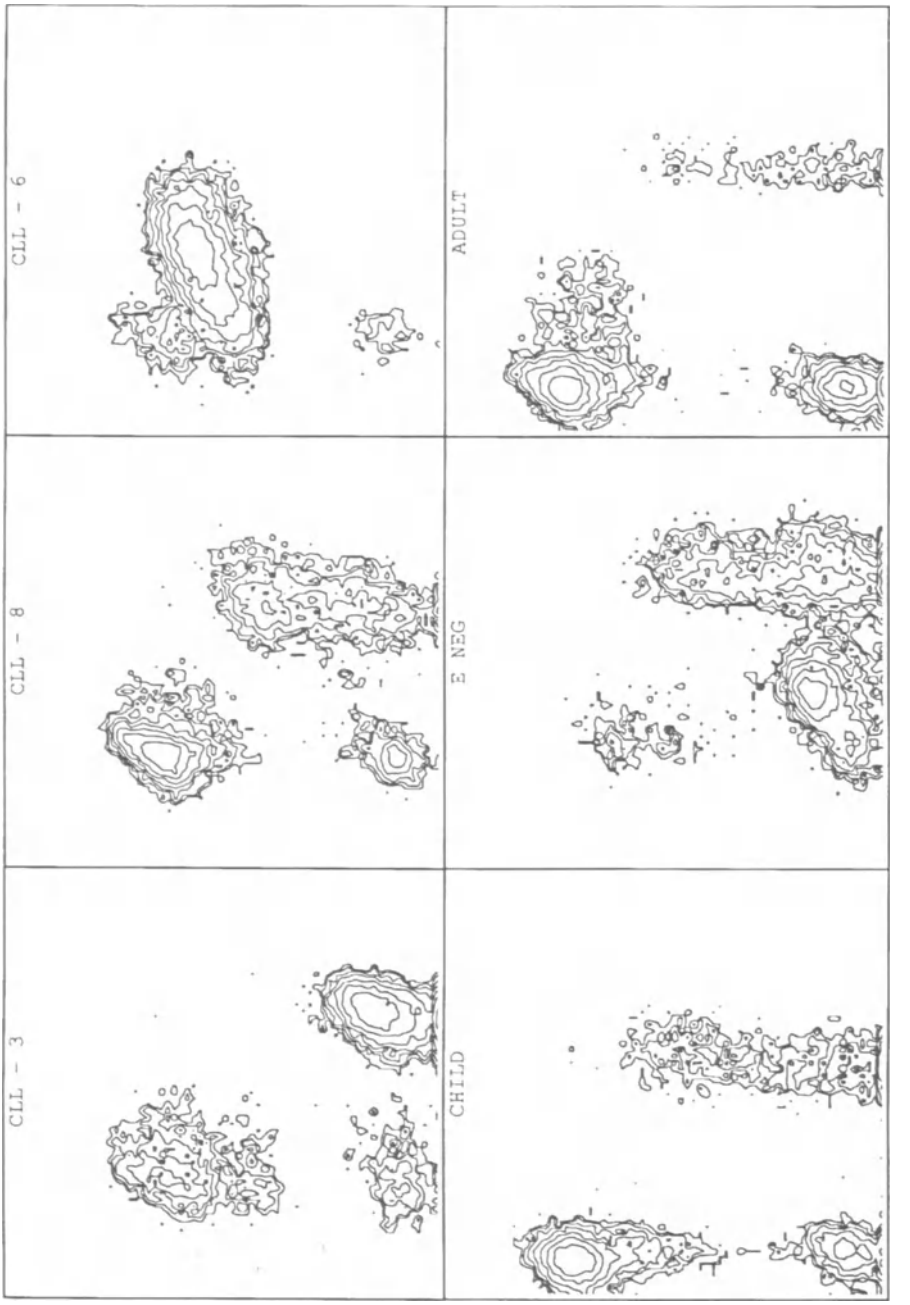


Figure 2. Synopsis of CD20 and CD5 expression. The top row is for three patients with B-CLL (monoclonal B lymphocytosis) with increasing size of the clone from left to right. The bottom row shows the same lymphocyte subsets in a child (left) and an adult (right). Enriched B lymphocytes (ENEG) are shown in the bottom middle panel.

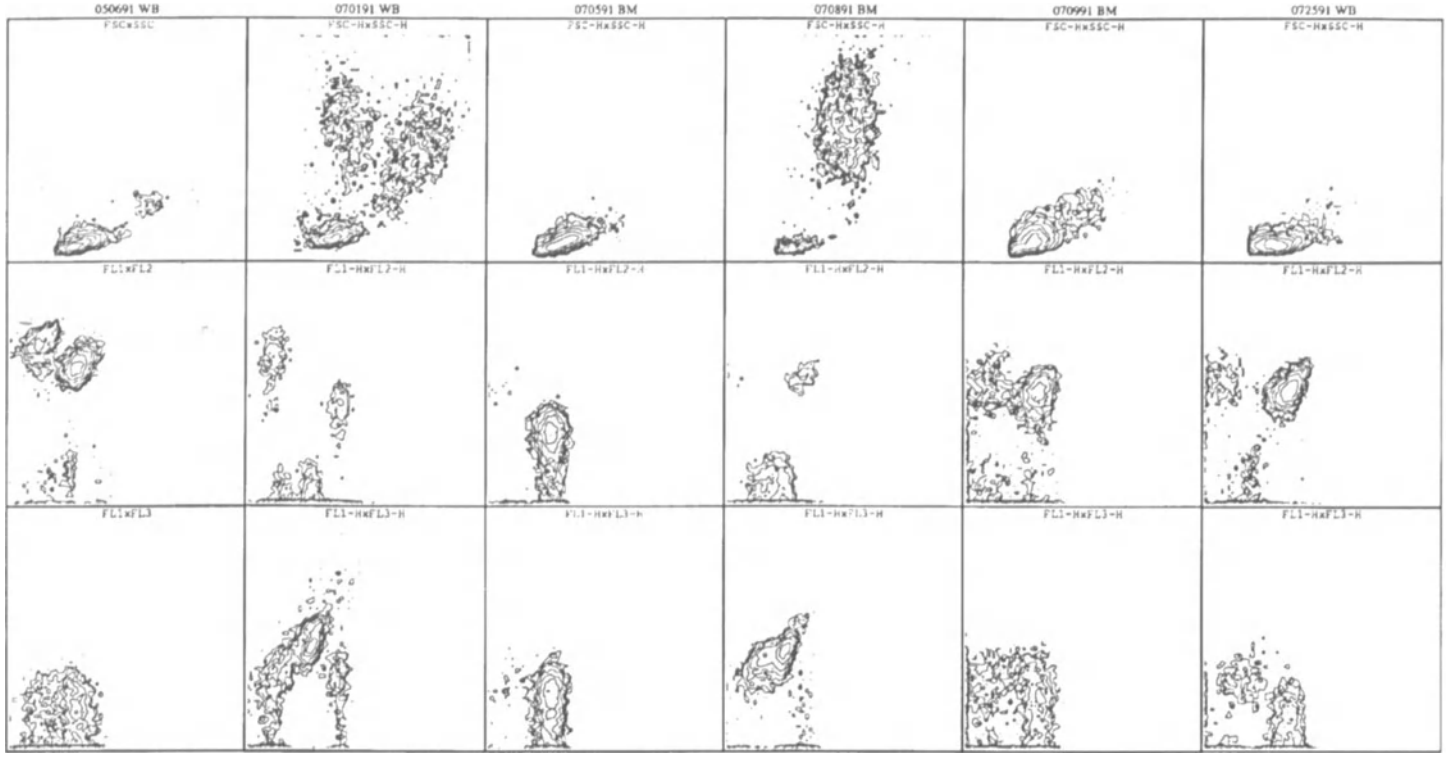


Figure 3. Selected summary of two parameter light scatter and fluorescent contour plots of CD19, CD5 and CD10 expression in B-CLL patients. The data is presented in groups of three panels from top to bottom. Light scatter (FSC x SSC), is in the upper panel; CD19, CD5 (FL1 xFL2) is in the middle panel; and CD19, CD10 (FL1 x FL3) are shown in the lower panel for each patient sample. Whole blood and bone marrow samples are indicated.

control. There were no difference in G0/G1, S or G2/M cells for either population. However, fractionation of the cells by size indicated a significant number of S phase cells in the large cell fraction. The B cell origin of these cells needs to be confirmed.

Further evaluation of the B-CLL clone was undertaken using a cluster analysis program (CAP, N plots, L. Barden, NIH) to dissect the isolated clone using the four parameter, two color CD5, CD20 data and the five parameter, three color CD19, CD 5, CD10 data. An example of this analysis is shown for a single patient in Fig 4. These data demonstrate a mathematical description of the long-suspected morphological and immunophenotypic heterogeneity of these clones as well as the distribution of the individual parameters and their relationship to one another. How does one interpret these findings? The variation of FSC compared to SSC is a good starting point. In fact it has been suggested that neither the linear nor log SSC signal is ideal, but rather that a polynomial transformation of the SSC signal leads to better resolution of lymphocyte subsets (9). The variation in FSC is striking and appears to correlate with CD20 FI. The FCM multiparameter image is a single snapshot at a given point is time. However, it is the sum analysis of all that has occurred or happened to the clone to date. Blood cells that have been just added or have remained and accumulated since the initial event of neoplastic transformation are contained in the analysis gate.

In a final experiment we have begun to use ICM to analyze the polymorphic appearance of B-CLL lymphocytes as seen on routine blood films. After digitization, each cell was numbered, pseudo colored and a threshold was set. Measurements consisted of nuclear perimeter, nuclear area, length of major and minor axes and mean optical density per individual cell. Figure 5 is representative of this approach applied to cell density. Assuming that these four cells all belong to the same clone and that all the artifacts of staining can be negated or neglected, there is a gradient in mean nuclear optical density that is inversely related to size. As the chromatin becomes more condensed (packaged, compacted), the nuclear size becomes smaller. Of further interest, the chromatin texture is related to both the nuclear size and mean optical density, i.e., greatest in the largest cell and the least in smallest cell. This is illustrated by the irregular pattern of density observed for the cells with mean nuclear optical densities of 182 and 214 compared to the cell with a mean nuclear density of 244.

Conclusions

WB lysis is adequate for the analysis of B-CLL lymphocytes. Light scatter patterns are usually complex for B-CLL lymphocytes but need to be further evaluated using cell sorting. In addition, the multiple scatter populations are associated with decreased expression of CD20 FI. A composite analysis of CD5, CD20 distributions suggests at least three steps in the development of B-CLL. An initial monoclonal B lymphocytosis develops which expands into a CD5 monoclonal B lymphocytosis with a progressive decrease in CD20 FI. Coexpression of the common CLL antigen (cCLLa) at the monoclonal B lymphocytosis stage is the earliest known marker of B cell neoplastic transformation (see references in [6, 8]). The large cells in this clone are CD10 positive and in the S phase of cell cycle. Cluster analysis suggests further heterogeneity not only between patients but within individual patients. The nuclear optical density of the B-CLL lymphocyte increases as the cell size gets smaller. Peripheral blood as the final compartment in B-CLL may show synchronous waves or a continuous addition of cells that are heterogeneous in many patients. Depending at which time point the B-CLL clone is sampled, based on the presence or absence of therapy and or terminal transformation, together with which lymphocyte sub population is transformed, all determine what flow and image cytometry sees.

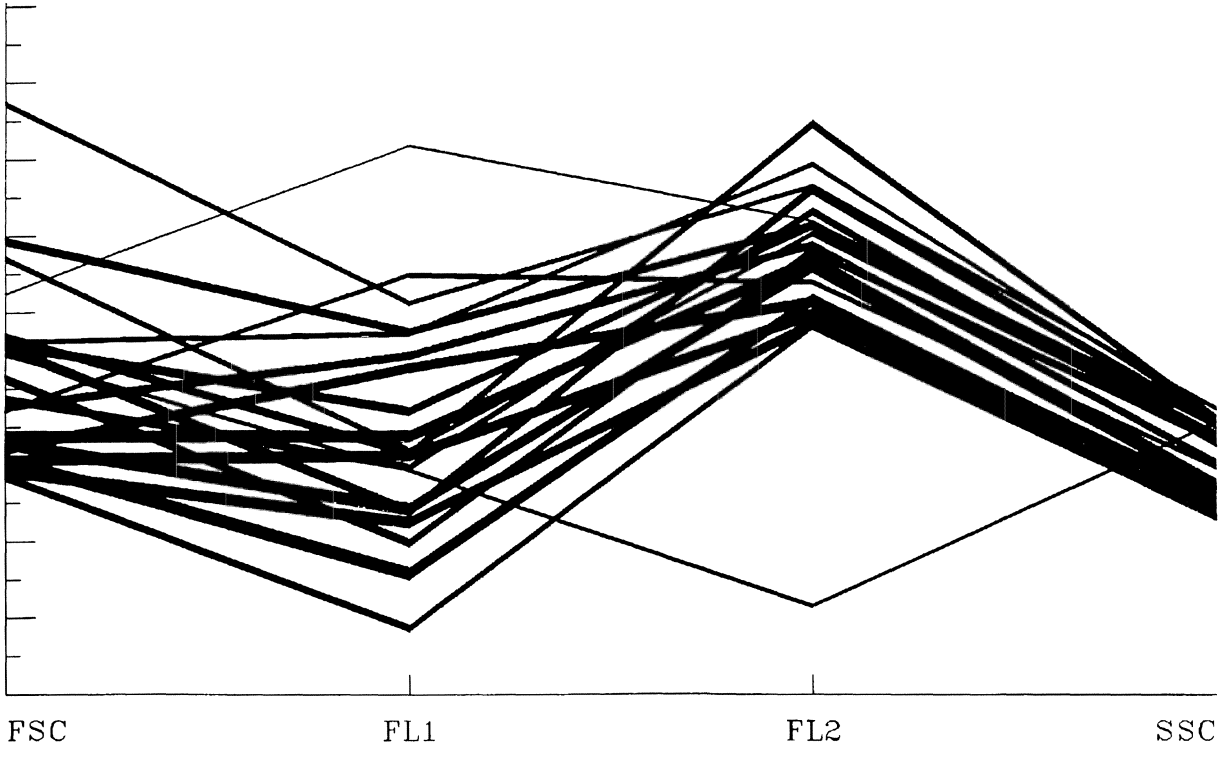


Figure 4. Selected example of cluster analysis for FSC, SSC, FL1 and FL2 for the coexpression of CD19 and CD20 on B-CLL lymphocytes. Each line represents a separate cluster while the thickness of the line indicates how many cells are in that particular cluster. This particular analysis was arbitrarily set at twenty clusters.

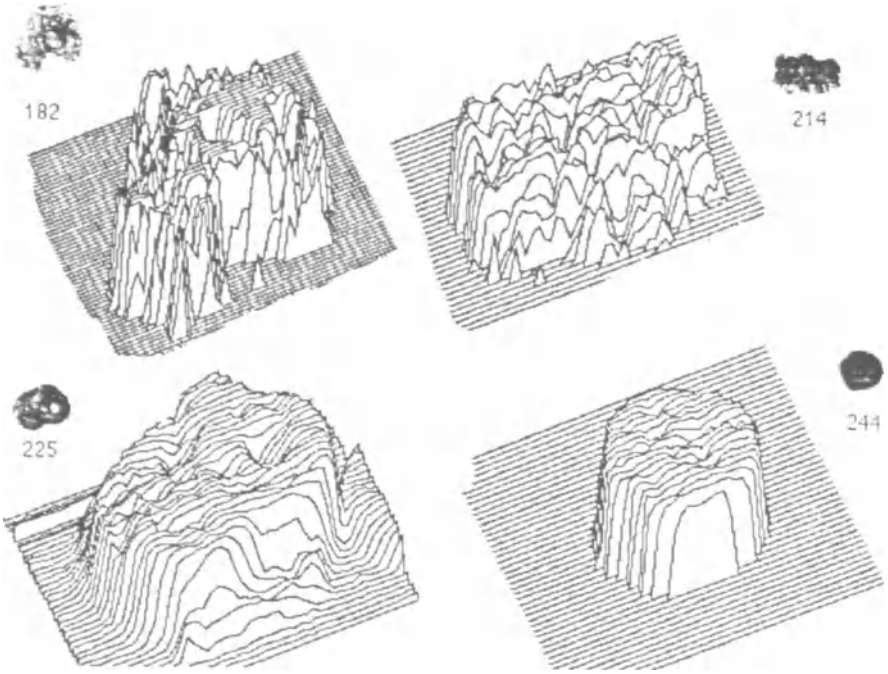


Figure 5. Summary of isometric displays of optical density and corresponding gray level image of four selected lymphocytes from a single patient with B-CLL. The three digit number is the mean optical density of the nuclear area measured.

Continued analysis and dissection of the peripheral blood compartment should yield further information about the natural history and the underlying molecular lesion(s) of this common lymphoproliferative disorder in older Western people.

References

1. Marti, GE and T. A. Fleisher, B Cell CLL Immunophenotypes (1987) in Lymphocytic Leukemia in Recent Progress, Future Directions, UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 59; Editors, R.P. Gale & K. Rai, Alan R. Liss, Inc., New York, NY pp 195-204.
2. Marti, G.E. and T.A. Fleisher. 1988. Application of lymphocyte immunophenotyping in selected diseases. Path. and Immunopath. Res. 7(5):319-328.
3. Marti, G.E., V. Zenger, N.E. Caproaso, M. Brown, G.C. Washington, P. Carter, G. Schecter, and P. Noguchi. 1989. Antigenic expression of B-cell chronic lymphocytic leukemia lymphocytes. Analytical and Quantitative Cytololy and Histology 11(5):315-323.
4. Caparoso, N.E., J. Whitehouse, P. Bertin, C. Amos, N. Papadopoulos, J. Miller, J. Whang-Peng, M.A. Tucker, T. A. Fleisher, and G.E. Marti. A 20 year clinical and laboratory study of familial B-chronic lymphocytic leukemia in a single kindred. Leukemia & Lymphoma, Vol. 3, pp 331-342,1991 .
5. Faguet G. B., Agee, J. F., Marti, G.E. CD5 and cCLLa expression in chronic lymphocytic leukemia (CLL) Demonstration of their relative prevalence and that of other common B-CLL markers. Leukemia and Lymphoma, *in press.*
6. Faguet G. B., Agee, J.F., .Marti, G.E. Clone emergence and evolution in chronic lymphocytic leukemia: characterization of clinical, laboratory and immunophenotypic profiles of 25 patients. Leukemia and Lymphoma, *in press.*
7. Marti G.E., Faguet, G., Bertin, P., Agee, J., Washington, G., Ruiz, S., Carter, P., Zenger, V., Vogt R., and Noguchi, P. CD20 and CDS expression in B-chronic lymphocytic leukemia (B-CLL). Proc. N.Y. Acad Sci. USA, *in press.*
8. Faguet G.B., Marti, G.E., Agee, J.F., and Bertin, P. CD5 positive and negative B-CLL: Evidence supporting phenotypic heterogeneity in B-chronic lymphocytic leukemia (B-CLL). Proc. N.Y. Acad. Sci. USA, *in press.*
9. Terstappen, L. W. M. M. et al. 1990. Increased light scattering resolution facilitates multidimensional flow cytometric analysis Cytometry 11(4):506-12.

p53 Mutations in B-Cell Chronic Lymphocytic Leukemia

S. El Rouby, W. Bayona, S. M. Pisharody and E. W. Newcomb

Department of Pathology, New York University and Kaplan Comprehensive Cancer Center, New York, N. Y. 10016

Introduction

The p53 gene is a nuclear phosphoprotein that can function as a tumor suppressor gene (Michalovitz et al. 1991). Frequent mutations of the p53 gene have been detected in several types of solid tumors including breast (Mackay et al. 1988), colon (Vogelstein et al. 1989), lung (Yokota et al. 1987), liver carcinoma (Hsu et al. 1991), astrocytoma (Fults et al. 1992), and gliomas (Frankel et al. 1992). In the majority of these tumors with p53 mutations, allelic loss of one copy of human chromosome 17p, the site of the p53 gene has been demonstrated. Such allelic deletion has often been shown to be associated with point mutation on the remaining allele of the p53 gene (for review see Levine et al. 1991). For hematologic malignancies, several reports have demonstrated structural alterations and point mutations of the p53 gene in blast crisis of chronic myelogenous leukemia (CML) (Feinstein et al. 1991) and in acute myeloid leukemia (AML) (Fenaux et al. 1991). In the case of T- and B-cell lymphoid malignancies, a high incidence of p53 mutation was found to be associated with Burkitt lymphoma, B-cell chronic lymphocytic leukemia (B-CLL) but not in other B-cell (Gaidano et al. 1991) or T-cell malignancies (Jonveaux et al. 1991). We have studied the role of the p53 gene in murine lymphoid tumors (Brathwaite et al. 1992) as well as some human astrocytomas (Frankel et al. 1992 and Lang et al. 1992). We describe in this report the analysis of p53 mutations in 92 patients diagnosed with B-cell CLL. We also compared the frequency of point mutations with that of the loss of heterozygosity for chromosome 17p in the same group of patients.

Frequency of p53 Mutations in B-CLL

p53 mutations in B-CLL patients were detected by single strand conformation polymorphism (SSCP) analysis, of polymerase chain reaction (PCR) products. Primer pairs for exons 4-9 were used to amplify p53 coding sequence using genomic DNA derived from CD5+ B cells separated from peripheral blood leukocytes. The PCR reaction mixture and the amplification conditions have been described in detail elsewhere (Frankel et al. 1992). The PCR products were electrophoresed through 8% polyacrylamide gels under non-denaturing conditions. Of the 92 B-CLL patients that we studied for p53 mutations, mutations were detected by SSCP analysis in 12 cases for an overall frequency of 13%. Similarly a frequency of 14% was

Current Topics in Microbiology and Immunology, Vol. 182

obtained in a smaller survey of 40 B-CLL cases by Gaidano et al. (1991). The mutations were identified in exons 4 through 8 of the p53 gene. No mutations were detected in exon 9. One patient had two mutations, one in exon 5 and one in exon 8 of the p53 gene. Although the p53 mutations in B-CLL patients occurred within the evolutionary conserved regions of the p53 gene, the frequency of mutations was relatively low (13%) as compared to other types of B-cell malignancies e.g. 33% of Burkitt's lymphoma and >50% of acute lymphoblastic leukemia (ALL) (Gaidano et al. 1991).

Confirmation of p53 Mutations by direct PCR-DNA Sequencing Analysis

Mutations in the 12 CLL cases with mobility shifts in PCR-SSCP analysis were confirmed by DNA sequencing. The direct genomic sequencing of double stranded PCR fragments was performed according to the protocol as described previously (Frankel et al. 1992). We found a perfect concordance between positive samples detected by SSCP and the presence of nucleotide base changes by PCR DNA sequencing analysis. Fig. 1 shows representative DNA sequences of p53 mutations occurring in codons 47, 241 and 249. Table 1 summarizes the p53 mutations that have been confirmed in 9 of 12 B-CLL samples to date by DNA sequence analysis. Nine of 10 mutations that have been analyzed were missense mutations resulting in an amino acid substitution. One sample (#55) contained an in frame deletion of 3 base pairs. The majority of mutations (67%, 6 of 9) were transitions occurring at CpG dinucleotides either C to T or G to A. The remaining 33% (3 of 9) of mutations were transversions (G to C, A to C, and A to T). The mutations detected in the p53 gene were not clustered in a specific codon or exon but were distributed over the regions of the p53 gene which are most highly conserved.

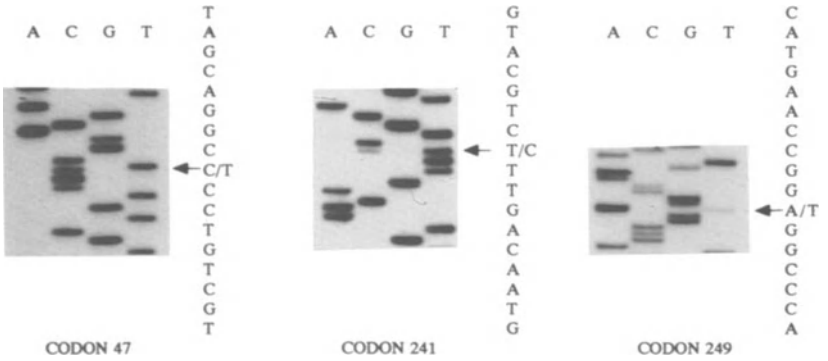


Fig. 1. DNA sequence analysis of p53 mutations detected in B-CLL cases. Representative DNA sequences are shown for codons 47, 241 and 249. Arrows indicate the position of the nucleotide change (Normal/Mutated) that result in an amino acid change.

Table 1. Summary of p53 Mutations in B-CLL Patients

Sample	Exon	Codon	Mutation	Amino Acid
2	7	241	TCC-T**T**C	SER to PHE
3	5	168	CAC-C**C**C	HIS to PRO
9	5	177	CCC-C**T**C	PRO to LEU
41	4	47	CCG-**T**CG	PRO to SER
54	6	209	AGA-A**C**A	ARG to THR
55	6	209	Deleted	
318	4	47	CCG-**T**CG	PRO to SER
325	7	249	AGG-**T**GG	ARG to TRP
407	5	177	CCC-C**T**C	PRO to LEU
	8	273	CGT-C**A**T	ARG to HIS

DNAs (100ng) were screened for mutations in exons 4-9 of the p53 gene using PCR-SSCP analysis. P53 mutations that were detected were confirmed by direct sequencing of PCR (500ng) amplified DNA.

RFLP Analysis To Assess 17p Loss of Heterozygosity

Loss of heterozygosity for chromosome 17p is one of the most frequent genetic alterations involved in the progression of human malignancies (Levine et al. 1991). To examine the allelic constitution of 17p in B-CLL patients, restriction fragment length polymorphism (RPLP) analysis was performed. Representative DNAs from patients known to be positive or negative for p53 mutations were digested to completion with *Hinf*1 enzyme, separated on 1.2% agarose gels and hybridized sequentially to two highly polymorphic variable tandem repeat probes from chromosome 17p (pYNZ22 and p144D6) as previously reported (Frankel et al. 1992). The results of such an analysis are presented in Fig. 2. Probe pYNZ22 detects two chromosome 17 alleles in all patients except lane 6. The filter was stripped and rehybridized to the second probe, p144D6. Again the patient in lane 6 was scored with only one chromosome 17 allele. Of 92 patients assessed for loss of heterozygosity for 17p, 85 patients (>90%) showed no loss.

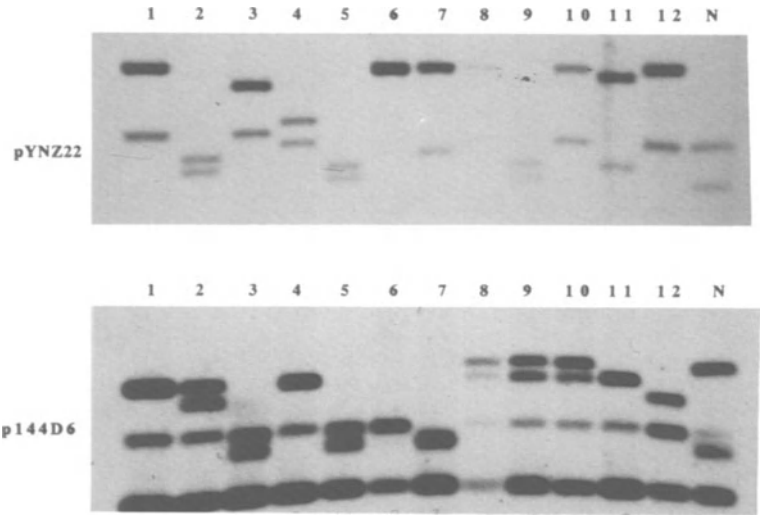


Fig. 2. RFLP analysis of p53 alleles. DNAs were digested with *Hinf* I and probed sequentially with two highly polymorphic probes for 17p. All patients, except in lane 6, demonstrated two 17p alleles with one or both probes.

Correlation Between Loss of Heterozygosity and p53 Mutation

Loss of heterozygosity on chromosome 17p and p53 mutation show a linked temporal association in many human malignancies (Fenaux et al. 1991, Fults et al. 1992, Mackay et al. 1988, Vogelstein et al. 1989). To determine whether the p53 point mutations in B-CLL patients occurred together with chromosome 17p allelic deletion, we compared the p53 mutation frequency with the loss of heterozygosity for 17p. The results are shown in Table 2. A total of 12 patients out of 92 contained mutations in the p53 gene and 25% (3 of 12) also demonstrated loss of heterozygosity for chromosome 17p. The frequency of p53 mutations was increased to >40% (3 of 7) in those patients with only one 17p allele. Taken together these findings indicate that although the loss of heterozygosity for 17p overall in B-CLL patients is infrequent, a higher frequency is observed in those patients with p53 point mutations.

Table 2. Comparison of LOH of 17p with Frequency of p53 mutations in B-CLL

No. of 17p alleles	No. of samples analyzed	No. with p53 mutations	Frequency
2	85	9	10.5%
1	7	3	43%

DNAs (5 μg) were digested with *Hinf* I, separated on 1.2% agarose gels and filters were hybridized sequentially with two highly polymorphic probes from chromosome 17p to determine loss of heterozygosity (LOH).

Concluding Remarks

p53 mutations have been identified in 13% of (12 of 92) B-CLL patients. The pattern of mutations observed is similar to that found in other malignancies. Point mutations occurred randomly within the evolutionary conserved regions, exons 4-8, of the p53 gene. None were detected in exon 9. The majority of mutations were transitions occurring at CpG dinucleotides, where most of the p53 gene mutations in other human cancers are also observed (Levine et al. 1991). Loss of heterozygosity for chromosome 17p was observed with increased frequency (43%) in patients with p53 mutations but is low compared to other malignancies such as colon cancer (Vogelstein et al. 1989). The role of p53 mutations in the biology of B-CLL disease and in tumor progression remains to be determined.

References

Brathwaite O, Bayona W, Newcomb E (1992) p53 mutations in C57BL/6J murine thymic lymphomas induced by γ-irradiation and N-methylnitrosourea. Cancer Res 52: in press

Feinstein E, Cimino G, Gale RP, Alimena G, Berthier R, Kishi K, Goldman J, Zaccaria A, Berrebi A, Canaani E (1991) p53 in chronic myelogenous leukemia in acute phase. Proc Natl Acad Sci USA 88:6293-6297

Fenaux P, Jonveaux P, Quiquandon I, Laï JL, Pignon JM, Loucheux-Lefebvre MH, Bauters F, Berger R, Kerckaert JP (1991) p53 gene mutations in acute myeloid leukemia with 17p monosomy. Blood 78:1652-1657

Frankel RH, Bayona W, Koslow M, Newcomb EW (1992) p53 mutations in human malignant gliomas: comparison of loss of heterozygosity with mutation frequency. Cancer Res 52:1427-1433

Fults D, Brockmeyer D, Tullous MW, Pedone CA, Cawthon RM (1992) p53 mutation and loss of heterozygosity on chromosomes 17 and 10 during human astrocytoma progression. Cancer Res 52:674-679

Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM, Dalla-Favera R (1991) p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc Natl Acad Sci USA 88:5413-5417

Hsu LC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC (1991) Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 350:427-428

Jonveaux P, Berger R (1991) Infrequent mutations in the p53 gene in primary human T-cell acute lymphoblastic leukemia. Leukemia 5:839-840

Lang FF, Frankel RH, Bayona W, Koslow M, Miller DC, Newcomb EW (1992) Comparison of p53 mutations and loss of heterozygosity of chromosomes 17p and 10 in low grade and high grade human astrocytomas. Cancer Res: Submitted

Levine AJ, Momand J, Finlay CA (1991) The p53 tumour suppressor gene. Nature 351:453-456

Mackay J, Elder PA, Steel CM, Forrest APM, Evans HJ (1988) Allele loss on short arm of chromosome 17 in breast cancers. The Lancet 2:1384-1385

Michalovitz D, Halevy O, Oren M (1991) p53 mutations: gains or losses? J Cell Biochem 45:22-29

Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R (1989) Allelotype of colorectal carcinomas. Science 244:207-211

Yokota J, Wada M, Shimosato Y, Terada M, Sugimura T (1987) Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc Natl Acad Sci USA 84:9252-9256

Burkitt's Lymphoma Cells Frequently Carry Monoallelic DJ Rearrangements

K. BHATIA, M. GUTIERREZ and I. T. MAGRATH

Pediatric Branch, National Cancer Institute, NIH

Introduction

In Burkitt's lymphoma, c-*myc* is most frequently translocated from chromosome 8 (q34) to the immunoglobulin heavy chain region on chromosome 14 (q32) where its expression is deregulated by virtue of its juxtaposition to enhancer sequences [1]. Because of the frequent location of the chromosomal breakpoint in DNA regions which are normally involved in the physiological recombination of immunoglobulin genes, it has been hypothesized that the immunoglobulin recombinases normally responsible for the creation of a functional immunoglobulin gene may mediate these translocations [2]. It has also been proposed that switch recombinases could mediate a proportion of translocations - presumably, those, or a fraction of those in which the breakpoint is within a switch region [3].

Since the heavy and light chain immunoglobulin rearrangements occur in a strictly hierarchical order [4], examination of the configuration of these loci provides a potential means of identifying the point in B cell differentiation at which the translocations of Burkitt's lymphoma occur. The first event in immunoglobulin gene rearrangement is the joining of a D region to a J region [4]. At least in murine B cell [5], D-J joining has been shown to occur on both alleles simultaneously; subsequently, an entire variable region is assembled on one allele by the juxtaposition of a V region to the joined D-J segment [4]. Burkitt's lymphomas essentially always synthesize immunoglobulin (IgM in the vast majority of cases) [6], indicating that one allele must have been productively rearranged.

It is clear from these considerations that in Burkitt's lymphoma both immunoglobulin alleles would be expected to be rearranged at the J_H region - one productively (VDJ juxtaposition), and the other because of prior D-J joining, prior unsuccessful VDJ joining and/or as a result of an 8;14 translocation.

Surprisingly, in characterizing breakpoint locations in 56 Burkitt's lymphomas (mostly biopsy specimens) by Southern blot analysis, we found a number of tumors (25 of 35 informative cases) in which the J_H region of one allele remained in a germline configuration. Such tumors had only a single rearranged band in addition to the germline band, excluding the possibility that the germline band was due to normal cells present in the tumor sample. In the present report we describe our findings in this regard, and discuss their implications.

Current Topics in Microbiology and Immunology, Vol. 182

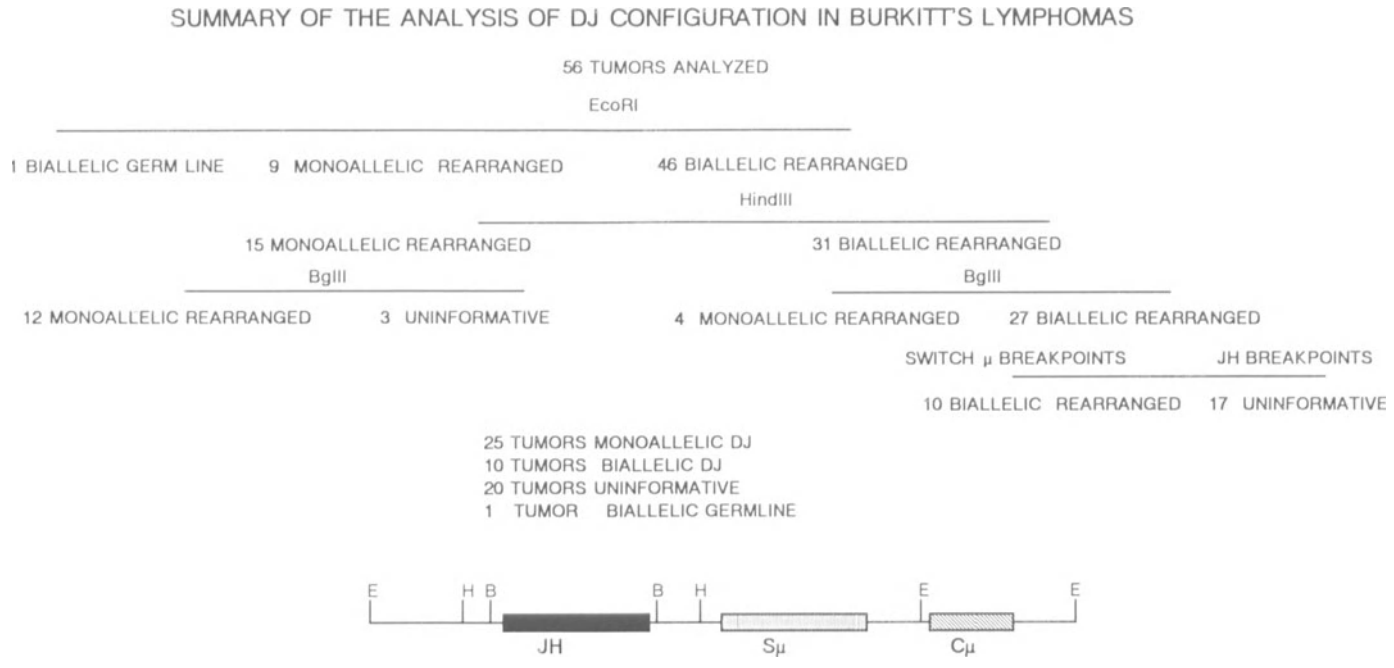


Fig 1: Summary of Southern Blot analysis of Burkitt's lymphoma for the assessment of DJ configuration. DNA from the tumors was initially digested with EcoR1 (E), DNA from tumors that were biallelically rearranged were subsequently restricted with HindIII (H) and those that showed two rearranged IgH fragments were finally digested with BglII (B) and electrophoresed on a 1% agarose gel. Following transfer to a nylon membrane, the blots were hybridized with a JH probe. For tumors that showed biallelic rearrangement of DJ following BglII digestion,information on the breakpoint location on chromosome 14 was further utilized to assess whether the rearranged bands truly represented a physiological DJ rearrangement. Thus in tumors with breakpoints within the JH locus the presence of two rearranged bands could not solely be ascribed to biallelic DJ rearrangements and hence these tumors were labelled uninformative.

Results

Ig producing human B cells have D-J rearrangements on both alleles

Since previous data demonstrating the concurrent rearrangement of D-J on both alleles is derived from studies using murine cells [5], we wished to confirm that this is also the case in human cells. We analyzed 19 mature B cell lines (EBV transformed peripheral lymphoblastoid cell lines) and all of them showed two rearranged bands in the EcoRI and HindIII digests hybridized with the J_H probe.

Ig producing Burkitt's lymphoma cells frequently have an unrearranged D-J segment on one allele

Fifty-six BLs (fig. 1) were subjected to similar analysis to determine the status of the J_H region on both alleles. In 9 tumors we observed only a single rearranged band along with a germline band in EcoRI digests probed with J_H. This germline band was of equal or greater intensity than the rearranged band. The presence of a germline D-J region in each of these cases was confirmed in HindIII digests. Bg1II digests were performed in 5 of these tumors and confirmed the germline/single rearranged band pattern in 4. The fifth tumor had an extra rearranged band which was probably due to a mutation that created an additional Bg1II site. In 46 tumors with more than one rearranged band in EcoRI digests, further analysis with HindIII revealed a single rearranged band and a germline band in 15 additional tumors. Twelve of these were examined with BglII and 9 had a single rearranged band and a germline band; the remaining 3 were considered uninterpretable. These 9 tumors and the 3 not tested with Bg1II were interpreted as having germline D-J configuration on the translocated allele, and a translocation breakpoint within EcoRI but outside HindIII (and, of course, BglII), thus accounting for the additional rearranged band in the EcoRI digest.

In the remaining 31 tumors, more than a single rearranged band was observed with both EcoRI and HindIII. In 4 of them Bg1II digest revealed only a single rearranged band, indicating a germline configuration of the J_H region and a breakpoint outside BglII and within the HindIII fragment. In ten tumors with a $S\mu$ breakpoint, i.e., in which no rearranged bands in EcoRI or HindIII digests could be ascribed to a breakpoint, the presence of two rearranged bands in EcoRI and HindIII digests (confirmed in BglII digests in 2 cases) suggests that the physiological joining of D to a J region occurred on both alleles. In all other tumors (17), we were unable to determine whether the tumor cells contained a germline allele because at least two rearranged bands were present in all three restriction digests. These tumors either had breakpoints within the BglII fragment, in which case a germline allele could have been masked, and/or a physiological rearrangement on both alleles.

In total, in 25 of the 35 informative tumors (72%) Southern blot analysis indicated the presence of a germline D-J segment. Because 20 tumors were uninformative, between 25/55 (45%) and 45/55 (83%) of tumors in this series had a monoallelic DJ recombination. It is highly probable that VDJ recombination is essential to the production of large amounts of IgM so that breakpoints beween V and $C\mu$ probably cause lack of expression. In fact, in many tumors our analysis has confirmed that the translocation is on the germline allele. In addition, in all tumors, it was shown that switch sequences comigrated with J_H sequences in at least one of the alleles (assumed

to be the productive allele). It appears highly likely, therefore, that the translocated allele is always, or nearly always, that which contains the germline D-J segment.

Discussion

While it has previously been suggested that the chromosomal translocations in Burkitt's lymphoma occur close to the time of immunoglobulin rearrangement (during VDJ or class switching) [2,3], the possibility that the translocation occurs before DJ joining has not been considered. It is still, therefore, generally assumed that the J region of the immunoglobulin heavy chain will always be rearranged in these tumors because D-J joining occur on both alleles simultaneously. In addition, the breakpoint on chromosome 14 in the 8;14 chromosomal translocation is sometimes in or close to either the J_H region or switch-μ region. Our finding that a high proportion of Burkitt's lymphomas carry a germline D-J segment on one allele is, therefore, surprising. Strong evidence for the presence of a germline D-J region in the tumor cells themselves was provided by the finding that in many tumors only one rearranged band was observed with at least two restriction enzymes (in some cases with three). Moreover, we found a germline D-J region in one of our cell lines that has been in continuous culture for many years, excluding the possibility that contaminating normal cells account for the finding.

Our findings with respect to the frequency of germline D-J segments in Burkitt's lymphoma differ markedly from those of published reports of other B lineage neoplasms, e.g., precursor B cell leukemia and other non-Hodgkin's lymphomas [7,8]. Even in acute precursor B cell leukemia [8], when there are other grounds for believing that the malignant clone arises in close proximity to Ig gene rearrangement, D-J regions examined by Southern blotting are nearly always both rearranged (rarely, both are germline) further confirming that DJ joining is usually bi-allelic, and emphasizing the uniqueness of Burkitt's lymphoma. Indeed, even in mouse plasmacytomas DJ rearrangement appears to be biallelic [9].

Our observations have a number of implications. Firstly, they strongly suggest that in the majority of Burkitt's lymphomas, the chromosomal translocation takes place *before* D-J joining has occurred on the allele in which the translocation occurs. If we accept the notion that D-J joining, in at least the vast majority of B cells, takes place simultaneously on both alleles, we must further conclude that the 8;14 translocation in Burkitt's lymphoma usually occurs before the initiation of immunoglobulin gene rearrangement, i.e., in a pro-B cell. As a corollary to this observation, we must also conclude that the translocation often prevents the D-J region on the same chromosome from undergoing subsequent rearrangement. This would clearly have to be the case when the breakpoint is between D and J, since these two segments would be on separate chromosomes. In tumors in which at least one J region remains on the same chromosome as D, D-J joining is at least physically possible. In such cases, D-J joining could sometimes occur after the translocations. If this is the case, translocations prior to D-J rearrangement could also have occurred in at least some of the tumors in which there was evidence of D-J rearrangements on both alleles. Only tumors with breakpoints downstream of J_H, including $S\mu$ breakpoints fall into this

category. Since some tumors with Sμ breakpoints still had a germline D-J segment, it is formally possible, that translocations always occur before D-J joining even in tumors with a Sμ breakpoint and rearranged D-Js. This is entirely consistent with demonstration that clones of the pro-B cell line FLEB 14 have a marked tendency to undergo chromosomal translocation, the breakpoint on chromosome 14 being in a switch region [10].

Translocation immediately before D-J joining has implications with respect to the sequence of pathogenetic events occurring in Burkitt's lymphoma. Depending upon the number of cell divisions that occur between the translocation and immunoglobulin rearrangement on the normal allele, there is the possibility that progeny cells could rearrange their immunoglobulin genes differently. This may particularly apply to light chain rearrangements, which are late in the hierarchy of Ig gene recombinational events. One might ask why Burkitt's lymphoma is not polyclonal or oligoclonal at the light chain locus. Presumably, the need for additional genetic lesions results in monoclonality - indicating that the translocation is itself insufficient to fully transform cells.

Just as translocation before D-J joining raises the issue of the polyclonality of Burkitt's lymphoma at the immunoglobulin loci, one is also obliged to question why, in the presence of a fallible system (only about one third of attempted rearrangements are successful), Burkitt's lymphomas which lack all immunoglobulin expression, are rarely observed. This suggests strongly that the production of immunoglobulin is an essential prerequisite for the genesis of a malignant clone. Since cells committed to B cell differentiation which fail to make a functional immunoglobulin molecule are eliminated under normal circumstances, probably by apoptosis [11], it is entirely feasible that even cells with an 8;14 translocation would be similarly eliminated if unable to make an immunoglobulin molecule.

While we favor the interpretation that translocation occurs before D-J joining, at least one other explanation for our findings could be proposed. It remains formally possible that DJ fails to rearrange simultaneously on both alleles, at least in a small fraction of pro-B cells. It is unlikely, however, that such an explanation could account for the high frequency of BLs with one germline DJ that we observed unless there is a predisposition for translocations to occur with much greater frequency into the unrearranged allele of a cell with one germline DJ segment.

The occurrence of translocations in a pro-B cell would not exclude the possibility that VDJ recombinases mediate the event. However, it does exclude the possibility that such translocations are mistakes of VDJ recombination.

References

1. Dalla-Favera R, Bergni M, Erickson J, Patterson D, Gallo RC, Croce CM (1982) Proc. Natl. Acad. Sci. USA 79:7824-7827
2. Haluska FG, Finver S, Tsujimoto Y, Croce C (1981) Nature 324: 158-161
3. Magrath I (1990) Adv Can Res 55:133-270

4. Tonagawa S (1983) Nature 302:575-581
5. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, Coffman B, Rosenberg N, Tonegawa S, Baltimore D (1984) EMBO J 3:1205-1219
6. Gunven P, Klein G, Klein E, Norin T, Singh S (1980) Int J Cancer 25:711-719
7. Griesser M, Tkachuk D, Reis MD, Mak WT (1989) Blood 73:1402-1415
8. Felix CA, Poplack DG, Reaman GH, Steinberg MS, Cole DE, Taylor BJ, Begley GC, Kirsch IR (1990) J Clin Oncol 8:431-442
9. Coleclough C, Perry PR, Kargalainen K, Weigert M. (1981) Nature 290:372-378
10. Altoik E, Klein G, Zech L, Uno M, Henriksson BE, Battat S, Ono Y, Ernberg I (1989) Proc Natl Acad Sci USA 86:6333-6337
11. Ales-Martinez JE, Cuende E, Martinez AC, Parkhouse RME, Pezzi L, Scott DW (1991) Immunology Today 12:201-205
12. Cohen JH, Revillard JP, Magaud JP, Lenoir G, Willaume M, Manel AM, Vincent C, Bryon PA (1987) J Natl Canc Inst 78:235-242

Centrocytic Lymphoma: a B-Cell Non-Hodgkin's Lymphoma Characterized by Chromosome 11 BCL-1 and PRAD 1 Rearrangements

M. E. WILLIAMS[1], S. H. SWERDLOW[2], C. L. ROSENBERG[3], and A. ARNOLD[3]

[1]Departments of Internal Medicine and Pathology, University of Virginia School of Medicine, Charlottesville, VA;
[2]Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH;
[3]Endocrine Unit, Massachusetts General Hospital, Boston, MA.

Introduction

Centrocytic lymphoma is a CD5-positive B cell neoplasm defined in the Kiel classification (Figure 1) [1]. This lymphoma, composed of cells resembling centrocytes (cleaved follicular center cells), is distinguished from other small cleaved cell lymphomas by the lack of transformed cells, the diffuse, vaguely nodular, or mantle zone growth pattern, and immunophenotypic differences (CD5+, CD10-, and strong surface IgM and/or IgD expression) [1,2]. Transformation to non-cleaved or centroblastic (large cell) lymphomas is rare. Patients are usually males 50-70 years of age, who generally present with diffuse lymphadenopathy, splenomegaly, and marrow involvement, and have a median survival < 4 years.

Previous work from this laboratory has demonstrated a strong association between centrocytic lymphoma and rearrangements at the chromosome 11 bcl-1 breakpoint loci designated MTC (major translocation cluster) and p94PS [3,4]. Recently the bcl-1 oncogene postulated to exist near the 11q13 breakpoint was identified as a cyclin gene (PRAD1) located approximately 110 kb telomeric of the MTC [5]. We now report the presence of rearrangements flanking the PRAD1 gene in a subset of centrocytic lymphomas, further supporting the hypotheses that this locus is pathogenetically important in the development of this neoplasm, and that centrocytic lymphoma represents a distinct clinicopathologic entity.

Methods

Twenty-seven patient samples were selected based upon a histologic diagnosis of centrocytic lymphoma; cases were included only after review by one of us (S.H.S.) without knowledge of Southern blot results. No karyotypic data was available for any of these cases. Tissue or peripheral blood cells were also analyzed from 80 other non-centrocytic B cell malignancies. All cases showed clonal rearrangement of the immunoglobulin genes.

Southern blot analysis was performed as previously described utilizing high molecular weight DNA digested with the restriction endonucleases Bam HI, Eco R1, Hind III, Sst I and Bcl I [6,7]. bcl-1 translocation breakpoint probes included the MTC, p94PS, p210 and p11EH probes as previously described (Figure 2) [4]. Three PRAD1 genomic probes included probe D, a 500 base pair fragment which lies approximately 15 kb

upstream of the first PRAD1 exon, a 1.65 kb Bam HI/Bgl II fragment which lies just upstream of PRAD1 exon 1 (probe A), and a 1.7 kb Bam HI/Eco R1 genomic fragment which includes the first PRAD1 exon (probe B). In selected cases hybridization was also performed with a PRAD1 cDNA probe corresponding to the λ Pl-4 insert described in reference [5].

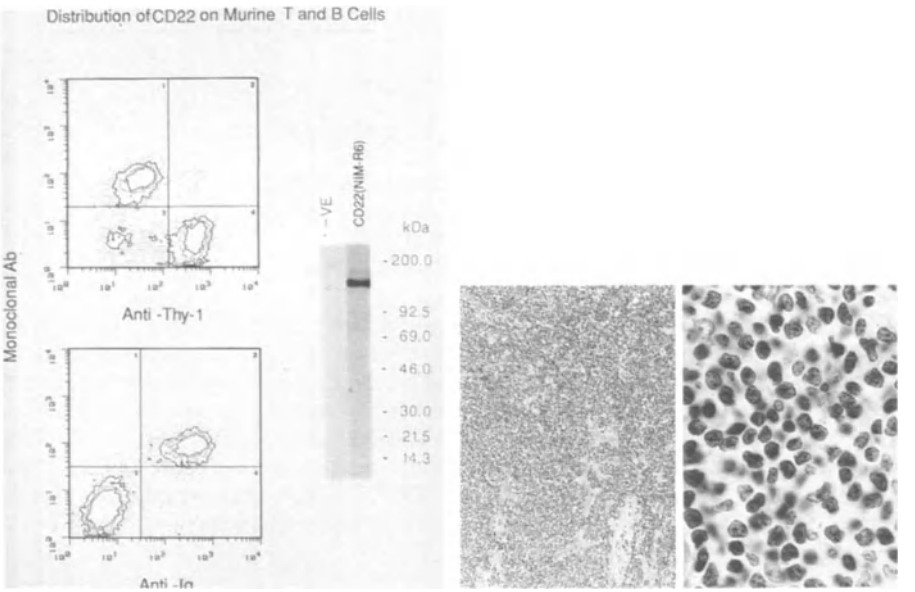


Fig. 1. Centrocytic lymphoma. At low power (75x, left) there is diffuse architectural effacement and focally prominent hyalinized blood vessels. At high power (750x, right) note the small lymphoid cells, many of which have irregularly shaped and clefted nuclei. (H & E stained sections).

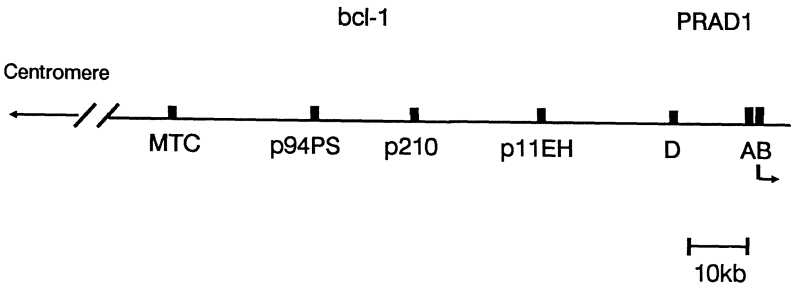


Fig. 2. Relative positions of the chromosome 11q13 bcl-1 breakpoint loci and PRAD1 probes A, B, and D. The first exon of the PRAD1 cyclin gene (arrow) is encoded within the 1.7 kb probe B genomic fragment.

Results and Discussion

Fifteen of the 27 centrocytic lymphoma patient samples showed rearrangement at bcl-1 breakpoints, nine at the MTC and six at p94PS; 23 of these samples were previously reported [4]. With the exception of one p94PS-rearranged case, all showed comigration of rearranged immunoglobulin JH bands, consistent with a t(11;14)(q13;q32). Five patient samples demonstrated PRAD1 rearrangement. Three were detected with probes A, B and the cDNA probe, while an additional two cases were detected with probe D (Table 1). One of these five samples rearranged with the p94PS probe on multiple restriction digests but lacking JH comigration also showed rearrangements with PRAD1 probes A, B and cDNA on Bam HI digest only; whether this PRAD1 rearrangement represents a Bam HI point mutation or a polymorphism could not be determined. However, the lack of identifiable polymorphisms in over 150 other human DNA samples with these PRAD1 probes and restriction enzymes supports the presence of an acquired point mutation. Each of the remaining four cases with PRAD1 rearrangement showed comigration with a rearranged JH band, consistent with an 11;14 translocation. Only one of the 80 non-centrocytic B cell neoplasms showed rearrangement, a mediastinal sclerosing large cell lymphoma rearranged at the bcl-1 MTC locus (Table 1). Notably, no case of chronic lymphocytic leukemia/small lymphocytic lymphoma showed rearrangement, in contrast to previous reports of 11;14 translocations and bcl-1 rearrangements in CLL [8,9]. It is likely that many of these previous cases represented centrocytic lymphoma in leukemic phase [10,11]. Multiple myeloma has also been reported to contain chromosome 11q13 karyotypic anomalies, including the t(11;14) [12]; failure to identify bcl-1 or PRAD1 rearrangements in this series may be a consequence of the small sample size, or may reflect involvement of alternative bcl-1/PRAD1 breakpoints or other 11q13 gene loci.

Table 1. Rearrangement at bcl-1 and PRAD1 loci in B cell neoplasms[1]

	n	bcl-1		PRAD1		Total (%)
		MTC	p94PS	D	B	
Centrocytic lymphoma	27	9	6[2]	2	3[2]	19(70)
Follicular lymphoma	15	0	0	0	0	0
Diffuse large cell lymphoma	22	1	0	0	0	1(4)
CLL/small lymphocytic lymphoma	18	0	0	0	0	0
Monocytoid B cell lymphoma	6	0	0	0	0	0
Multiple myeloma	12	0	0	0	0	0
Small noncleaved cell lymphoma	7	0	0	0	0	0

[1] Immunoglobulin gene rearrangement present in all cases.
[2] One case showed rearrangement with both p94PS and PRAD1 probes (see text).

These analyses thus extend the strong association of 11q13 breakpoint rearrangements and centrocytic lymphoma. The rarity of such rearrangements in other B cell neoplasms is consistent with the hypothesis that this locus is pathogenetically important in the development of centrocytic lymphoma. PRAD1 encodes a cyclin gene (cyclin D1) which may play an important role in cell cycle regulation [5]. The gene is highly conserved throughout evolution, and has been isolated from yeast cells, murine macrophages, human parathyroid adenomas, and human lymphoma and squamous

carcinoma cell lines [13-18]. PRAD1 mRNA expression appears to be low in non-centrocytic lymphoma samples, whereas expression is at high levels in centrocytic lymphoma cells and in lymphoid cell lines carrying the 11;14 translocation [16,19]. PRAD1 overexpression is postulated to be a consequence of juxtaposition with the immunoglobulin promoter and enhancer elements, although these breakpoint sites most commonly lie at the MTC and p94PS loci, 85-110 kb upstream of the PRAD1 gene. Nevertheless, deregulated expression from translocations similarly distant from the relevant oncogene has been reported for c-myc in cases of endemic Burkitt's lymphoma [20]. Overexpression of a normal-size 4.5kb PRAD1 transcript has been shown in centrocytic lymphoma [19], and restriction mapping of the PRAD1 rearrangements in the present series indicates that none disrupt the coding region itself (data not shown). Furthermore, the observed breakpoints are similar to the chromosome 11 inversion breakpoints identified in parathyroid adenomas, which also show increased PRAD1 expression [18].

Centrocytic lymphoma has been recognized as a distinct entity only in the Kiel classification. However, it is similar and probably identical to lymphocytic lymphoma of intermediate differentiation (IDL) as currently defined [21]. In view of the confusing terminology and overlapping categorization among the various classification schemes, it has been proposed that centrocytic lymphoma, IDL, intermediate lymphocytic lymphoma and mantle zone lymphoma be reclassified under the term "mantle cell lymphoma" [22]. However, it will be important to take into account other B cell neoplasms which may arise from mantle zone cells, as proposed for B-CLL [23]. In addition, it will be useful to establish the precise cell of origin for this lymphoma.

Thus, 11q13 bcl-1 and PRAD1 rearrangements are strongly associated with centrocytic lymphoma. Identification of this molecular marker will be useful in classification of this lymphoma subtype. Because rearrangements are heterogeneous and require multiple probe and enzyme combinations for efficient detection, demonstration of PRAD1 overexpression at the RNA or protein level may ultimately prove more efficacious. Polymerase chain reaction methodology also can be utilized to identify translocation breakpoints at the MTC, which lie within an approximately 60 base pair span and represent the most frequently rearranged site [24]. Finally, centrocytic lymphoma should serve as an important model for understanding the role of cell cycle deregulation in cancer pathogenesis. Such insights will have implications in a variety of solid tumors, such as breast and squamous cell carcinomas, in which PRAD1 and possibly other genes are overexpressed by 11q13 amplification [25].

References

1. Swerdlow SH, Habeshaw JA, Murray LJ, Dhaliwal HS, Lister TA, and Stansfeld AG (1983) Centrocytic lymphoma: a distinct clinicopathologic and immunologic entity. Am J Pathol 113:181-197
2. Hollema H, Poppema S (1988) Immunophenotypes of malignant lymphoma centroblastic-centrocytic and malignant lymphoma centrocytic: An immunohistologic study indicating a derivation from different stages of B cell differentiation. Hum Pathol 19:1053
3. Williams ME, Westermann CD, and Swerdlow SH (1990) Genotypic characterization of centrocytic lymphoma: frequent rearrangement of the chromosome 11 bcl-1 locus. Blood 76:1387-1391
4. Williams ME, Meeker TC, and Swerdlow SH (1991) Rearrangement of the chromosome 11 bcl-1 locus in centrocytic lymphoma: analysis with multiple breakpoint probes. Blood 78:493-498

5. Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, and Arnold A (1991) A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 350:512-515
6. Williams ME, Innes DJ, Borowitz MJ, Lovell MA, Swerdlow SH, Hurtubise PE, Brynes RK, Chan WC, Byrne GE Jr, Whitcomb CC, and Thomas CY IV. (1987) Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. Blood 69:79-86
7. Lee JT, Innes DJ Jr, and Williams ME (1989) Sequential bcl-2 and c-myc oncogene rearrangements associated with the clinical transformation of non-Hodgkin's lymphoma. J Clin Invest 84:1454-1459
8. Tsujimoto Y, Yunis J, Onorato-Showe L, Erikson J, Nowell PC, and Croce CM (1984) Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukemias with the t(11;14) chromosome translocation. Science 224:1403-1406
9. Juliusson G, Oscier DG, Fitchett M, Ross FM, Stockdill G, Mackie MJ, Parker AC, Castoldi GL, Cuneo A, Knuutila S, Elonen E, and Gahrton G (1990) Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. New Engl J Med 323:720-724
10. Raffeld M, Jaffe ES (1991) bcl-1, t(11;14), and mantle cell-derived lymphomas. Blood 78:259-263
11. Kluin PM, van Krieken JHJM (1991) The molecular biology of B-cell lymphoma: clinicopathologic implications. Ann Hematol 62:95-102
12. Gould J, Alexanian R, Goodacre A, Pathak S, Hecht B, and Barlogie B (1988) Plasma cell karyotype in multiple myeloma. Blood 71:453-456
13. Xiong Y, Connolly T, Futcher B and Beach D (1991) Human D-type cyclin. Cell 65:691-699
14. Lew DJ, Dulic V, and Reed SI (1991) Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell 66:1197-1206
15. Matsushime H, Roussel MF, Ashmun RA, and Sherr CJ (1991) Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65:701-713
16. Withers DA, Harvey RC, Faust JB, Melnyk O, Carey K and Meeker TC (1991) Characterization of a candidate bcl-1 gene. Mol Cell Biol 11:4846-4853
17. Schuuring E, Verhoeven E, Mooi WJ, Michalides RJAM (1992) Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene 7:355-361.
18. Rosenberg CL, Kim HG, Shows TB, Kronenberg HM, and Arnold A (1991) Rearrangement and overexpression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors. Oncogene 6:449-453
19. Rosenberg CL, Wong E, Petty EM, Bale AE, Tsujimoto Y, Harris NL, and Arnold A (1991) PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. Proc Natl Acad Sci 88:9638-9642
20. Magrath I (1990) The pathogenesis of Burkitt's lymphoma. Advances Cancer Res 55:133-270.
21. Lardelli P, Bookman MA, Sundeen J, Longo DL, Jaffe ES (1990) Lymphocytic lymphoma of intermediate differentiation. Am J Surg Path 14:752-763
22. Banks PM, Chan J, Cleary ML, Delsol G, De Wolf-Peeters C, Gatter K, Grogan TM, Harris NL, Isaacson PG, Jaffe ES, Mason D, Pileri S, Ralfkiaer E, Stein H, Warnke RA (1992) Mantle cell lymphoma: a proposal for unification of morphologic, immunologic and molecular data. Am J Surg Path (in press)
23. Schena M, Larsson LG, Gottardi D, Gaidano G, Carlsson M, Nilsson K, Caligaris-Cappio F (1992) Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. Blood 79:2981-2989
24. Williams ME, Swerdlow SH, Meeker TH (1992) Chromosome t(11;14)(q13;q32) breakpoints in centrocytic lymphoma (CL) are highly localized at the bcl-1 major translocation cluster (MTC) locus. Clin Res 40:171A
25. Lammie GA, Peters G (1991) Chromosome 11q13 abnormalities in human cancer. Cancer Cells 3:413-420

Acknowledgements: This work was supported in part by NIH grants CA 46723 (to M.E.W.) and DK 11794 and CA 55909 (to A.A.). C.L.R. received a Postdoctoral/Postresidency Cancer Fellowship from the American Cancer Society, Massachusetts Division, and A.A. is the recipient of a Faculty Research Award from the ACS. We thank Drs. C. Croce, P. Leder, T. Meeker and Y. Tsujimoto for providing probes utilized in these studies, and Drs. Donald Innes, Jr. and Marsha Kinney for contributing cases of centrocytic lymphoma. We also acknowledge Patricia Ennis, Holly Dressman and Sue Likowski for expert technical assistance and Lisa Morris for manuscript preparation.

Two Surface Antigen Targets for Immunotoxin-Mediated Elimination of Normal and Neoplastic Murine B Cells

R. M. E. PARKHOUSE[1,2], L. SANTOS-ARGUMEDO[1], C. TEIXEIRAL[1], R. V. HENRY[3] and E. WAWRZYNCZAK[3]

[1]National Institute for Medical Research, London, N.W.7.I.A.A, U.K.;
[2]Institute for Animal Health, Pirbright, Surrey, GU24 ONF, U.K.;
[3]Institute of Cancer Research: Royal Cancer Hospital, Surrey SM2 SNG, U.K.

Introduction

The idea of selective toxic agents for the elimination of neoplastic cells *in vivo*, first proposed by Ehrlich, has been the subject of considerable interest over the last decade (reviewed by Uhr, 1991: Wawrzynczak, 1991). Particular attention has been focussed on the use of immunotoxin conjugates composed of ricin and monoclonal antibodies (reviewed by Vitetta and Thorpe, 1991). Indeed, a limited success has recently been reported using ricin-anti-CD22 conjugates for treatment of patients with B cell lymphomas (Vitetta et al., 1991). As all B cells, both normal and neoplastic, bear surface CD22, then the treatment must severely deplete the B cell compartments. The issue then arises: what is the nature of the newly emerging B cell system after such treatment? This question cannot, of course, be systematically approached experimentally in man, but the availability of a recently described murine CD22 homologue, both the gene and a corresponding monoclonal antibody (Torres et al., 1992), now provides an appropriate experimental system. As a prelude to such studies, this monoclonal anti-murine CD22 antibody has been coupled to ricin A chain and tested *in vitro*, using both normal B cells and the B cell lymphoma BCL_1. In addition, another monoclonal antibody recognizing a surface protein present on BCL_1 cells, but undetectable on normal B cells, has been similarly tested. The latter system provides an unusual example for highly restricted tumor cell elimination.

Materials and Methods

Mice and Tumors

Specific pathogen free mice were used at 6-8 weeks of age for preparation of cell suspensions. The spleen seeking BCL_1 tumor was maintained *in vivo* in Balb/c mice.

Tissue Culture

Tissue culture was done in RPM1-1640 medium (Flow Labs) supplemented with non-essential amino acids (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma), 1 mM Sodium pyruvate (Sigma), 2 mM Glutamine (Sigma) and 5% Fetal Bovine Serum. Splenic B cells were isolated using anti-Thy-1 monoclonal antibody ascites (NIM-R1) (Chayen and Parkhouse, 1982) plus idubiose A37 (IBF Biotechnics)—absorbed guinea pig complement to kill T lymphocytes, followed by percoll (Pharmacia) separation to purify the small resting B cells. The population with $p<1.080$ was >90% slg^+. BCL_1 cells were similarly prepared. Cells were cultured at 1×10^6 cells/ml, and assayed for proliferation after 2 days *in vitro* by uptake of radioactive thymidine. Purified B cells were stimulated with LPS (50 μg/ml) and BCL_1 cells were stimulated by recombinant IL-5

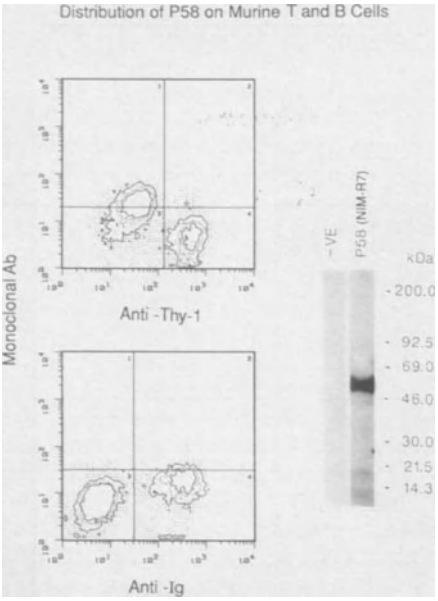


Fig. 1. Distribution of NIM-R6 anti-CD22 (left side) and NIM-R7 anti-P58 (right side) on murine splenocytes. Spleen cells were stained with rat Mab-PE-anti-rat-Ig as indicated and counterstained with FITC-goat-anti-mouse Ig or FITC rabbit-anti-mouse-Thy-1 as indicated. Coprecipitation of relevant surface antigens recognized by NIM-R6 (left) and NIM-R7 (right). Surface labelled BCL_1 cell lysates were reacted with either a control, irrelevant Mab, anti-CD22 (NIM-R6) or anti-P58 (NIM-R7). Soluble complexes were captured with protein-G for SDS-PAGE analysis.

(10 U/ml). For analysis of [^{3}H]-thymidine uptake, cultures of 10^5 cells in 200 µl in flat-bottom tissue culture plates were labelled for 4 h with 0.5 µCi of [^{3}H]-thymidine before being harvested and counted. Splenic T cells were purified with nylon wool columns, stimulated with Concanavalin A (1 µg/ml) and proliferation assays were done on day 2 of culture as above.

Preparation, Characterization and Purification of Monoclonal Antibodies

BCL_1 lymphoma B cell plasma membranes were prepared (Snary et al., 1976), dissolved in 2% (w/v) sodium deoxycholate – 50 mM Tris HCl – 50 mM NaCl, pH 8.3 (DOC-Tris), and then passed over a column of Lentil lectin-Sepharose (Pharmacia), equilibrated with DOC-tris. The absorbed lymphocyte plasma membrane glycoproteins were eluted with 0.1 M α-methylmannoside-DOC-Tris, dialyzed and injected into footpads of Lou strain rats (Kearney et al., 1981). The resulting immune popliteal lymph nodes were fused with the J.K. mouse myeloma cell line (Kearney et al., 1979), and antibodies were selected by positive reactions with splenic B cells.

Lactoperoxidase catalyzed surface iodination of splenic CBA x C57 B cells, immune coprecipitation and SDS-PAGE were done by standard procedures as described (Abney and Parkhouse, 1974).

Purified splenic B and T lymphocytes were activated with LPS (50 µg/ml) or Concanavalin A (1 µg/ml), respectively. Resting and activated cells were stained with the rat monoclonal antibody followed by specifically absorbed goat anti-rat Ig-phycoerythrin (PE) (Southern Biologicals, Birmingham, Alabama, U.S.A.) and then counter stained, either with specifically

absorbed goat anti-mouse fluorescein (FITC) (Southern Biologicals, Birmingham, Alabama, U.S.A.) or with a rabbit anti-purified Thy-1 antigen-FITC (given to us by Dr. Alan Williams, Oxford University, England). The cells were then analyzed in a Beckton-Dickinson Facscan machine with the appropriate settings for small dense lymphocytes or the larger activated cells.

Monoclonal Antibody Purification

All the monoclonal antibodies were raised *in vitro* in tissue culture medium. Each sample was dialyzed extensively with 10 mM MES (Sigma) pH 5.6 and then loaded at 0.5 ml/minute into a (7.75 mm x 10 cm) Bakerbond ABx gold column (J.T. Baker). After 10 minutes in 10 mM MES, the sample was eluted with a continuous linear gradient from 0 to 50 % with 1 M Sodium acetate (Sigma) pH 7, for 40 min at 0.5 ml/minute.

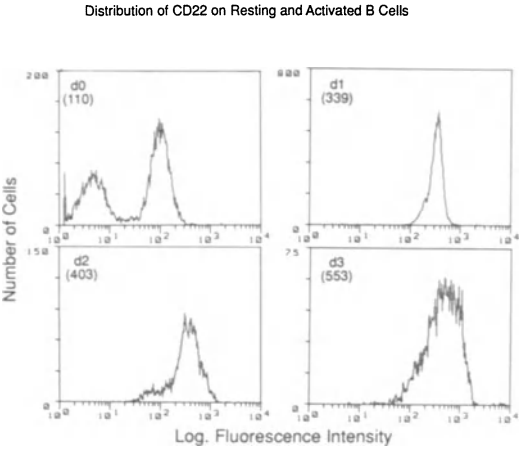


Fig. 2. Distribution of CD22 on resting and activated B cells. Spleen cells depleted of T cells were activated with LPS and stained on d0, d1, d2 and d3 with Mab NIM-R6 anti-CD22 and PE-goat anti-rat Ig. For FACS analysis small lymphocytes were analyzed on day 0 and day 1 and larger, activated cells on day 2 and day 3. The numbers in parentheses are the mean intensity fluorescence values.

Preparation of Monoclonal Antibody- Ricin A Chain Conjugates

Purified rat monoclonal antibodies NIM-R6 (anti-CD22), NIM-R7 (anti-P58) and an irrelevant rat Mab (ICR-12) were coupled to ricin A chain and gel filtered through Sephacryl S200 (Cumber and Wawrzynczak, 1992). The fraction selected contained predominantly 1:1 conjugate and lesser amounts of 1:2 conjugate and unconjugated antibody.

Results

The characteristics of monoclonal antibodies NIM-R6 (anti-CD22) and NIM-R7 (anti-P58) presented in Fig. 1. As can be seen, NIM-R6 stains the Ig-positive-Thy-1 negative population of spleen cells, but not the Ig negative-Thy-1-negative or Thy-1 positive populations. It precipitates an antigen of ~140 kDa from surface labeled splenic B cells (Fig. 1, left), demonstrated to be the murine homologue of human CD22 (Torres et al., 1992). Upon activation, the density of CD22 expressed by B cells is increased (Fig. 2). The other antibody, NIM-R7 does not stain splenic lymphocytes, but does react with BCL_1 cells, and precipitates a surface antigen of 58 kDa molecular weight (Fig. 1, right).

Both antibodies were coupled to ricin A chain and the resulting immunotoxins were titrated into two assays. In the first, the effect was assessed on LPS-stimulated proliferation of normal splenic B cells. As can be seen (Fig. 3, top), the CD22 (NIM-R6)-ricin A chain conjugate was

highly inhibitory; whereas, the anti-p58 (NIM-R7) immunotoxin was without effect. In contrast, both of these two immunotoxins abrogated IL-5-stimulated proliferation of the BCL_1 tumour cell line. The control, rat monoclonal antibody (ICR-12)-ricin A chain conjugate was without effect in both proliferation assays. Finally, none of the three immunotoxins tested affected the Concanavalin A-induced proliferation of splenic T cells.

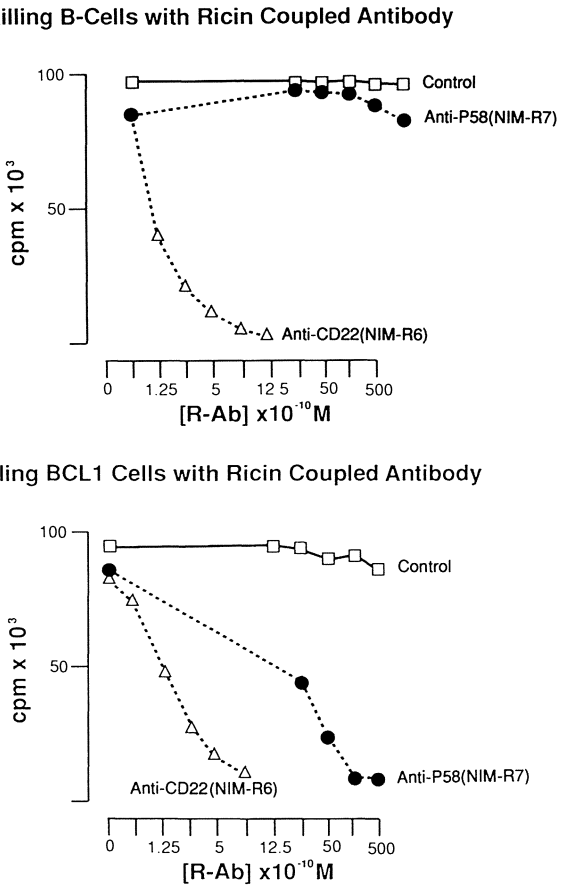


Fig. 3. Killing normal splenic B cells (top) and BCL_1 cells (bottom) with monoclonal antibody-ricin A chain conjugates. The immunotoxins were titrated with the assays as described in the Materials and Methods. After 3 and 2 days, respectively, proliferation was assessed by uptake of radioactive thymidine.

Discussion and Conclusions

The results presented show that normal murine splenic B cells and the murine Ly-1-positive B cell lymphoma BCL_1 can be killed *in vitro* by an immunotoxin composed of anti-murine CD22 and ricin A chain. As such conjugates are currently undergoing clinical trial for elimination of B cell lymphomas *in vivo* in man, the availability of the murine counterpart will be of considerable interest as a model system to investigate the dynamics and characteristics of depletion and regeneration of the B cell compartment.

The second system tested is also of interest, as the NIM-R7 antibody in question identifies a rare 58 KDa B cell antigen expressed by the BCL_1 tumour cell line but not by detectable numbers of normal splenic B cells. The antibody, therefore, provides an example of a "tumor specific antigen," and will provide another useful system for the exploration of immunotoxin therapy.

References

Abney ER and Parkhouse RME (1974) A candidate for immunoglobulin D present on murine B lymphocytes. Nature 252:600-602

Chayen A and Parkhouse RME (1982) Preparation and properties of a cytotoxic monoclonal anti-mouse Thy-1 antibody. J Immunol Methods 49:17-22

Cumber AJ and Wawyrzynczak EJ (1992) Preparations of cytotoxic antibody-toxin conjugates. In: Manson M (ed) Immunochemical protocols, pp 283-293 (Methods in molecular biology, vol 10)

Kearney JK, Barletta R, Quan ZS and Quintans J (1981) Monoclonal vs. heterogeneous anti-H-8 antibodies in the analysis of the anti-phosphorylcholine response in BALB/c mice. Eur J Immunol 11:877-883

Kearney JK, Radbruch A, Leisengang B and Rajewsky K (1979) A new mouse myeloma cell line that has lost immunoglobulin expression bet permits the construction of antibody-secreting hybrid cell lines. J Immunol 123:1548-1550

Snary D, Woods F and Crumpton M (1976) Disruption of solid tissue for plasma membrane preparation. J Analyt Biochem 74:457-465

Torres RM, Law C-L, Santos-Argumedo L, Kirkham PA, Grabstein K, Parkhouse RME and Clark EA (1992) Identification and characterization of the murine homologue of CD22, a B-lymphocyte-restricted adhesion molecule. J Immunol In press

Uhr JW (1991) Immunotoxins: an overview. Seminars in Cell Biology 2, 1-6

Vitetta ES and Thorpe P (1991) Immunotoxins containing ricin or its A chain. Seminars in Cell Biology 2, 47-58

Vitetta ES, Uhr J, Stone M, Fay J, May R, Till M, Amlot P, Newman J, Cunningham D, Clark P, Chetje V and Thorpe PE (1991) A phase I immunotoxin trial in patients with B cell lymphoma. Cancer Res 51:4052-4058

Wawrzynczak EJ (1991) Systemic immunotoxin therapy of cancer: advances and prospects. Br J Cancer 64:624-630

Acknowledgements

L. Santos-Argumedo thanks the Wellcome Trust for the award of a travel grant.

Generation and Differentiation Potential of Retrovirus-Immortalized Mature Murine B Cells

Y. W. CHEN, K. A. VORA and M.-S. LIN

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia PA 19140

Introduction

Given the availability of recombinant lymphokines, antibodies specific for various lymphokines and lymphocyte cell-surface molecules, and functional antigen-specific Th clones, homogeneous and functionally responsive B cell lines and clones would be valuable tools for studying T cell-B cell interactions and B lymphocyte differentiation. However, most of the available B cell lines to date have limited potential for differentiation. Generation of functional B cell lines has become an urgent task.

Acute transforming retroviruses or retroviral vectors carrying oncogenes have been used successfully by many laboratories to immortalize cells of the B lineage. Thus, murine Abelson leukemia virus was shown to immortalize pre-B cells [1,2]. Under certain culture conditions, Abelson leukemia virus infection would result in IgM^+ or IgG^+ B cells [3,4]. Kirsten sarcoma virus pseudotyped with an amphotropic helper virus was shown to immortalize IgM^+ B cells [5]. Surface IgM^+ B cell lines can be obtained by infecting bone marrow cells [6] with J-2 virus [7], a retroviral vector carrying v-myc and v-raf/mil oncogenes pseudotyped with leuk Moloney leukemia virus. However, the functional status of these B cells is not known. In this report, we describe immortalization of mitogen-activated, small, resting B lymphocytes using J-2 virus. The immortalized B cells were shown to express a mature B cell phenotype, to respond to lymphokines and B cell mitogens, and to interact with and respond to T helper clone cells. Thus, the J-2 virus-immortalized B cells, which resemble normal B lymphocytes both phenotypically and functionally, will be useful tools for dissecting molecular events involved in T cell-B cell interactions and B cell differentiation.

Results

Figure 1 depicts the protocol employed to immortalize B lymphoblast cells. Coculture of murine B lymphoblast cells with irradiated J-2 virus-producing NIH/3T3 cells resulted in cell lines in about one month. These cell lines have been carried in vitro continuously for more than one year, thus, we considered these cell lines to be immortal. Table 1 summarizes the surface phenotype of the immortalized B cells. It was noted that most of the immortalized B cells coexpress IgM and IgD. Thus, the surface phenotype of the immortalized B cells resembles that of the starting splenic mature B cell population. The B cell nature of the immortalized cells was further confirmed by Southern blot and Northern blot analysis. Both alleles of JH and κ light chain were rearranged in all the lines and clones analyzed, whereas λ light chain and β chain of T cell receptors were in germline configuration. Analysis of RNA isolated from immortalized B cells with μ and δ sequence-specific probes detected both the membrane and secreted forms of μ mRNA and the membrane form of δ mRNA. These data further strengthen the notion that the immortalized cells are of B cell origin.

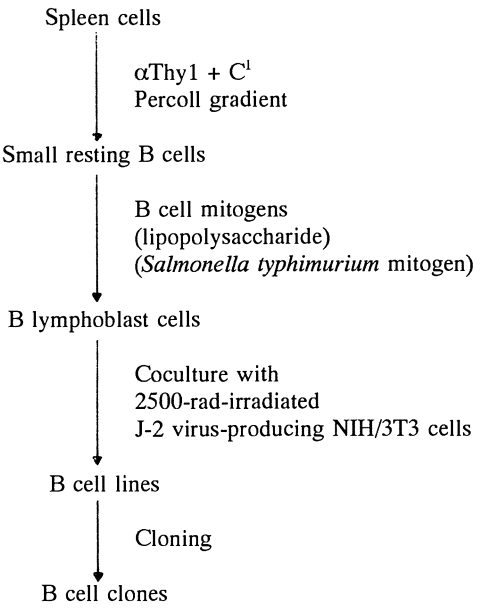


Fig. 1. Protocol for immortalization of B cells

Table 1. Cell surface phenotype of the immortalized B cells

Cells	IgM	IgD	Ia	FcR	CD45	Thy1
V2P3 (line)	+	+	+	+	+	-
V2P32B (clone)	+	+	+	+	+	-
V5M3 (line)	+	+	+	+	+	-
V5M3C6 (clone)	+	+	+	+	+	-
V7K2 (line)	+	+	+	+	+	-

Integration of J-2 virus into the genomes of the immortalized B cells was assessed by Southern blot analysis with a v-raf probe. Analysis of several clones indicated that most clones containing a single unique J-2 virus integration with a few clones contained more than one provirus. Expression of the introduced v-myc and v-raf oncogenes in these cells was evaluated by Northern blot analysis. Both genomic and subgenomic J-2 RNA as well as myc and raf mRNA were detected. The raf gene product was implicated as the downstream effector of mitogen signal transduction in many cell types including lymphocytes [8]. Therefore, expression of v-raf (perhaps, in conjunction with v-myc) may be the driving force for immortalization of these cells. To assess whether the immortalized cell lines produce any retrovirus particles, the

supernatants from the V2P3, V5M3 and V7K2 lines were measured for reverse transcriptase activity. Supernatants from the V5M3 line, but not from the V2P3 or V7K2 lines contained a high level of reverse transcriptase activity. Thus, only the V5M3 line, but not the V2P3 or V7K2 cell line, produced retrovirus particles. It was further shown that infection with supernatant from the V5M3 cells produced retrovirus particles in the SC-1 cells (a mouse embryo line susceptible to ecotropic, polytropic and xenotropic retroviruses), but not in the Mv 1 Lu cells (a mink lung cell line susceptible to polytropic and xenotropic but not ecotropic retroviruses). Therefore, the V5M3 cells were most likely producing either the helper virus (leuk Moloney leukemia virus) alone or both the helper and J-2 viruses, but not polytropic or xenotropic viruses.

Proliferative and differentiative responses of the immortalized B cells to polyclonal B cell activators were assessed. Treatment of cells with lipopolysaccharide or *S. typhimurium* mitogen resulted in an accelerated proliferation in a dose-dependent manner. When the immortalized B cells were cultured with 80 μg/ml of lipopolysaccharide for two days, the number of cells recovered was twice that recovered following cultured in medium alone. Similar results were obtained when cells were treated with *S. typhimurium* mitogen. Lipopolysaccharide and *S. typhimurium* mitogen also induced the immortalized B cells to differentiate into IgM-secreting cells. Thus, incubation of B cell lines with lipopolysaccharide or *S. typhimurium* mitogen at 25 μg/ml for five days induced a 3- to 5-fold increase in IgM secretion as compared to incubation in medium alone. These data demonstrated that the retrovirus-immortalized cells respond to B cell polyclonal activators in a fashion similar to that of normal B lymphocytes. These cells are also IL-4-responsive. Treatment of these cells with IL-4 resulted in an increase in cell-surface Ia expression as measured by flow cytometry.

In order to determine whether the immortalized B cells can interact with and respond to T helper cells, a superantigen-responsive Th2 clone, D10 G4.1 [9,10] was used. Addition of the superantigen staphylococcal enterotoxin B to cultures consisting of D10 G4.1 cells and the immortalized B cells should promote T cell-B cell interactions. These interactions are mediated by the Ia of the immortalized B cells and Vβ of T cell receptors of D10 G4.1 cells, thus, mimicking the cognate T cell-B cell interactions during the normal immune response. In the presence of staphylococcal enterotoxin B, 15-30% of the immortalized B cells form conjugates with D10 G4.1 cells. Supernatants collected from these cultures stimulated the IL-2- and IL-4-responsive cell line HT-2 to proliferate. This lymphokine activity can be blocked by anti-IL-4 antibody but not by anti-IL-2 receptor antibody, indicating that this lymphokine activity is due to the presence of IL-4. This data indicated that the Ia^+-immortalized B cells can present staphylococcal enterotoxin B to D10 G4.1 cells and activate these cells to secrete IL-4. Expression of germline transcripts of immunoglobulin constant region genes prior to heavy-chain isotype switching has been demonstrated in many systems [11,12]. When staphylococcal enterotoxin B was added to the cultures containing D10 G4.1 cells and the immortalized B cells, germline γ1 transcripts could be detected by reverse trancription polymerase chain reaction using a pair of primers based on the DNA sequence of published germline γ1 transcripts [13]. The identity of the reverse transcription polymerase chain reaction products was further confirmed by restriction enzyme digestion and Southern blot analysis using an internal oligonucleotide probe. Most significantly, supernatants collected from day-five cultures consisting of D10 4G.1 cells, immortalized B cells, and staphylococcal enterotoxin B contained not only IgM but also IgG1 molecules, as detected by radioimmunoassay. No immunoglobulin of other classes or subclasses can be detected under these culture conditions. This data clearly indicate that T cell-B cell interactions promoted by staphylococcal enterotoxin B not only lead B cells to differentiate into IgM-secreting cells, but also induce B cells to switch from IgM to IgG1 production.

In summary, we have demonstrated that J-2 virus can infect and immortalize dividing B lymphoblast cells. The immortalized B cells resemble normal B lymphocytes both phenotypically and functionally. These cell lines and clones will be useful tools for study of B cell biology.

References

1. Rosenberg N, Baltimore D, Sher CD (1975) In vitro transformation of lymphoid cells by Abelson murine leukemia virus. Proc Natl Acad Sci USA 37:1932
2. Ramakrishnan L, Rosengerg N (1988) Novel B-cell precursors at the stage of DJ_H recombination. Mol Cell Biol 8:5216
3. Serunian LA, Rosenberg N (1986) Abelson virus potentiates long-term growth of mature B lymphocytes. Mol Cell Biol 6:183
4. Runnels J, Serunian L, Thursby M, Rosenberg N (1991) Moleney leukemia virus immortalizes B lymphocytes in vitro. J Virol 65:5549
5. Litchman AH, Reynolds DS, Faller DF, Abbas AK (1986) Mature murine B lymphocytes immortalized by Kirsten sarcoma virus. Nature 324:489
6. Rapp UR, Cleveland JL, Fredrickson TN, Holems KL, Morse HC III, Jansen HW, Patschinsky T, Bister K (1985) Rapid induction of hemopoietic neoplasms in newborn mice by a raf(mil)/myc recombinant murine retrovirus. J Virol 55:23
7. Principato M, Cleveland JL, Rapp UR, Holems KL, Pierce JH, Morse HC III, Klinken SP (1990) Transformation of murine bone marrow cellls with combined v-raf-v-myc oncogenes yields clonally related mature B cells and macrophages. Mol Cell Biol 10:3562
8. Rapp UR, Troppmair J, Carroll M, May S (1990) Role of raf-1 protein kinase in IL-3 and GM-CSF-mediated signal transduction. Curr Top Microbiol Immunol 166:129
9. Kaye J, Porcelli S, Tite J, Jones B, Janeway CA Jr (1983) Both a monoclonal antibody and antisera specific for determinants unique to individual clones helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. J Exp Med 158:836
10. Yagi J, Baron J, Buxser S, Janeway CA Jr (1990) Bacterial proteins mediate the association of a defined subset of T cell receptor:CD4 complexes with class II MHC. J Immunol 144:892
11. Berton MT, Uhr JW, Vitetta ES (1989) Synthesis of germline γ1 immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon γ. Proc Natl Acad Sci USA 86:2869
12. Stavnezer J, Radcliffe G, Lin Y-C, Nietupski J, Berggren L, Sitia R, Severinson E (1988) Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. Proc Natl Acad Sci USA 85:7704
13. Xu M, Stavnezer J (1990) Structure of germline immunoglobulin heavy-chain γ1 transcripts in interleukin-4 treated mouse spleen cells. Dev Immunol 1:11

The Role of Growth Factors in Human Lymphomas

R. J. FORD[1], A. TAMAYO[1], and J. L. AMBRUS, JR.[2]

[1]University of Tx-MD Anderson Cancer Ctr., Houston, Tx. 77030 and
[2]Washington Univ. Med School, St. Louis, MO., 63110

Introduction

Human lymphomas are a common, heterogeneous group (>20 types) of lymphoid neoplasms, primarily derived (>80%) from the B lymphocytic lineage[1]. While the B cell non-Hodgkin's lymphomas (NHL-B), show a considerable morphologic spectrum of neoplastic B lymphocytes, the NHL-B are classified primarily on the basis of clinical behavior, into low, intermediate, and high grade lymphomas in the International Working Formulation (IWF) [2]. While this classification system is woefully inadequate scientifically and considerably out of date clinically, it does emphasize the point that NHL-B can be generally thought of as being either low grade (indolent) or high grade (aggressive), (with the gratuitous hedge of being "intermediate" for those lymphomas that do not quite fit into the extremes). This yin-yang type of clinical behavior correlates quite well with the proliferative potential of the various subtypes of lymphoma cells, suggesting a cause-effect relationship. This type of correlation also suggests that very different types of growth regulation is present in high vs. low grade NHL-B. A variety of arguments have been made for the role(s) of several proto-oncogenes in the dysregulation of growth control in these neoplasms. Activation and rearrangement of the *myc* proto-oncogene in high grade NHL-B (esp. Burkitt's type) seems to be at least one factor in the prodigious growth potential of these cells, that may in fact be further potentiated by inactivation of the tumor suppressor gene p53, by point mutations or other mechanisms[3]. In the low grade NHL-B, the central importance of the *bcl-2* proto-oncogene has been emphasized, due to its consistent association with the disease process in follicular lymphoma. While the exact mechanism for its apparent involvement in the development of follicular lymphoma is still obscure, the transgenic experiments suggest that abrogation of apoptosis mediated by *bcl-2* expression in B lymphocytes may contribute to the pathogenesis[4]. Although a variety of studies have shown that cell kinetics play a role in the clinical behavior of the NHL-B, the biologic basis of growth regulation in these human lymphoid tumors is still obscure. Our basic hypothesis has been that malignant human B cells, which retain most of the immunophenotypic cell surface molecular characteristics of their normal B cell counterparts, retain the capacity to respond to the same or very similar cytokine growth factor signals[5]. Differences observed in the regulation of neoplastic B cell growth, relate at least in part to the presence of potential cellular sources (e.g. accessory immune cells, etc.)

Current Topics in Microbiology and Immunology, Vol. 182

and subsequent availability of the growth factor(s) that control the proliferative potential of these tumors. Our studies[6] have shown that the only growth factors that show consistent stimulatory activity on NHL-B are the human B cell growth factors (BCGFs), as shown in Table 1. These cytokine growth factors, which are normally produced by activated human T lymphocytes, are also similar or identical to autocrine growth factors (AGF) produced by high grade NHL-B. While most of the early members of the Interleukin group of cytokines (IL1-7) have been reported to have at least some stimulatory activity on normal human B cells, most of these factors have subsequently been shown to preferentlally stimulate various subsets of T cells or other non-lymphoid cells (e.g. IL-4 functions as a Mast cell growth factor) rather than human B lymphocytes (Table 2).

Table 1. Human B cell growth factors (BCGFs).

HUMAN B CELL GROWTH FACTORS					
Factor	Source	Molecular Wt	Bioassay	P/D	Reference
LMW-BCGF	T+ PHA	12 kD	αμ B (PBL) SAC, αCD 40 B cell lines	+/-	FORD et al.
50 kD-BCGF	T+ PHA+ Az	50 kD	αμ B, SAC B cell lines	+/-	VAZQUEZ et al. OPPENHEIM et al.
HMW-BCGF	NAMALVA BL NHL-B LINES T-ALL LINES	60 kD	SAC B (PBL) αμB, *Autocrine*	+/-	AMBRUS et al. FORD et al.
30-kD-BCGF	B-PBL+ SAC	30 kD	SAC B (PBL)	+/-	AMBRUS et al.

Table 2. Other cytokines reported to have BCGF-like activity.

FACTORS THAT WILL PUTATIVELY STIMULATE HUMAN B CELL GROWTH IN VITRO					
Factor	Source	MW	Bioassay	P/D	Reference
I L-2	CD 4+ T cells	15 kD	αμB, Sac B (PBL)	+/+	Taniguchi et al.
I L-3	Recombinant (T cells)	15-30 kD	pre-B (fetal liver)	+/ ?	Ucken et al.
I L- 4	CD 4+ T cells Recombinant	15-20 kD	αμB αCD 40, FcR/ L	+/ + +/ -	Yakota et al. Banchereau et al.
I L- 5	Recombinant	13-45 kD	αμB, Sac B	+/ ?	Yakota et al.
I L- 6	Recombinant Monocytes	30-35 kD	EBV+ B lines C ess B line	+/ + +/ ?	Tosato et al. Hirano et al.
I L- 7	Recombinant (Stromal cells)	20-28 kD	BM preB cells PBL B cells	+/ ? + / -	Namen et al.
I L-10	Recombinant	20kD	Tonsil B cells	+/ +	Rousset et al.
γ-IFN	T cells, recomb	20-25 kD	αμB (PBL)	+/ +	Defrance et al.
Lactic Acid	EBV cell SN	<5000	EBV+ B lines	+/-	Tosato et al.

Low Grade Lymphomas.

Low grade lymphomas are the most common form of NHL-B in the US, usually behaving as indolent lymphomas in relatively elderly patients, with a slowly progressive clinical course over a decade or more. The main types of lymphomas seen in this group of NHL-B are the follicular small cleaved cell type(FSCL) and the small lymphocytic(SLL) form, usually considered to be similar to or the tissue counterpart of chronic lymphocytic leukemia (CLL). Both these types of lymphoma show very low proliferative capacities on cell kinetic analysis with S phase values around 5%. As stated above, much has recently been written regarding the involvement of the *bcl -2* proto-oncogene in the FSCL with the characteristic t(14-18) chromosomal translocation, regarding the possible elimination of spontaneous apoptosis or programmed cell death in the lymphoma cells accounting for an extended tumor cell life span. This type of cellular phenomenon could lead to an increased tumor cell numbers without the usual cell kinetic dependence on an increased cellular proliferative capacity. Our in vitro studies [6] with freshly explanted, purified FSCL and SLL indicate that none of the currently described Interleukin growth factors (i.e. IL 1-7) will reproducibly stimulate cell growth in the low grade B cell lymphomas. We have found that both the high and low molecular weight human B cell growth factors (BCGFs) will stimulate modest amounts of tritiated thymidine incorporation, with extended presence of viable cells for up to 6 weeks in these cultures. Actual lymphoma cell growth is however minimal, and in no case have we seen the cells undergo immortalization into a permanent cell line. Recently however, Levy's group has reported that human recombinant IL-3 can stimulate FSCL growth in vitro [7], and we have confirmed that this cytokine does stimulate some thymidine incorporation but little actual cell growth in these lymphomas in vitro. These findings suggest that cell growth in the low grade B cell lymphomas may be controlled by paracrine secretion of cytokine growth factors by the ubiquitous T cell populations usually found in and around the lymphomatous lesions. Like CLL, this group of common lymphoid neoplasms presents the conundrum of malignancy in the face of minimal neoplastic cell growth. Perhaps a cogent question here relates to the possiblity of a cryptic location for a potential "proliferating pool" of low grade lymphoma cells. While we and others have generally studied the lymphomatous cells found in involved peripheral lymph nodes, spleens, etc., it may be that another anatomic site contains tumor cells with a higher proliferative capacity. A likely candidate could be the bone marrow, (that is almost always involved in these diseases), that may harbor a malignant cell population with larger percentage of dividing cells, giving rise to peregrine lymphoma cells that subsequently traffick into the B cell dependent anatomic domains in lymphoid tissue, such as the germinal centers, giving rise to the pathologic pattern of FSCL.

High Grade Lymphomas.

High grade B cell lymphomas are also common human lymphoid neoplasms, that generally fulfill most of the commonly held general concepts about cancer cells much better than their low grade NHL-B counterparts. These lymphomas usually display aggressive clinical characteristics, local invasiveness, and a high proliferative capacity on cell kinetic analysis. High grade human B cell lymphomas are generally classified as either large cell type or small non-cleaved cell (Burkitt's) type by the IWF, although some less aggressive presentations of large cell types can fall into the inevitable "Intermediate" category. The AIDS related lymphomas (ARL) have recently emerged as one significant group in this category, with the large cell component usually being of the "Immunoblastic" subtype[8]. High grade NHL-B are heterogeneous not only with regard to histopathologic criteria, but also clinically and biologically, as they appear to represent the neoplastic manifestations of several biologic or immunologic pathophysiologic processes. First they represent the apparent eventual end stage in the natural history of some forms of low grade NHL-B, if the patients can be followed long enough (often >10 years) to undergo transformation to the large cell (aggressive) phenotype[9]. Secondly, they are the lymphoid neoplasms associated with the immune deficient state, either natural or iatrogenic, being frequently observed as the apparent consequence of immune suppression as in AIDS or as part or a post-transplant syndrome[10]. Thirdly, these lymphomas can arise de novo, without any of the previous associations being evident., suggesting that multiple etiologic pathways to high grade lymphomagenesis exist. The relationship between high grade NHL-B and the low grade NHL-B is probably best demonstrated in the first example above, where the "transformation" process is not only a morphologic event, possibly analogous to blast transformation in normal B cell activation, but also a herald of a completely different clinical and biologic behavior in these patients, most likely secondary to the expression of a new program of genes in the lymphoma cells. One of the most striking differences observed in the "transformed" high grade NHL-B is autorcrine cell growth, giving rise to the prodigious lymphoma growth often observed clinically in these patients, and reflected in vitro by the propensity of these tumors to immortalize into permanent cell lines by methods that we have described[11]. In these studies on high grade B cell lymphomas, we provide purified BCGF to freshly explanted, biopsy-derived NHL-B as an exogenous source of growth factor, to stimulate the growth of the lymphoma cells while they are acclimating to the initial shock of in vitro culture conditions, and to over-ride the inherent tendency of the freshly explanted NHL-B cells to undergo apoptosis during the ensuing culture crisis period. (Fig. 1). Once cell growth has been sustained through this crisis period, exogenous growth factor can be discontinued and biologically active AGF/BCGF is found in the culture supernatants.

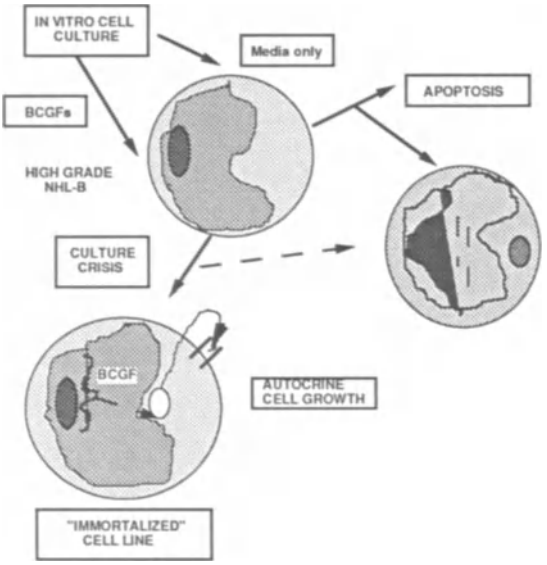


Fig. 1. In vitro cell culture techniques for generating long term cell lines from freshly explanted high grade NHL-B cells.

Human B Cell Growth Factors

Recent studies in our laboratory have shown that BCGFs can also be isolated from clinical specimens in which the cytokines are secreted into malignant pleural or other effusions. BCGFs isolated from these effusions show growth stimulatory activity on both autochthanous lymphoma cells as well as other normal and neoplastic B cells in vitro[12]. These studies suggest that actively secreted AFG/BCGF(s) are involved in the in vivo control of malignant cell growth. In our model, the candidate genetic changes for mediating these phenomena include the expression of a possible family of growth control genes that include the human BCGFs. These genes, which are usually expressed by activated T cells, do not appear to be expressed in normal B cells or in low grade NHL-B. In collaboration with Dr Julian Ambrus (Washington Univ.), we have recently isolated cDNA clones for the HMW (60kD) BCGF from a Burkitt's lymphoma cell line[13]. Recombinant growth factor (rBCGF), derived from expression of these cDNAs in prokaryotic vectors has shown growth factor activity on both normal and neoplastic human B cells in vitro. Our model predicts that the "transformational " event(s) from a normal B cell or low grade NHL-B leading to high grade NHL-B, the nature of which is completely unknown, leads to expression of these growth-associated genes, giving rise to autocrine lymphoma cell growth. Various proto-oncogenes such as *myc* and *bcl -2* may also be activated as part of the pathogenetic mechanism. (Fig.2)

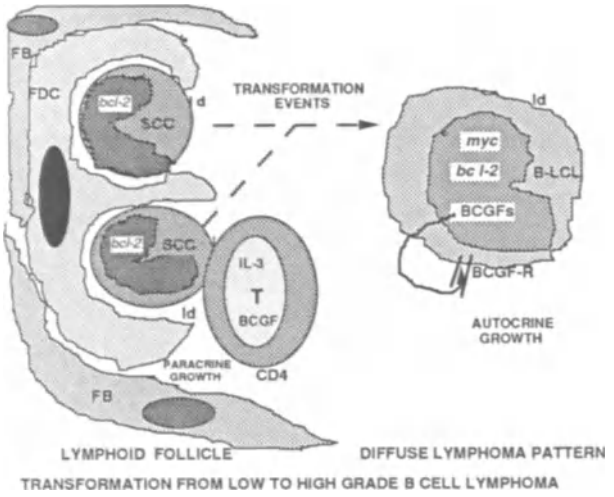


Fig. 2. Hypothetical mechanism for the transformation of low grade follicular NHL-B to high grade B cell lymphoma (see text).

This model implies a possible biotherapeutic appoach to these common lymphomas, that can often be refractory to conventional chemotherapy at presentation or after relapse, leaving the clinician with no effective therapeutic options. A tumor dependent on an autocrine loop type growth mechanism can at least theoretically be attacked therapeutically at a one or more of a variety of points that can be thought of as "weak links", by interdicting the growth factor "loop". Hybrid growth factor-toxins[14], growth factor analogues, anti-sense oligos for growth factors and / or their receptors, are but a few of the potential strategies that can be considered for the next generation of therapeutic modalities for these lymphomas.

References.

1. Magrath IT (ed.) (1990) The Non-Hodgkin's Lymphomas. Williams & Wilkens, Baltimore.
2. NCI Non-Hodgkin's lymphoma classification project (1982), Cancer 49:2112-2135.
3. Gaidano G, Ballerini P, Gong JZ, Neri A, Newcombe EW, Magrath IT, Knowles DM, Dalla-Favera R. (1991) p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc Natl Acad Sci (USA) 88:5413-5417.
4. McDonnell TJ, Deane N, Platt F, Hockenbery D, London L, Mckearn JP, Korsmeyer SJ (1989) bcl-2 -immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 57: 79-88.
5. Ford RJ, Kwok D, Quesada J, Sahasrabuddhe CG. (1986) Production of B cell growth factors by neoplastic B cells from hairy cell leukemia patients. Blood 67:573-577.
6. Ford RJ, Kouttab N, Sahasrabuddhe CG, Davis FM, Mehta SR. (1985) Growth factor-mediated proliferation in B cell non-Hodgkin's lymphomas. Blood 65:1335-1341.
7. Clayberger C, Lee JE, Pillai A, Campbell M, Levy R, Krensky AM. (1992) Interleukin 3 is a growth factor for human follicular B cell lymphoma. J Exp Med 175:371-376.
8. Knowles DM, Chamuluk GA, Subar M, Dalla-Favera R. (1988). Lymphoid neoplasia associated with acquired immune deficiency syndrome (AIDS): The New York University experience. Ann Intern Med 108: 744-753.
9. Horning SJ, Rosenberg SA. (1984) The natural history of initially untreated low-grade non-Hodgkin's lymphomas. N Eng J Med 311:1471-1475.
10. Swerdlow SH. (1992) Post-transplant lymphoproliferative disorders: a morphologic, phenotypic and genotypic spectrum of disease. Histopathol 20:373-385.
11. Ford RJ, Goodacre A, Ramiriz I, Mehta SR, Cabanillas F. (1990). Establishment and characterization of human B cell lymphoma cell lines using B-cell growth factor. Blood 75:1311-1318.
12. Ford RJ, Tamayo A , Ambrus JL (1992) Isolation of autocrine B cell growth factors from effusions of patients with high grade B cell lymphomas. Manuscript submitted for publication.
13. Ambrus JL, Fauci AL, Tamayo A, Li DJ, Ford RJ. (1992) Cloning of a c-DNA for high molecular weight B cell growth factor (HMW-BCGF). Submitted for publication.
14. Kreitman RJ, Fitzgerald D. Pastan I. (1992) Targeting growth factor receptors with fusion toxins. Int J Immunopharmac 14:465-472.

Production of Interleukin-6 in High Grade B Lymphomas

D. EMILIE, O. DEVERGNE, M. RAPHAEL*, L. J. COUMBARAS, and P. GALANAUD

INSERM U131, Clamart, and
*Département d'hématologie, Hôpital de la Pitié, Paris, France.

A number of reports have outlined the role of IL-6 in the growth of malignant B lymphocytes in multiple myeloma. From these studies, it can be asked whether IL-6 is also able to stimulate the growth of malignant B lymphocytes frozen at other stages of maturation. Such an hypothesis requires however that IL-6 is available for malignant cells *in situ* and that these latter cells express the corresponding receptor.

The present study was undertaken to investigate the *in situ* production of IL-6 in high grade B lymphomas. We particularly focused this work on AIDS lymphomas as high grade B lymphomas are frequent in the course of HIV infection and as HIV interferes with the biology of IL-6. Indeed, HIV induces IL-6 production both *in vitro* and *in vivo*, and IL-6 is involved in the chronic stimulation of B lymphocytes from HIV-infected patients (1-3).

IL-6 Gene-Expressing Cells in High Grade B Lymphomas.

Twenty four high grade B lymphomas were included in this work. Their main characteristics are summarized in table 1. Eighteen of them were from HIV-infected patients and corresponded to the most frequent pathological forms of lymphomas observed during HIV infection (Burkitt's, diffuse large cell and immunoblastic lymphomas).

Case n°	1	2	3	4	5	6	7	8
Pathology (a)	BL	BL	BL	BL	BL	BL	BL	BL
EBV (b)	+	+	NT	+	-	NT	NT	-
HIV (c)	+	+	+	+	+	+	+	+
Case n°	9	10	11	12	13	14	15	16
Pathology	BL*	BL	BL	DLCL	DLCL	DLCL	DLCL	DLCL*
EBV	+	NT	NT	+	+	-	NT	NT
HIV	+	-	-	+	+	+	-	-
Case n°	17	18	19	20	21	22	23	24
Pathology	DLCL*	DLCL*	IB*	IB*	IB*	IB*	IB*	IB*
EBV	+	-	+	+	+	-	NT	NT
HIV	+	+	+	+	+	+	-	-

Table 1. Main characteristics of lymphomatous tissues.
a: BL: Burkitt's lymphoma; DLCL: diffuse large cell lymphoma; IB: immunoblastic lymphoma.
*Presence of an immunoblastic component within the malignant clone.
b: Presence of the EBV genome in pathological samples was analyzed by Southern blot analysis.
NT: not tested
c: HIV serology of patients.

Current Topics in Microbiology and Immunology, Vol. 182

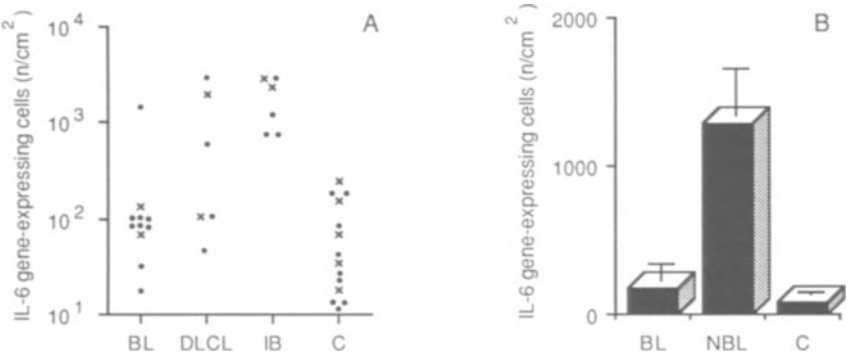


Fig.1. IL-6 Gene Expression in High Grade B Lymphomas

BL: Burkitt's lymphoma; DLCL: diffuse large cell lymphoma; IB: immunoblastic lymphoma; C: follicular hyperplasia; NBL: non-Burkitt's lymphoma (DLCL or IB).
● and × : tissues from HIV infected and uninfected patients, respectively.
Results are expressed as the number of IL-6 gene-expressing cells /cm^2 of tissue section.

The presence of IL-6-producing cells in these lymphomas was first assessed by *in situ* hybridization experiments. Positive cells were detected in all lymphomas, either related to HIV or not. However, the number of IL-6 gene-expressing cells was highly heterogeneous between cases. Interestingly, this number correlated with pathological findings.

In 10 out of the 11 Burkitt's lymphomas, IL-6 gene-expressing cells were present in limited number. This amount was not increased as compared to control lymph nodes displaying a benign follicular hyperplasia and involved in an anti-infectious immune response. In contrast, the 6 immunoblastic lymphomas tested all contained particularly large numbers of IL-6 gene-expressing cells. Three out of the 7 diffuse large cell lymphomas also contained numerous IL-6 gene-expressing cells (Fig.1a). Overall, the number of IL-6 gene-expressing cells was significantly lower in the Burkitt's lymphomas than in the non-Burkitt's lymphomas ($p<0.01$ using a Mann-Whitney's U test) (Fig.1b).

The IL-6 Gene is Expressed at a High Level in Lymphomas Containing Malignant Immunoblasts.

Although the level of IL-6 gene expression and the classification of the lymphoma according to the Working Formulation (4) were clearly related, analysis of individual cases showed that this correlation was not absolute. We thus asked whether the level of IL-6 gene-expressing cells could be related to parameters other than the pathological group of the lymphoma.

No correlation was found between the HIV status of patients and the level of IL-6 gene expression (see Fig.1a). Similarly, the level of IL-6 production in AIDS patients was not correlated with their circulating CD4+ cell count (data not shown).

As EBV can upregulate IL-6 production (5), we asked whether those lymphomas containing the EBV genome expressed the IL-6 gene at a higher level. No correlation was observed (Fig.2a). For example, those Burkitt's lymphomas which

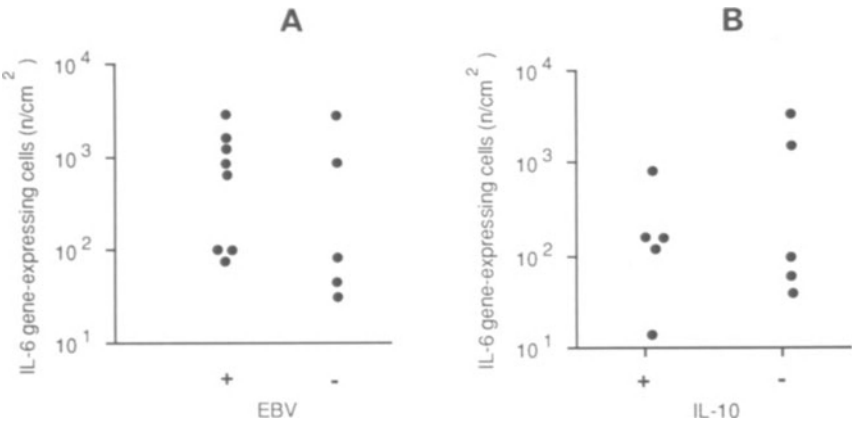


Fig.2. Absence of correlation between IL-6 production and either EBV infection or IL-10 production in high grade B lymphomas.

contained the EBV genome did not expressed the IL-6 gene at a high level. On the other hand, one immunoblastic lymphoma contained no EBV genome but a high number of IL-6 gene-expressing cells. Although this finding does not rule out a role for EBV in IL-6 production, it indicates that EBV is neither required nor sufficient to account for increased IL-6 production in some high grade lymphomas.

IL-10 can down-regulate IL-6 production (6), and we recently showed that this gene is expressed by malignant cells in a number of AIDS lymphomas (7). We thus looked for an inverse correlation between IL-6 and IL-10 productions. Such a correlation was not detected (Fig.2b).

In contrast, increased expression of the IL-6 gene was strongly associated with the presence of immunoblasts whithin the malignant clone. Only one case of Burkitt's lymphoma was associated with an increased IL-6 gene expression as compared to benign control lymph nodes. This case differed histologically from the other 10 Burkitt's lymphomas as it was the only one to contain malignant immunoblasts in association with small non-cleaved cells (Fig.3a), a feature already

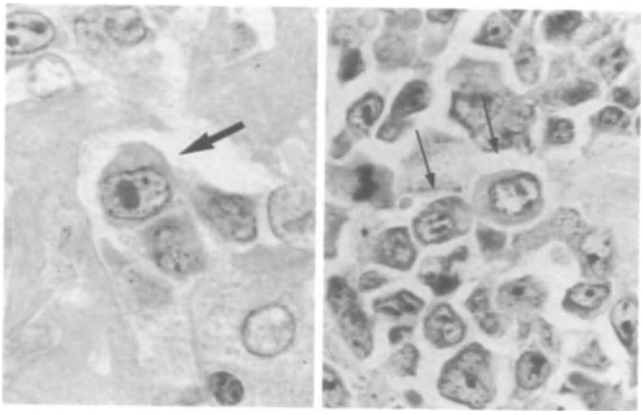


Fig.3. Immunoblasts in a Burkitt's lymphoma (A) and in a polymorphic diffuse large cell lymphoma (B).

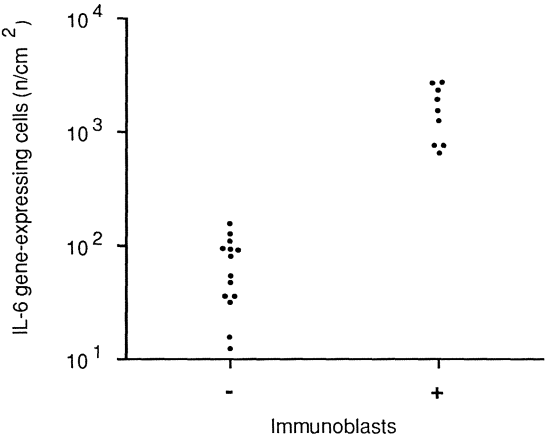


Fig.4. Increased Expression of IL-6 in Immunoblastic-Containing Lymphomas

reported in some Burkitt's lymphomas, and particularly in Burkitt's lymphomas associated with HIV infection (8-10). Of the non-Burkitt's lymphomas, the group of diffuse large cell lymphomas appeared heterogeneous with respect to IL-6 gene expression. Only 3 of the 7 cases expressed the IL-6 gene at a high level. These 3 cases were histologically different from the 4 others as they were polymorphic, associating immunoblasts and centroblasts (Fig.3b). The other 4 cases were monomorphic, containing no immunoblasts (8).

The presence of malignant immunoblasts in the lymphomatous tissue was thus the major parameter associated with an increased IL-6 gene expression. Altogether, the mean number of IL-6 gene-expressing cells was 23 times higher in the 10 lymphomas containing immunoblasts than in the 14 lymphomas containing no immunoblasts ($p<0.001$)(Fig 4).

Paracrine Production of IL-6 in High Grade B Lymphomas.

We next analyzed whether IL-6 was produced by malignant B lymphocytes themselves or by stromal cells. Although the morphology of IL-6-producing cells could not be accurately determined by *in situ* hybridization experiments for most positive cells, some of them undoubtly corresponded to endothelial cells (Fig 5a). This finding was confirmed in experiments in which the presence of IL-6-producing cells was assessed by immunohistochemistry using an anti-IL-6 mAb (kindly provided by Dr Jo Van Damme). These experiments confirmed the link between an increased IL-6 production and the presence of immunoblasts, as stained cells were only detected in those lymphomas which contained immunoblasts. These studies also showed that a number of IL-6-producing cells were indeed endothelial cells. In addition, tumor-infiltrating macrophages were found to contain IL-6. In contrast, virtually all malignant cells were unstained with the anti-IL-6 mAb, and the rare malignant cells which scored positive were very weakly stained (data not shown). Therefore, IL-6 was mainly if not exclusively produced by reactive, non malignant cells in high grade B lymphomas. This result contrasted with the production of IL-6

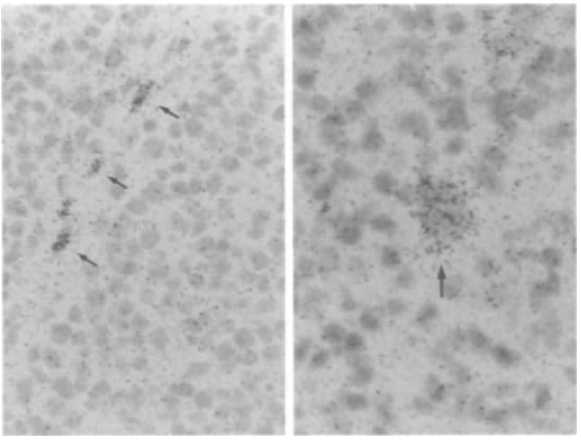


Fig.5. IL-6 production by endothelial cells (arrows) in high grade B lymphomas (a) and by Reed-Sternberg cells (arrow) in a HIV-related Hodgkin's disease (b).

by Reed-Sternberg cells in 4 cases of HIV-associated Hodgkin's disease (Fig.5b), a finding already reported in HIV-unrelated Hodgkin's disease (11).

Expression of the IL-6 Receptor by Malignant Immunoblasts.

Expression of the IL-6 receptor was assessed by immunohistochemistry using a mAb (kindly provided by Dr T. Kishimoto) directed toward the 80KD chain of the IL-6 receptor. Four cases were studied, which all contained malignant immunoblasts. Three were from HIV-infected patients and one was from an HIV-seronegative patient. In the 4 cases, malignant immunoblasts were shown to express the p80 chain of the IL-6 receptor (data not shown), thus suggesting that the IL-6 produced in the vicinity of malignant immunoblasts may affect their behaviour.

Discussion

Taken together, these findings show that the IL-6 gene is expressed *in situ* at a high level in some but not all high grade B lymphomas. This level of expression does not correlate with the HIV status of patients but is strongly linked to the pathological features of the lymphoma: an increased production of IL-6 is only observed in lymphomas in which an immunoblastic component is present within the malignant clone. In these cases, IL-6 is produced mainly if not only by stromal cells.

These findings raise two different questions. The first one is the mechanism linking an increased IL-6 production by stromal cells and the presence of malignant immunoblasts. Different hypotheses could account for this observation (discussed in 12). Interferon-γ stimulates the production of IL-6 by macrophages and

endothelial cells, and we previously showed that interferon-γ-producing cells are present *in situ* in lymphomas (13). One can hypothesize that in Burkitt's lymphomas, which express only low amounts of adhesion molecules due to c-Myc gene deregulation, malignant cells cannot be recognized by immune cells, leading to an absence of *in situ* production of cytokines, including interferon-γ and IL-6. However, we do not favour such an hypothesis as we recently observed that the level of *in situ* interferon-γ production was correlated neither with the pathological type of the lymphoma nor with the level of IL-6 production (unpublished results). Alternatively, malignant B lymphocytes may be able to directly stimulate IL-6 production by their microenvironment. In this case, they would be able to do this only if they have reached a given stage of maturation along the B lymphocyte lineage. The striking similarity between immunoblastic lymphomas and multiple myelomas with respect to the induction of IL-6 production by stromal cells (14-16) may in then reflect the advanced stage of differentiation of malignant B lymphocytes in both conditions. In contrast, Burkitt's cells and monomorphic large cells, which correspond to less differentiated cells, may not be able to trigger IL-6 production by the microenvironment.

The second issue raised by our results is whether IL-6 play any role in the growth of high grade B lymphomas. This is suggested by the effect of IL-6 in the growth of myeloma cells. Involvement of IL-6 in the proliferation of some high grade B lymphomas would however not exclude a role for additional growth factors. Previous reports have indeed documented the synergistic effects of various mediators in the IL-6-induced proliferation of myeloma cells. In high grade B lymphomas, IL-10 may be one of these additional factors as it can stimulate the growth of B lymphocytes (17) and as it is produced *in situ* by malignant cells in EBV-related lymphomas of AIDS patients (7).

Definition of the growth requirements of malignant cells in high grade B lymphomas is a critical issue as it may allow alternative therapeutic approaches in the clinical situations which poorly respond to conventional chemotherapy. This is particularly the case of AIDS lymphomas. Our demonstration that in some AIDS lymphomas IL-6 is produced *in situ* in a paracrine fashion and that malignant cells express the IL-6 receptor indicates that, as in multiple myeloma (18), a positive effect of therapies interfering with IL-6 effects may be obtained in this condition. Our results indicate that such a therapeutic approach should primarily be evaluated in those lymphomas which contain malignant immunoblasts.

References

1. Birx DL, Redfield RR, Tencer K, Fowler A, Burke DS, Tosato G. (1990) Induction of interleukin-6 during Human Immunodeficiency Virus infection. Blood 76:2303-2310.
2. Emilie D, Peuchmaur M, Maillot MC, Crevon MC, Brousse N, Delfraissy JF, Dormont J, Galanaud P. (1990) Production of interleukins in Human Immunodeficiency Virus-1-replicating lymph nodes. J Clin Invest 86:148-159.
3. Amadori A, Zamarchi R, Veronese ML, Panozzo M, Barelli A, Borri A, Sironi M, Colotta F, Mantovani A, Chieco-Bianchi L. (1991) B cell activation during HIV-1 infection. Cell-to-cell interactions and cytokine requirement. J Immunol 146:57-62.
4. Rosenberg S, Berard CW, Brown BW. (1982) National Cancer Institute sponsored study of classification of non-Hodgkin's lymphoma: summary and description of a working formulation for clinical usage. Cancer 49:2112-2135.

5. Tosato G, Tanner J, Jones KD, Revel M, Pike SE. (1990) Identification of Interleukin-6 as an autocrine growth factor for Epstein-Barr virus-immortalized B cells. J Virol 64:3033-3041.
6. de Waal Malefyt R, Abrams J, Bennet B, Figdor C G, de Vries J.E. (1991) Interleukin-10 inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J. Exp. Med. 174:1209-1220.
7. Emilie D, Touitou R, Raphael M, Peuchmaur M, Devergne O, Rea D, Coubaras J, Crevon MC, Edelman L, Joab I, Galanaud P. (1992) *In vivo* production of IL-10 by malignant cells in AIDS lymphomas. Submitted for publication.
8. Hui PK, Feller AC, Lennert K. (1988) High-grade non-Hodgkin's lymphoma of B-cell type, I: Histopathology. Histopathology 12:127-143.
9. Sigaux F, Berger R, Bernheim A, Flandrin G. (1984) Malignant lymphomas with band 8q24 abnormality: a morphologic continuum extending from Burkitt's to immunoblastic lymphoma. Br J Haematol 57:393-405.
10. Raphael M, Gentilhomme O, Tulliez M, Byron PA, Diebold J. (1991) Histopathologic features of high-grade non-Hodgkin's lymphomas in acquired immunodeficiency syndrome. Arch Pathol Lab Med 115:15-20.
11. Jücker M, Abts H, Schindler R, Merz H, Günther A, von Kalle C, Schaadt M, Diamanstein T, Feller AC, Krueger GRF, Diehl V, Blankenstein T, Tesch H. (1991) Expression of interleukin-6 and interleukin-6 receptor in Hodgkin's disease. Blood 77:2413-2418.
12. Emilie D, Coumbaras J, Raphael M, Devergne O, Delecluse HJ, Gisselbrecht C, Michiels JF, Van Damme J, Taga T, Kishimoto T, Crevon MC, Galanaud P. (1992) Interleukin-6 production in high grade B lymphomas: Correlation with the presence of malignant immunoblasts in acquired immunodeficiency syndrome and in human immunodeficiency virus-seronegative patients. Blood. In press.
13. Peuchmaur M, Emilie D, Crevon MC, Brousse N, Gaulard P, D'Agay MF, Galanaud P, Solal)Celigny P. (1991) Interleukin-2 and interferon-γ production in follicular lymphomas. Am J Clin Pathol 95:55-62.
14. Klein B, Zhang XG, Jourdan M, Content J, Houssiau F, Aarden L, Piechaczyk M, Bataille R. (1989) Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. Blood 73:517-526.
15. Calligaris-Cappio F, Bergui L, Gregoretti MG, Gaidano G, Gabolli M, Schena M, Zallone AZ, Marchisio PC. (1991) Role of bone marrow stromal cells in the growth of human multiple myeloma. Blood 77:2688-2693.
16. Portier M, Rajzbaum G, Zhang XG, Attal M, Rusalen C, Wijdenes J, Mannoni P, Maraninchi D, Piechaczyk M, Bataille R, Klein B. (1991) In vivo interleukin 6 gene expression in the tumoral environment in multiple myeloma. Eur J Immunol. 21:1759-1762.
17. Rousset F., Garcia E, Defrance T, Péronne C, Vezzio N, Hsu D-H, Kastelein R, Moore K W, Banchereau J. (1992) Interleukin-10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc. Natl. Acad. Sci. USA. 89:1890-1893.
18. Klein B, Wijdenes J, Zhang XG, Jourdan M, Boiron JM, Brochier J, Liautard J, Merlin M, Clement C, Morel-Fournier B, Lu ZY, Mannoni P, Sany J, Bataille R. (1991) Murine anti-interleukin-6 monoclonal antibody therapy for a patient with plasma cell leukemia. Blood 78:1198-1204.

Lymphomas: EBV, AIDS Associated Lymphomas

Mutational Analysis of the Transforming Function of the EBV Encoded LMP-1

R. MOORTHY and D. A. THORLEY-LAWSON

Dept. of Pathology, Tufts University School of Medicine, 136, Harrison Ave., Boston MA 02111

Introduction

Epstein-Barr virus, a member of the herpesvirus group, infects B cells in vitro and in vivo. In vitro the virus infects small resting B cells and drives them to become immortalized, latently infected, proliferating B lymphoblasts. This process has been employed as a model system for studying the mechanisms of herpesvirus latency, B cell activation and oncogenic transformation. To date nine virus encoded proteins have been detected in latently infected B cells (reviewed in Kieff and Liebowitz 1989) consisting of six nuclear proteins (EBNA's) and three membrane proteins (LMP-1, TP-1 and TP-2). Of these only LMP-1 has been demonstrated to have the function of a transforming oncogene in rodent fibroblasts such as Balb 3T3 (Baichwal and Sugden 1988) and Rat 1 cells (Wang et al 1985).

LMP-1 is localised in the plasma membrane and a diagrammatic representation of its orientation and structure within the membrane is presented in Figure 1. It has a short, hydrophilic amino-terminal domain and a long hydrophilic carboxy-terminal domain, both of which project

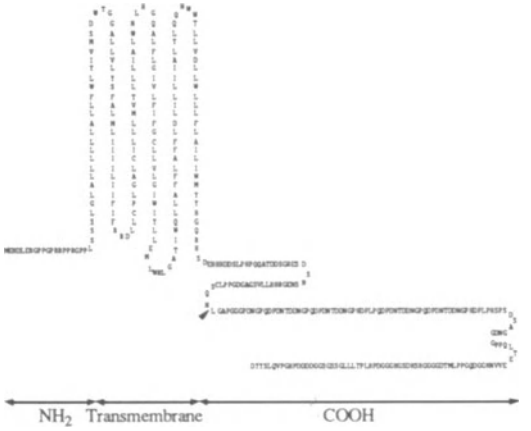


Fig. 1. Diagrammatic Representation of the Orientation of LMP-1 in the Plasma Membrane.

The position of the three major structural domains is indicated. The amino-and carboxy-terminal domains are on the cytoplasmic side of the plasma membrane The arrow head marks the point at which LMP-1 is cleaved to release p25 into the cytoplasm.

into the cytoplasm. The two ends are linked by six membrane-spanning hydrophobic sequences connected by short hydrophilic loops. LMP-1 is synthesized and translocated to the plasma membrane where it becomes associated with the cytoskeleton as a phosphoprotein (Baichwal

Current Topics in Microbiology and Immunology, Vol. 182

and Sugden 1987, Mann and Thorley-Lawson 1987). This process occurrs with a half-life of 1-2 hrs. The protein is then turned over with a short half-life of 2-3hrs., through a specific single proteolytic cleavage that results in the carboxy-terminal domain being released into the cytoplasm as a 25kD phosphoprotein (p25) (Moorthy and Thorley-Lawson 1990). Both intact LMP-1 and p25 are phosphorylated on serine and threonine residues at a ratio of about 6:1.

Previous analysis has suggested that the amino-terminal and transmembrane domains are both required for the transformation of rodent fibroblasts, but the carboxy terminal domain is dispensable (Baichwal and Sugden 1988). The amino-terminal domain has also been reported to be essential for cytoskeleton association and to be required to demonstrate the toxic effects of LMP-1 seen when it is transfected into various cell types (Hammerschmidt et al 1989). The strong selective pressure exerted by this toxic effect, when stable transfectants are being derived, has not been well recognised in interpreting such experiments. Thus, it has been demonstrated that the stable expression of LMP-1 in EBV-negative B cell lines is associated with a cellular phenotype more typical of an EBV lymphoblastoid cell line including the induction of bcl-2 (Henderson et al 1991). These experiments have been interpreted to demonstrate that LMP-1 is inducing the expression of this phenotype. However, the only cell type that tolerates LMP-1 well is the EBV lymphoblast, therefore it is highly possible that LMP-1 is selecting for, rather than inducing the phenotypic changes observed. This interpretation is also consistent with the observation that LMP-1 expression occurs just prior to entry of the cells into S phase (Mann et al 1985), too late to induce the blastoid phenotype and more consistent with the notion that LMP-1 cannot be tolerated until the cells have become activated.

The studies reported here were initiated to attempt to correlate the biochemical and functional properties of LMP-1 by generating and testing a series of deletion and point mutations of LMP-1.

Analysis of Deletion Mutants.

We have analyzed the transforming properties and biochemical processing of three deletion mutants compared to wild type LMP-1. The deletions were of the first 43 amino-terminal amino-acids (NΔ43), the transmembrane sequences (Δ27-212) and the last 144 carboxy-terminal amino-acids (cD242-386) respectively. CΔ242-386 was engineered so as to terminate at the point where wild type LMP-1 is normally cleaved to release p25 and thus should represent what remains in the membrane after cleavage. NΔ43 and Δ27-212 were kindly provided by Dr. Bill Sugden. The mutants and their properties are summarized in Table I.

Rat-1 cells were transfected with plasmids containing the appropriate mutant. In the case of NΔ43 and Δ27-212, which do not contain a selectable marker, the plasmids are all derivatives of the pSV2BNLF1 construct and were cotransfected with the pSV2gpt plasmid. The CΔ242-386 mutant was constructed by placing a translational stop codon at the appropriate position in the pSV2gptLMP construct (kindly supplied by Dr. Elliot Kieff). Drug resistant colonies were then grown up, stained with methylene blue and scored, to obtain a measure of transfection efficiency. Subsequently, transformed foci were counted. In parallel, individual colonies were picked grown up and LMP-1 expression checked by Western blot. The phosphorylation status of LMP-1 and p25 was then assessed by performing immuneprecipitation on cells metabolically labelled with ^{32}P.

The most important conclusion that can be drawn from this study is that all three major domains are necessary for LMP-1 to function as a transforming oncogene in our experiments. The analysis of mutant Δ27-212 demonstrates two points of interest. Since this mutant lacks the transmembrane sequences it does not localize to the plasma membrane thus membrane localization is not essential for LMP-1 to become phosphorylated, but is required for cleavage and turnover.

Table I. Summary of the Findings of the Biochemical Processing and Transforming Efficiency of Mutants Deleted in the Three Major Domains of LMP-1

MUTANT		Pi [1]	CLEAVE [2]	TRANSFORM [3]
WT		+	+	+
NΔ43*		+	+	-
Δ27-212*		+	-	-
CΔ242-386				-

* Kindly supplied by Dr. Bill Sugden.

1. Phosphorylation was confirmed by immuneprecipitation from cells metabolically labelled with 32Pi.
2. Cleavage was confirmed by detecting p25 in cells metabolically labelled with 32Pi or 3H-proline.
3. Transformation was assesed by counting transformed foci in double blind experiments repeated at least three times. + indicates an average of 30-40 foci per plate, - an average of >1.

Biochemical Localization of the Major Sites of Phosphorylation.

The p25 molecule contains a number of serine and threonine residues that could act as targets for phosphorylation. To narrow down the number of possibilities we took advantage of the fact that p25 has unique CNBr (met) and trypsin (lys) cleavage sites at amino-acids 339 and 330 respectively.

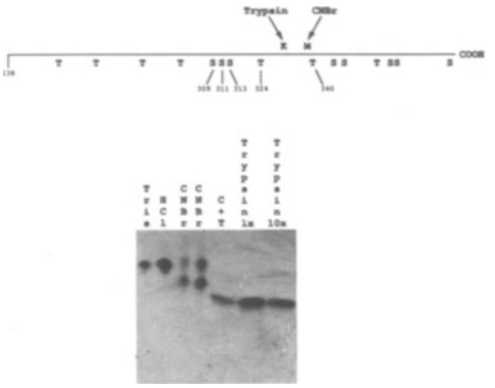


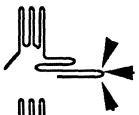
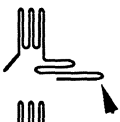
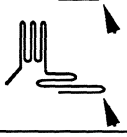
Fig 2. Localization of the phosphorylation sites by cleavage of p25.
The upper part of the figure demonstrates the p25 sequence with the unique CNBr (M) and trypsin (K) sites and the threonine (T) and serine (S) residues identified. The lower part of the figure demonstrates the result of single and double cleavage of p25. Note that doubly cleaved p25 has the same size as cleaved with trypsin alone, smaller than CNBR alone. This localizes the phosphorylation sites amino-terminal to the trypsin site.

By cleaving ^{32}P labelled p25 singly or in combination we were able to localize (Fig. 2) the phospho-aminoacids to a sequence between amino-acid 242 (the site of cleavage for release of p25) and the lysine residue at position 330. This narrowed down the site of serine phosphorylation to three serines (position 309, 311 and 313) and a single threonine (position 324) since the remaining threonines reside in a repeat motif.

Mutational Analysis of the Serine Phosphorylation Site.

To precisely identify the site of serine phosphorylation the serine residues at position 309, 311 and 313 were mutated to glycine residues either singly or in pairs or all three were mutated. Glycine was picked as a conservative change because the sequence of p25 is rich in glycine residues. The mutants were then analyzed biochemically and functionally as described for the deletion mutants above. A summary of the results is given in Table II.

Table II. Summary of the Findings of the Biochemical Processing and Transforming Efficiency of Mutants in the Three Serine Residues 309, 311 and 313.

MUTANT			Pi [1]	CLEAVE [2]	TRANSFORM [3]
WT			+	+	+
SALL	S > G		-	+	+
S309	S > G		+	+	+
S311	S > G		+	+	+
S309/311	S > G		+	+	+
S313	S > G		-	+	+
S313	S > D		TOXIC		

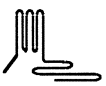
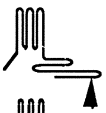
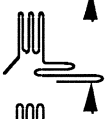
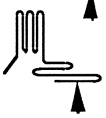
1,2 and 3. For details see legend to Table I.
TOXIC indicates that, in multiple experiments with different DNA preparations , no drug resistant colonies were obtained.
► Denotes site of mutation.
> Denotes amino-acid change.

The results demonstrate several points of interest. First, all mutations that changed serine 309 or 311, either singly or in combination, had no effect on the phosphorylation status of either LMP-1 or p25. Secondly, all mutations that involved a change in serine 313 resulted in no demonstrable phosphorylation of either LMP-1 or p25. Thus, the major site of serine phosphorylation resides at serine 313. The second key observation is that all mutants that included conversion of serine 313 to glycine were still transforming and processed as normal in the absence of detectable phosphorylation. This indicates that phosphorylation is not required for the transforming function of LMP-1. Furthermore, although these mutants do not get phosphorylated they still become associated with the cytoskeleton since they require boiling in SDS in order to be solubilized. Lastly, when serine 313 was mutated to aspartate, in order to appear constitutively phosphorylated, the mutant was toxic. Since the region around serine 313 is highly acidic it is possible to speculate that this toxicity is a specific consequence of the mutation. One possible explanation could be that this mutation cannot be turned over like wild type LMP-1 and therefore accumulates to toxic levels.

Mutational Analysis of the Threonine Phosphorylation Site.

With the exception of threonine 324, all of the threonine residues within the phosphorylated fragment of p25 were due to a repeat motif that contains a single threonine residue. For this reason only threonine 324 was analyzed. Threonine 340, which lies outside this region, was also mutated as a negative control. The results are summarized in Table III.

Table III. Summary of the Findings of the Biochemical Processing and Transforming Efficiency of Mutants at Threonine Residues 324 and 340.

MUTANT			Pi [1]	CLEAVE [2]	TRANSFORM [3]
WT			+	+	+
T324	T > G		+	+	+
T324	T > E		-	+	-
T340	T > G		+	+	+

1 and 2. For details see legend to Table I.

3. Transformation was assessed as described in the legend to Table I except in this case + indicates an average of 25-30 foci per plate.

►Denotes site of mutation.

> Denotes amino-acid change.

Mutation of threonine 324 to glycine had no detectable effect on phosphorylation. This was expected as the major site of phosphorylation is serine. However, mutation to glutamate, so as to appear constitutively phosphorylated, gave a striking result. This mutant did not become phosphorylated at all however, unlike the serine 313 mutant, it was non-transforming. It is possible that the phenotype of this mutant is due to alteration in the folding of the protein rather than a property of the threonine/glutamate change per se. This interpretation is unlikely however, since this particular threonine residue lies adjacent to two glutamate residues. Therefore, the addition of a third should not greatly impact the electrostatic charge and therefore the structure of this region. A more attractive interpretation is that the glutamate residue behaves as a constitutively phosphorylated thrteonine and as such is inactive. This leads to the conclusion that phosphorylation at threonine 324 results in an LMP-1 molecule that is inactive and dephosphorylation of threonine 324 activates LMP-1 to give its transforming signal.This interpretation would also explain the measurements of the relative extent of phosphoserine to phosphothreonine (6:1). Since there is only a single phosphoserine residue this means that only a fraction of LMP-1 molecules are threonine phosphorylated, consistent with the idea that LMP-1 is only able to signal for part of its life-time. Furthermore, since LMP-1 turns over so quickly (half-life of 2-3 hrs.) the activation of LMP-1 through dephosphorylation may be regulated in a cell cycle specific fashion.

Model for LMP-1 Function.

Figure 3 presents one possible model of LMP-1's function that attempts to incorporate all of the observations that have been derived through the analysis described here and previously.

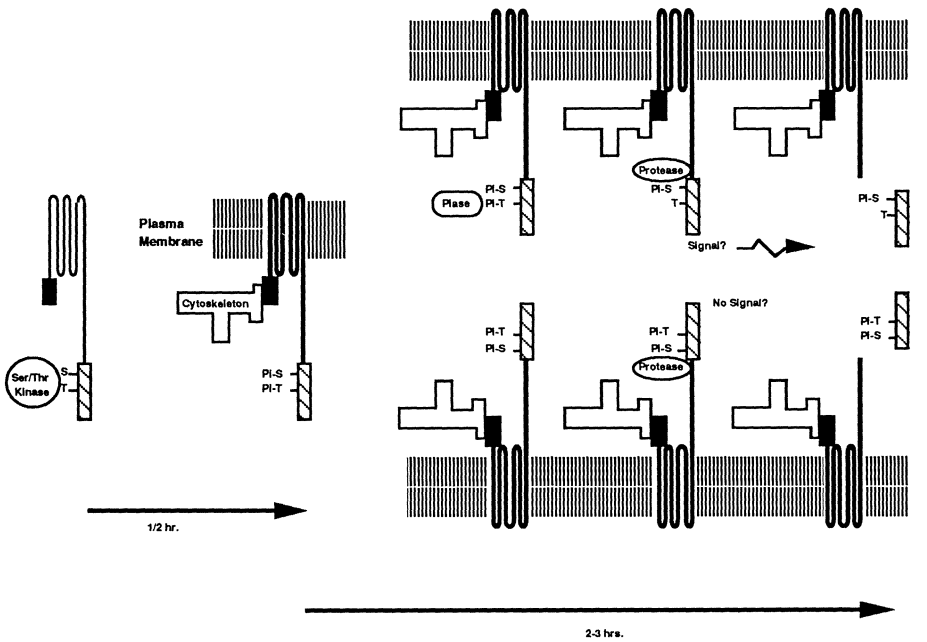


Figure 3. Schematic representation of a possible model for LMP-1 function based on the mutational and biochemical analysis described in this paper.

In this model LMP-1 is synthesized, becomes phosphorylated and associates with the cytoskeleton with a half-life of half an hour. Our results with the mutant Δ27-212 demonstrate that LMP-1 does not need to be in the plasma-membrane in order to become phosphorylated, thus phosphorylation could occur prior to arrival at the plasma membrane, however our previous studies indicated that all of the phosphorylated LMP-1 is cytoskeleton associated (Mann et al 1985) consistent with the idea that phosphorylation occurs just prior to or concomitant with cytoskeletal association. Based on the S313>G mutant it is apparent that phosphorylation is not a signal or in any other way required for cytoskeletal association nor is it required in order to generate the transforming signal. Phosphorylation occurs at serine 313 and threonine 324 and in this form LMP-1 is unable to signal (based on the non-transforming properties of T324>E). At a certain time, perhaps dependant on the stage of the cell cycle, threonine 324 is dephosphorylated and this form gives the active transforming/growth signal (based on the ability of T324>G to transform). The LMP-1 molecule is then deactivated due to cleavage (based on the inability of CΔ242-386 to transform) and p25 is released into the cytoplasm. LMP-1 needs to be localized to the plasma membrane in order to be cleaved, however, neither phosphorylation nor cytoskeleton association is required for the cleavage reaction to occur (see mutants NΔ43 and Δ27-212).

In summary therefore, we suggest that phosphorylation/dephosphorylation of threonine 324 provides an on/off switch for LMP-1 function. The role of phosphorylation at serine 313 remains unclear at present although it is clearly not essential in the Rat-1 transformation system.We are currently analyzing the biochemistry of the toxic S313>D mutant using inducible promoters and are attempting to construct viruses containing the mutations described here in order to study their effect on B cell transformation.

Acknowledgements.

This work was supported by Public Health Service Grants AI-15310 and CA-28737.

References.

Baichwal VR, Sugden B (1987) Posttranslational processing of an Epstein-Barr virus encoded membrane protein expressed in cells transformed by Epstein-Barr virus. J Virol 61:866-875

Baichwal VR, Sugden B (1988) Transformation of Balb-3T3 cells by the BNLF-1 gene of Epstein-Barr virus. Oncogene 2:461-468

Hammerscmidt W Sugden B Baichwal VR (1989) The transforming domain alone of the latent membrane protein of Epstein-Barr virus is toxic to cells when expressed at high levels. J Virol 63:2469-2475

Henderson S Rowe M Gregory C Croom-Carter D Wang F Longnecker R Kieff E Rickinson A. (1991) Induction of bcl-2 expression by Epstien-Barr virus latent membrane protein protects cells from programmed cell death. Cell 65:1107-1115

Kieff E, Liebowitz D (1989) Epstein-Barr Virus and its Replication In: Virology. Fields B. and D. Knipe, eds. Raven Press, New York, p. 1889-1920.

Mann KP Staunton D Thorley-Lawson DA (198) An Epstein-Barr virus encoded protein found in the plasma membranes of transformed cells. J Virol 55:710-720

Mann KP and Thorley-Lawson DA (1987) Posttranslational processing of the Epstein-Barr virus encoded p63/LMP protein. J Virol 61:2100-2108

Moorthy RK and Thorley-Lawson DA (1990) Processing of the Epstein-Barr virus encoded latent membrane proteinp63/LMP. J Virol 64:829-837

Wang D, Liebowitz D, Kieff E (1985) An EBV membrane protein expressed in immortalised lymphocytes transforms established rodent cells Cell 43:831-840

Integration of EBV in Burkitt's Lymphoma Cells

H. J. DELECLUSE[1], S. KOHLS[2], J. BULLERDIEK[2] and G. W. BORNKAMM

[1]Hämatologikum der GSF Institut für Klinische Molekularbiologie undTumorgenetik, D 8000 München,
[2]Zentrum für Humangenetik und Genetische Beratung, D 2800 Bremen

Burkitt's lymphoma is a highly malignant B cell tumor which occurs at high frequency in children in Equatorial Africa and New Guinea and at 20 to 50 fold lower incidence in all other parts of the world. The tumor is characterized by chromosomal translocations involving the c-myc locus on Chromosome 8 and the immunoglobulin heavy or light chain loci on chromosomes 14, 2 or 22. Activation of the c-myc protooncogene by jutaposition to regulatory elements of the Ig loci is a necessary although not sufficient event for the development of Burkitt's lymphoma (Bornkamm et al.1988). Other cofactors have therefore to be considered. EBV is associated with almost all of the cases occuring in Africa and New Guinea and 15 to 20% of the cases in low incidence areas. Tumor cells harbor multiple copies of the viral DNA and express at least one viral antigen, EBNA 1, which is involved in episomal replication of the viral DNA. EBV is assumed to be a prime candidate for providing cooperative functions for the development of Burkitt's lymphoma. Paradoxically, viral gene functions which are required to induce proliferation of normal human B cells, like EBNA2 and LMP, are not expressed in Burkitt lymphoma cells *in vivo* (Rowe et al. 1986; Rowe et al. 1987). Since large part of the immune response to EBV is mediated through EBNA2 and/or LMP, nonexpression of these antigens may help to explain immunological escape of EBV carrying Burkitt lymphoma cells, it does not help to understand, however, the role of EBV in the development of this tumor.

Viral integration is a recurrent theme of tumor virus research. In many naturally occurring as well as experimentally induced cancers viral integration plays a crucial role, either by activation of cellular protooncogenes, by inactivation of tumor suppressor genes or by abolishing the function of a viral transcriptional repressor. Taking into account the general importance of integration for virally induced cancers, surprisingly little is known about integration of EBV DNA in EBV associated tumors. The reason for this is primarily a technical one: within tumor cells EBV is present in multiple episomal copies (Lindahl et al. 1976; Kaschka-Dierich et al. 1976). The presence of additionalintegrated copies has been suggested (Adams et al. 1973). However the large number of episomal copies has precluded a definite proof of integration. Only a few cell lines with exceptional low copy have been studied for and have revealed integrated viral genome copies. The best studied example is the Burkitt lymphoma line Namalwa which was shown to harbor two integrated viral copies residing on chromosome 1p35 (Henderson et al. 1983; Matsuo et al. 1984; Lawrence et al. 1988). Integration has also been reported for the EBV immortalized cell line IB4 (Henderson et al. 1983; Hurley et al. 1991b), and for EBV negative Burkitt lymphoma lines which have been converted to EBV positivity by infection with exogenous EBV (Hurley et al. 1991a).

When we have studied expression of the viral terminal protein gene in Burkitt lymphoma cells, we have made the observation that aberrant transcripts of the

terminal protein gene could be detected in Burkitt lymphoma cells (Zimber-Strobl et al. 1991). These transcripts comprised the first exon of TP spliced to unknown sequences. Since the same type of aberrant TP transcripts had been detected in Namalwa and BL41-B95-8 cells, we reasoned that such transcripts might represent fusion transcripts of viral and cellular sequences which might be indicative for viral integration. This prompted us to reexamine the question of viral integration in Burkitt lymphoma cells in a more general fashion. It was important to find an approach (i) which would allow the detection of one integrated in a majority of non-integrated copies, and (ii) which would yield conclusive data not only in the case of integration but also if integration was not a general feature of Burkitt lymphoma cells. To this end, we have used fluorescence in situ hybridization of EBV DNA to metaphase chromosomes combined with chromosomal banding to assign hybridization signals to specific chromosomal loci. We assumed that doublet signals which are symmetrically oriented along the chromatids might represent integrated copies. If so, they should be located at specific chromosomal sites in many metaphases.

Variable EBV DNA Copy Number in the Nonproducer Line Raji

We started the analysis with in situ hybridization of metaphase spreads and interphase nuclei of the Burkitt lymphoma line Raji, which is known to contain about 50 episomal copies per cell (Hudewentz et al. 1980). Additionally integration has also been reported to occur in Raji cells, but is less well established. The viral genome of Raji cells is characterized by two deletions which preclude lytic replication and keep the copy number fairly constant (Polack et al. 1984). Despite the constant overall copy number, there is considerable variation, if the copy number is studied at a single cell level (Fig. 1).

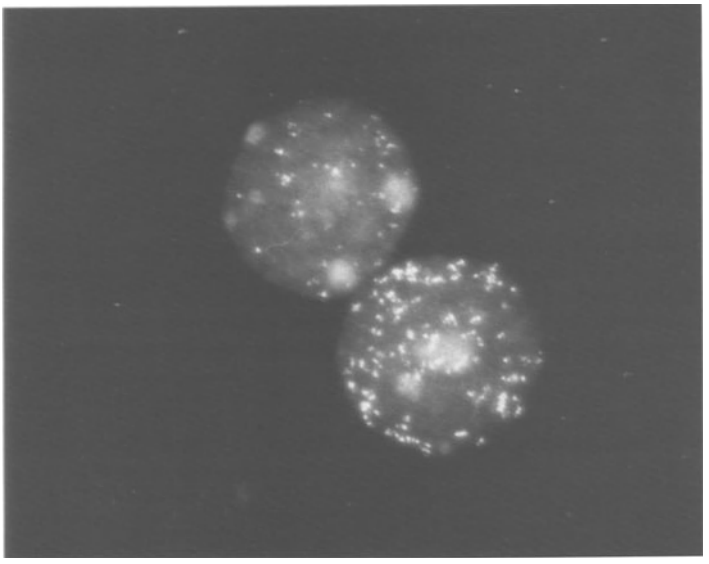


Fig 1: Fluorescent in situ hybridization on raji cells nuclei using the whole EBV as a probe. The variable numbers of copies in the two different cells is obvious.

The variation is much more than twofold excluding physiological variation within the cell cycle. Similar variations have been observed by studying the copy numbers of Raji single cell clones (Yamamoto et al. 1979; Bister et al. 1979). The observed variation in copy number raises the question whether replicated viral genomes are segregating to daughter cells in an ordered fashion or whether the viral genomes are randomly distributed during cell division.

Symmetrical Association of Episomal Viral DNA with Chromatids

It is well established that Raji cells contain a large number of viral episomal copies and only one or a few, if any, integrated copies. It was thus surprising to note that a large number of hybridization signals appear as symmetrically oriented doublet signals on both chromatids (Fig. 2).

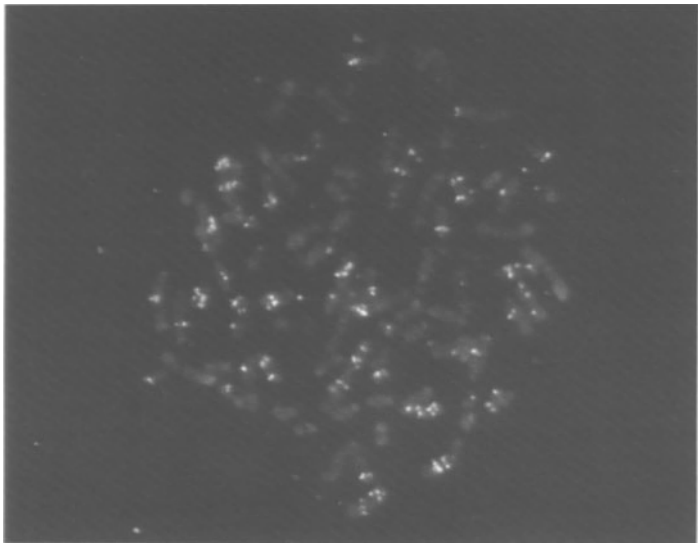


Fig 2: Fluorescent in situ hybridization on metaphase chromosomes from raji cells using the whole EBV genome as a probe. Note the symetrical orientation of the majority of the signals.

The number of doublet signals is varying from mitosis to mitosis. The doublet signals are not localized at specific sites if different metaphases are compared consistent with the assumption that they represent episomal viral DNA. The frequent association of viral episomal copies to both chromatids in mitosis argues for an ordered segregation of at least part of the episomal copies during cell division. Starting from single cell clones of EBV immortalized cell lines we are now going to study segregation of viral genomes and copy number stability in a more systematic fashion. Because of the large number of viral genome copies we have not pursued viral integration in the Raji cell line.

Two other african BL cell lines, Jijoye and LY91, presented similar problems. As Raji cells, both cell lines are highly aneuploid and harbor even more than 50 genome copies per cell. In LY91 and Jijoye, the situation is still complicated by the fact that a number of cells sustain a lytic cycle in culture and produce virus particles (Fig. 3).

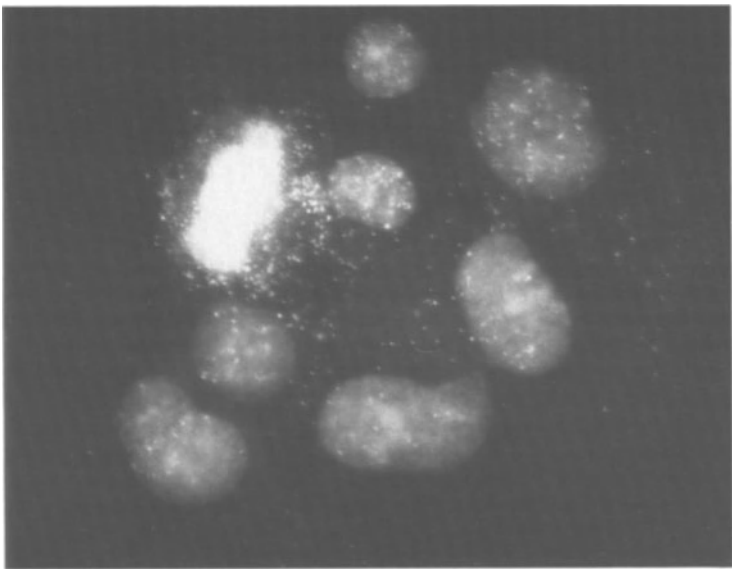


Fig 3: Active replication in LY91 cells.

If the virus is able to reinfect the remaining cells in culture, as it is known to occur in Jijoye's daughter cell line P3HR-1, the analysis of viral integration would be obscured not only be an excess of episomal but also of linear viral DNA (Heston et al. 1982; Miller et al. 1985).

After studying Raji, Jijoye and LY91 cells, we decided to make a selection for cell lines (i) with a low degree of aneuploidy, and (ii) with copy numbers not exceeding 15 to 20.

Integration of EBV in Burkitt Lymphoma Cells

Following the criteria described above it was in fact possible to identify integrated copies in EBV positive Burkitt lymphoma lines. Remarkably, integrated copies - as defined by symmetrical association with specific chromosomal loci in many metaphases - did coexist with episomal copies in the same cells. An example of a metaphase hybridized to EBV DNA and a corresponding banding pattern is shown in Fig. 4.

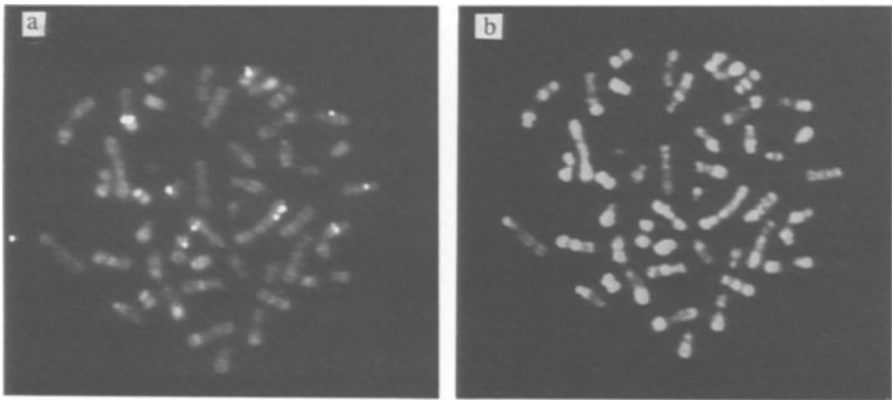


Fig 4: a: fluorescent in situ hybridization on metaphase chromosomes from LY 47 cells using the whole EBV as a probe. Note the strong symmetrical double signal at the top right of the figure.
Fig 4: b: R banding of the same mitosis.

In LY47 cells, EBV DNA was found to be integrated at one chromosomal locus on the long arm of chromosome 5. Three other Burkitt lymphoma lines also revealed viral integration, although to distinct sites. In two EBV immortalized cell lines established by infection of cord blood lymphocytes with B95-8 and M-ABA virus, respectively, only episomal and no integrated copies have been detected.

Is the Intensity of a Doublet Hybridization Signal Indicative for Viral Integration?

Studying a large number of metaphases hybridized to labeled EBV DNA we have observed pronounced differences in the intensity of hybridization signals. Symmetrical signals on both chromatids usually display the same intensity, the difference in intensity between individual pairs may vary, however, tremendously. The difference in intensity does not only reflect statistical variations in hybridization efficiency, since the integrated copies always displayed signals of high, and episomal copies usually signals of low intensity. One possible interpretation might be that EBV is not integrated as a single copy of the viral genome but rather as a cluster of repeated copies. Alternatively, the low intensity of episomal copies might reflect the intrinsic property of supercoiled circular molecules to reanneal during the hybridization procedure which might preclude efficient hybridization to the labeled probe. This might have an impact for studying by fluorescence in situ hybridization the viral genome copy number and chromosomal association of episomal copies since part of the episomal copies might even remain undetected. It might be possible to explore this property to fully suppress hybridization of episomal copies by prehybridization in the absence of a labeled probe. Alternatively, for counting copy numbers, it might be necessary to treat the chromosomes or nuclei with endonucleases to expose the viral sequences for hybridization to the labeled probe. These problems will be addressed more systematically in the future.

PERSPECTIVE

We have demonstrated that fluorescence in situ hybridization can be applied successfully to address the question whether EBV is integrated in Burkitt lymphoma cells. We have illustrated some of the problems which we have encountered. It became apparent that, at the present stage, aneuploidy and high viral copy numbers make the analyis and the interpretation of data very difficult. Therefore we have focused on cell lines with copy numbers between 5 and 20 and a low degree of aneuploidy. In four BL cell lines we were able to detect ingrated viral DNA at one chromosomal site each, however, the integration sites were different in all cell lines. Thus, if a cellular gene is affected by viral integration, there should be several cellular genes which might accomplish an equivalent function during tumor development. Alternatively, integration might affect a viral gene. It will be of prime importance to see whether integration is a phenomenon occurring in the Burkitt lymphoma cells *in vivo* or after establishment as cell lines in culture. We are now attempting to study metaphase chromosomes prepared from fresh tumor biopsies to answer this question.

References

Adams, A., Lindahl, T. and Klein, G. (1973) Linear association between cellular DNA and Epstein-Barr virus DNA in a human lymphoblastoid cell line. Proc.Natl.Acad.Sci.U.S.A. 70:2888-2892

Bister, K., Yamamoto, N. and zur Hausen, H. (1979) Differential inducibility of Epstein-Barr virus in cloned, non-producer Raji cells. Int.J.Cancer 23:818-825

Bornkamm, G.W., Polack, A. and Eick, D. c-myc deregulation by chromosomal translocation in Burkitt's lymphoma. In: *Cellular oncogene activation*, edited by Klein, G. New York and Basel: 1988, p. 223-273

Henderson, A., Ripley, S., Heller, M. and Kieff, E. (1983) Chromosome site for Epstein-Barr virus DNA in a Burkitt tumor cell line and in lymphocytes growth-transformed in vitro. Proc.Natl.Acad.Sci.U.S.A. 80:1987-1991

Heston, L., Rabson, M., Brown, N. and Miller, G. (1982) New Epstein-Barr virus variants from cellular subclones of P3J-HR-1 Burkitt lymphoma. Nature 295:160-163

Hudewentz, J., Bornkamm, G.W. and zur Hausen, H. (1980) Effect of the diterpene ester TPA on Epstein-Barr virus antigen-and DNA synthesis in producer and nonproducer cell lines. Virology 100:175-178

Hurley, E.A., Agger, S., McNeil, J.A., et al. (1991a) When Epstein-Barr virus persistently infects B-cell lines, it frequently integrates. J.Virol. 65:1245-1254

Hurley, E.A., Klaman, L.D., Agger, S., Lawrence, J.B. and Thorley Lawson, D.A. (1991b) The prototypical Epstein-Barr virus-transformed lymphoblastoid cell line IB4 is an unusual variant containing integrated but no episomal viral DNA. J.Virol. 65:3958-3963

Kaschka-Dierich, C., Adams, A., Lindahl, T., et al. (1976) Intracellular forms of Epstein-Barr virus DNA in human tumour cells in vivo. Nature 260:302-306

Lawrence, J.B., Villnave, C.A. and Singer, R.H. (1988) Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. Cell 52:51-61

Lindahl, T., Adams, A., Bjursell, G., Bornkamm, G.W., Kaschka-Dierich, C. and Jehn, U. (1976) Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J.Mol.Biol. 102:511-530

Matsuo, T., Heller, M., Petti, L., O'Shiro, E. and Kieff, E. (1984) Persistence of the entire Epstein-Barr virus genome integrated into human lymphocyte DNA. Science 226:1322-1325
Miller, G., Heston, L. and Countryman, J. (1985) P3HR-1 Epstein-Barr virus with heterogeneous DNA is an independent replicon maintained by cell-to-cell spread. J.Virol. 54:45-52
Polack, A., Hartl, G., Zimber, U., et al. (1984) A complete set of overlapping cosmid clones of M-ABA virus derived from nasopharyngeal carcinoma and its similarity to other Epstein-Barr virus isolates. Gene 27:279-288
Rowe, D.T., Rowe, M., Evan, G.I., Wallace, L.E., Farrell, P.J. and Rickinson, A.B. (1986) Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. EMBO J. 5:2599-2607
Rowe, M., Rowe, D.T., Gregory, C.D., et al. (1987) Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. 6:2743-2751
Yamamoto, N., Bister, K. and zur Hausen, H. (1979) Retinoic acid inhibition of Epstein-Barr virus induction. Nature 278:553-554
Zimber Strobl, U., Suentzenich, K.O., Laux, G., et al. (1991) Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene. J.Virol. 65:415-423

The Oncogenic Potential of Epstein-Barr Virus Nuclear Antigen 1 in Transgenic Mice

J. B. WILSON[1] and A. J. LEVINE[2]

[1]Institute of Biotechnology, Department of Genetics, Glasgow University, Glasgow G11 5JS, U.K.
[2]Department of Molecular Biology, Princeton University, Princeton N.J.08544, U.S.A.

Introduction

The human herpes virus, Epstein-Barr virus (EBV) is highly prevalent among all communities. Although largely asymptomatic prior to puberty it is the etiologic agent of the lymphoproliferative disorder, infectious mononucleosis. The virus is also tightly associated with two major forms of human B-cell malignancy: endemic Burkitt's lymphoma (BL) and the lymphomas to which immunosupressed individuals are prone (for review see Epstein and Achong, 1986).

BL is a multifactorial disease. Karyotypic analyses have revealed that BL (sporadic and endemic) tumour cells harbour one of three characteristic chromosomal translocations. The distal fragment of chromosome 8 carrying the *c-myc* locus is involved in each, being adjoined to one of the three immunoglobulin loci, most frequently the heavy chain (IgH) locus on chromosome 14 and in 10% of cases, either of the light chain loci on chromosomes 2 and 22 (for review see Klein, 1983). Expression of *c-myc* is accordingly induced. The specific cluster regions of the breakpoints differ in sporadic and endemic BL, the significance of which is as yet unclear. Mutations in the p53 gene have also been observed in a number of BL derived cell lines of both endemic and sporadic origin (Farrell et al., 1991). The geographically high incidence (endemic) areas of BL occurence are those where malaria is also prevalent, in equatorial Africa and New Guinea. The evidence also implicating EBV as factorial in endemic BL is strong, particularly in that while >95% of endemic Burkitt tumours are EBV-genome positive (Lenoir and Bornkamm, 1986), <1% of the total B-cell pool from the malaria carriers is detectably infected with EBV (Moss et al., 1983).

The ability of EBV alone to induce B-cell proliferation is further evidenced by the efficient transformation of normal, resting, human B-lymphocytes into permanent lymphoblastoid cell lines (LCLs), upon *in vitro* infection by the virus. The viral genome is maintained as an autonomously replicating episome in these "latently" infected cells, expressing eight defined genes constitutively. In contrast to this, only one of these eight viral gene products, the EBV nuclear antigen 1 (EBNA1), has been consistently detected in BL biopsy tissue and BL cell lines retaining the tumour biopsy phenotype (Rowe et al., 1987).

EBNA1 is a DNA binding protein which binds to specific sequences within and 5' to the latent origin of viral DNA replication (oriP) (Rawlins et al., 1985). The protein is required for episomal maintenance and replication of oriP containing plasmids in human and primate cells (Yates et al., 1984 and 1985). It has also been shown to promote transcriptional transactivation via the repeated binding sequences 5' to oriP (Reisman and Sugden, 1986) and therefore probably plays a critical role in the regulation of viral gene expression and replication in the latent state. The absence of any data attributing oncogenic activity to this protein to date may simply reflect the specificity of the protein's action and the lack of a suitable assay system in culture.

In this study, the oncogenic potential of EBNA1 has been addressed by expressing the protein in transgenic mice. As such, *in vivo* action in B-cells can be specifically examined. We show that EBNA1 may predispose B-cells to lymphomagenesis. The heritable predisposition to lymphoma of a line of mice described here will allow an analysis of the pre-neoplastic events generated by expression of EBNA1. As a consequence, the contribution of EBV to lymphomagenesis specifically through EBNA1 expression can be evaluated.

Results

Production of Transgenic Mice

Transgenic mice were generated as described by Brinster et al. (1985), using F2 generation zygotes from the mouse strains C57Bl/6J and DBA/2J (B6D2) and injecting the 3.2 kbp XbaI-AatII fragment from the plasmid EμEBNA1 (Fig. 1). These sequences incorporate the coding region of EBNA1 derived from the B95-8 strain of EBV, from a Sau3A site 20 bp upstream of the ATG to the PvuII site 239 bp downstream of the poly-A addition signal (303 bp 3' to the EBNA1 stop codon). The EBV sequences were linked to expression control sequences designed to enhance expression in B-lymphocytes, comprising the Polyomavirus (Py) early promoter and mouse immunoglobulin heavy chain (IgH) enhancer (Wilson et al., 1990). DNAs from tails of potentially transgenic mice were screened by Southern blot analysis using a 3' EBNA1 gene DNA probe (not shown). The internal repeat sequences of the EBNA1 gene demonstrate a marked degree of cross hybridisation with mouse cellular sequences and were avoided in the screening process.

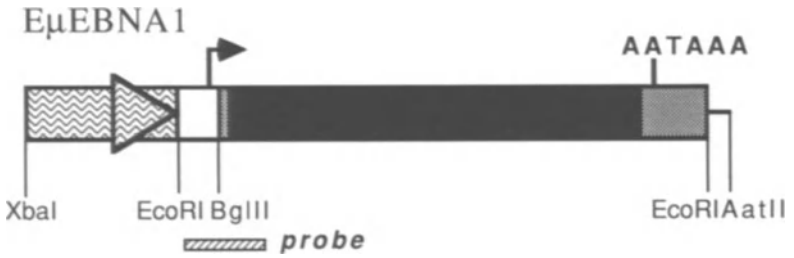


Fig. 1. The microinjected transgene. The XbaI to AatII 3.2 kb EμEBNA1 fragment was used for microinjection into mouse zygotes. B95-8 EBV sequences are stippled with the EBNA1 coding region in black.The Polyomavirus promoter sequences are white and the mouse immunoglobulin sequences are shaded with striated lines. The defined enhancer sequence is represented by a striated triangle. The major Polyomavirus early RNA start site is depicted with an arrow and the poly-A addition signal of the EBNA1 gene is indicated. The probe fragment shown beneath represents that used for the RNase protection analysis presented in Fig.5.

Of 58 offspring produced following injection of EμEBNA1 sequences into zygote pronuclei, 12 were found to carry the transgene (founder female numbers: 26, 62, 63, 64, 66, and founder male numbers: 59, 60, 61, 65, 67, 68 and 69). 10 of these (baring 65 and 68) transmitted the transgene through the germ line to generate transgenic lines (backcrossed to strain C57Bl/6J). Four lines have been bred to homozygosity for the transgene (lines: 59, 60, 62 and 66). All lines, except for line 60, harbour at least one intact copy (by restriction and blotting analysis) of the transgene, with between 1 and 10 copies, predominantly in the frequently observed configuration of head to tail tandem arrays. Line 60 appears to harbour one copy that has undergone rearrangement. The internal EcoRI fragment of 2446 bp (Fig. 1) encompassing the Py and EBV sequences is not detected on restriction of line 60 genomic DNA (unlike all other lines). Instead, probing with Py and 5' EBNA1 sequences detects an EcoRI fragment of 1.1 kbp, while 3' EBNA1 sequences hybridize to a 9.5 kbp band (not shown). This then indicates that while the transgene sequences are present in line 60, they have undergone a major rearrangement, separating the 5' and 3' EBNA1 sequences onto different EcoRI fragments in an unknown configuration.

One Line Develops Early Onset Lymphocytic Lymphoma

All positive mice of line 26 (which included the founder female) succumb to an invasive lymphoma, becoming moribund within an age range of 4-12 months. The hall mark of the disease is a massive enlargement of the spleen (up to 20 times the normal weight). Accompanying this is

either a massively enlarged, invaded liver and slightly expanded lymph nodes (Fig. 2), or (with roughly equal frequency) the reverse, massively enlarged lymph nodes with slightly expanded liver. Variable invasion of other tissues is frequently observed. The enlarged organs are found to be replete with a neoplastic infiltrate of poorly differentiated lymphoid cells and the blood and bone marrow contain large numbers of similar cells. The typical pathological diagnosis is therefore lymphocytic lymphoma with associated leukaemia. This has remained consistent when backcrossing the line into two different strain backgrounds, C57Bl/6J or Balb/cBy.

The lymphoid organs of young mice in this line (1 month old) appear histologically within normal limits. However as the mice age, a preleukaemic state becomes apparent, where the white pulp of the spleen and the lymph nodes are seen to be expanded with a population of immature lymphocytes. The age of onset of frank neoplasia is variable.

Transplantation of the neoplastic cells into syngeneic (non-transgenic) mice established that the disease is highly malignant. Rapidly growing tumours arose in B6D2 F1 hybrids after injection of splenocytes derived from line 26 mice with neoplasia, the recipients becoming moribund at 8-10 weeks post intravenous injection. Subsequent transplantation passages reduced this latency period to 6 and then as little as 4 weeks. Neoplastic cells transplanted in this fashion maintained the original "homing" pattern, with a bias to a liver or lymph node distribution in conjunction with the splenic invasion.

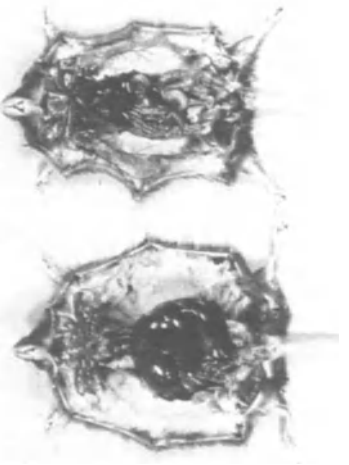


Fig. 2. Lymphocytic lymphoma with associated leukaemia. A line 26 male (26.6) is presented (left) with a transgenically negative sibling (right) sacrificed at four months of age. The massively enlarged spleen and liver, clearly evident in the positive mouse, is replete with neoplastic lymphocytes. Lymph nodes are also slightly expanded with the neoplastic infiltrate in the positive mouse, seen here particularly in the superficial cervical and superficial inguinal lymph nodes.

Monoclonal B-cell Lymphomas

In order to investigate the cell lineage and clonality of the lymphoid tumours, rearrangements of the endogenous immunoglobulin and T-cell receptor loci were analysed. DNAs were prepared from different tissues with infiltrated lymphoma from different positive mice of the line 26. The DNAs were digested with restriction enzymes, gel electrophoresed, Southern blotted and probed with fragments identifying the different loci. Rearrangements at the IgH locus were detected with a heavy chain joining region (J_H) DNA fragment as probe (Fig. 3 and 4A). The germ line 6.5 kb EcoRI fragment is still apparent in all of the samples as the tissues are a mixture of neoplastic and normal cells. As well as the germ line fragment, all tumour samples contain one or two other bands which are consistent across the different tissues for a single mouse. This pattern is indicative of diversity-joining region recombination at one or both IgH alleles within a single B-cell, demonstrating the monoclonality of the tumour. Rearranged loci generating a fragment of the same size in different mice were not uncommon (for example Fig. 3; 26.30 and 26.7, where both rearranged fragments are apparently the same size), possibly reflecting preferred D-to-J combinations, as have been previously observed (Langdon et al., 1986).

All tumour tissues analysed displayed monoclonal rearrangements at the IgH locus, while none have exhibited rearrangements at the T-cell antigen receptors ß or γ loci (not shown), demonstrating that the lymphomas are of B-cell origin.

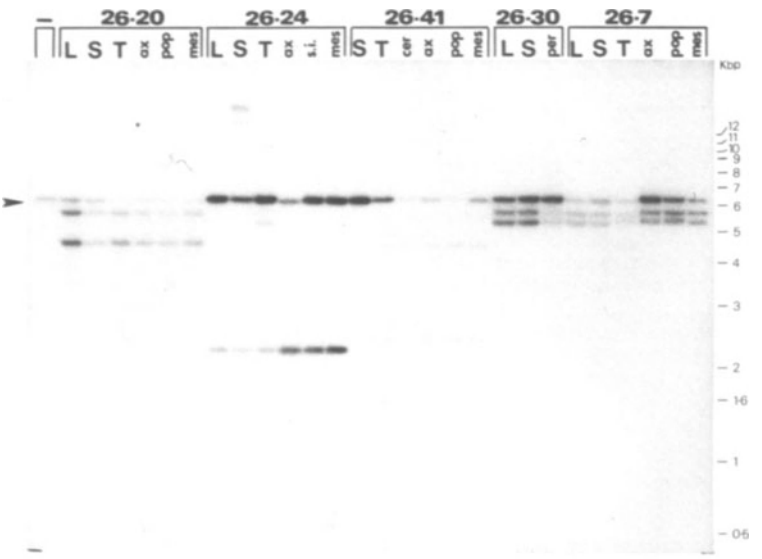


Fig. 3. Rearrangements of the IgH locus within tumours. DNAs were prepared from tissues of five transgenically positive mice of the line 26 (26.20, 26.24, 26.41, 26.30, 26.7), succumbing to lymphoma at ages between 4 and 10 months old, and a negative control (-). 4 μg of DNA was digested with EcoRI, electrophoresed and Southern blotted. The blot was probed with a heavy chain joining region DNA fragment which hybridizes to a 6.5 kb EcoRI germ line band, evident in all tissues. 1 or 2 other bands, indicative of monoclonal rearrangements at the IgH loci are present in all of the tumour samples. L=liver, S=spleen, T=thymus; lymph nodes: ax=axillary, pop=popliteal, mes=mesenteric, s.i.=superficial inguinal, cer=cervical, per=a mixture of peripheral nodes.

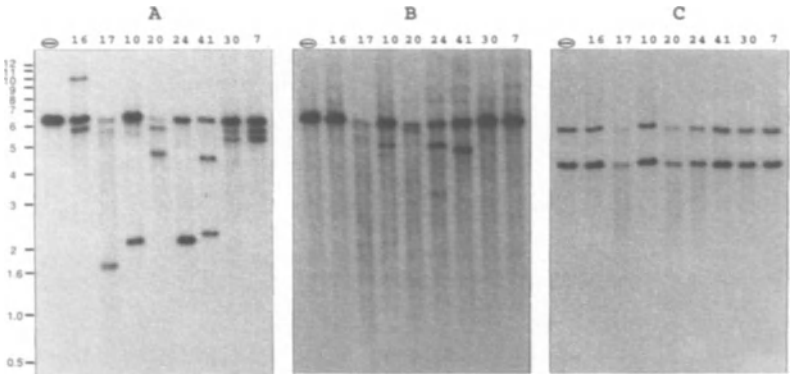


Fig. 4. Rearrangements of the heavy and light chain loci within tumours. 5 μg of lymph node DNAs from 8 transgenically positive mice of the line 26 (26.16, 26.17, 26.10, 26.20, 26.24, 26.41, 26.30, 26.7) and a negative control (-) were restriction digested, Southern blotted and probed with immunoglobulin loci fragments.
A: DNAs were digested with EcoRI and probed with an IgH joining region fragment (as for Fig. 3.), detecting a 6.5 kb germ line fragment and rearrangements in all positive samples. **B**: DNAs were digested with EcoRI and BamHI and probed with a joining-enhancer-constant region fragment of the κ light chain locus, detecting a 6.5 kb germ line fragment and rearrangements in 5 samples 26.17, 26.10, 26.20, 26.24 and 26.41. **C**: DNAs were digested with EcoRI and probed with a variable-joining-constant region fragment of the λ light chain locus, detecting the λ1 and λ2 loci with no detectable rearrangements in any sample.

Of 8 tumours analysed for immunoglobulin light chain rearrangements, 5 displayed rearrangements of the κ chain locus, while none demonstrated rearrangements at the λ locus (Fig. 4B and C), suggesting that at least some tumour cells could not express a functional immunoglobulin. The tumours may therefore arise around the time of light chain rearrangement (in the differential progression of the B-cells) or reflect that B-cells of varying differential stages (late pre-B and B) may progress to neoplasia.

A Second Line Displays a Lymphocytic Phenotype

A further line generated with the same transgene, line 59, displays a more subtle lymphoid phenotype. At ages when a pre-neoplastic change is evident in mice of line 26 (3 months and onward), mice of line 59 have histologically normal lymphoid organs. However in mice of line 59 of 8 months old and older, expansion of the splenic white pulp may be observed. Furthermore, B-cell lymphomas of very long latency (at two years of age) have also arisen in positive mice of line 59, which have not been observed in transgenically negative siblings. The degree of penetrance and statistical significance of this phenotype is currently being assessed.

Expression of the EBNA1 Transcript

Transcription from the transgene was analysed in the different lines by RNase protection (Fig. 5). An antisense ribo-probe encompassing the 5' sequences of the EBNA1 coding region and part of the Py promoter, as depicted in Figs. 1 and 5 (including the viral major early RNA start site) was protected from RNase digestion by hybridisation to 40 μg samples of total splenic RNA. The entire 418 base (b) undigested probe is shown. As a control, in lane 2, a spleen/lymphoma RNA derived from an unrelated transgenic mouse (19.63) harbouring a different transgene with the same promoter sequences, is seen to protect the 162 b Py-only portion as predicted. Also as control, total RNA from the EBV positive, human B-cell line IB4 protects EBNA1 specific sequences within the probe, the entire EBNA1 fragment of 190 b and also, consistently, three smaller discrete fragments of 180, 172 and 168 b, probably representing internal splice acceptor sites, 5' of the EBNA1 coding frame, in the viral RNAs.

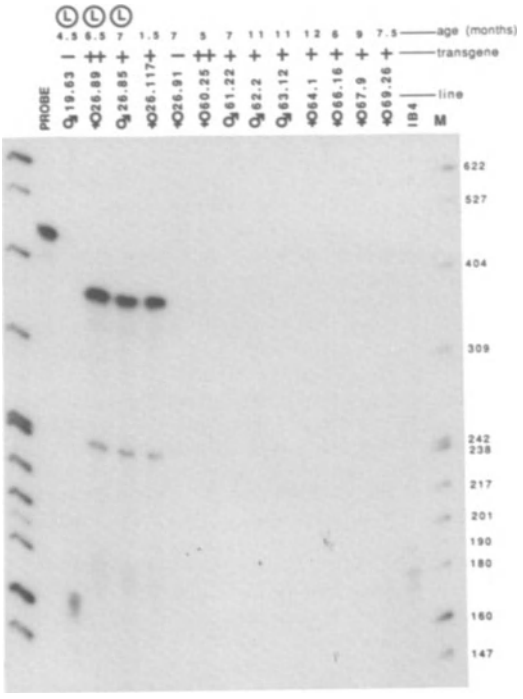
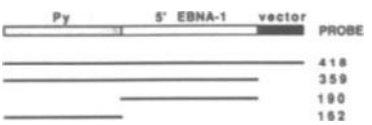


Fig. 5. Transgene transcription. Transgene specific transcripts were detected in samples using a Py-EBNA1 5' antisense probe: intact in track 1, depicted below and delineated in Fig. 1. 40 μg of total splenic RNAs were hybridized to the probe and digested with RNase. Total RNA from the EBV+ cell line IB4 (last track) was used as a positive control for EBNA1 specific fragments (of 190 bases and less, see text) and sample 19.63 as a control for Py specific fragments (162 bases, see text). Transgene specific transcript fragments of 359 bases and 240 bases represent upstream initiated RNA or RNA initiated from the Py promoter (respectively). Samples with malignant lymphoma (L) are indicated; age in months of each mouse is given; transgenic status is shown: -=negative, +=hemizygous, ++=homozygous. Representatives of 9 EμEBNA1 lines are presented, lines: 26, 60, 61, 62, 63, 64, 66, 67 and 69.



RNA samples from three line 26 mice demonstrate the same pattern of expression, with two predominating RNA fragments protected. The more abundant fragment of 359 b, indicating protection of the entire transgene sequences of the probe, reflects transcription initiating upstream of the probe sequences, 5' to the Py promoter. The second fragment of 240 b, maps a transcriptional start site within the Py promoter region to just 3' of the Py TATAA box at Py nt. 126 (Tooze, 1981). Two of the three line 26 RNA samples were from lymphoma infiltrated spleens (26.89 and 26.85). The former of these was derived from a mouse bred to homozygosity for the transgene and shows the predicted 2 fold level of expression over the latter from a hemizygous mouse. The third sample (26.117) is splenic RNA derived from a line 26 mouse at one month old, prior to the onset of the malignancy, demonstrating that transgene transcription precedes the disease phenotype. 26.91 is a transgenically negative litter mate (with normal phenotype) from the line as control.

No transgene specific transcription could be detected in spleens of mice from lines 61, 62, 63, 64, 66, 67 and 69 (Fig. 5), all of which were of normal phenotype. Included in this assay is an RNA sample from a line 60 homozygote, which as described above, harboured a rearranged transgene. At very long exposure of the autorad presented in Fig. 5 (not shown), a protected fragment of 240 b is also detected, but not of 340 b. As described below, and probably due to the rearranged transgene structure, expression at the protein level was not observed and again the line developed no phenotype. However, this does indicate that the transcriptional start site at Py nt. 126 is a characteristic of the transgene and that upstream initiation may be unique to line 26, possibly originating in the adjacent cellular sequences. Transgene transcription within line 59 tissues has not been addressed in this assay, however a direct analysis of expression at the protein level is detailed below.

Expression of the EBNA1 Protein

Expression of the EBNA1 protein product was analysed by immunoprecipitation of tissue extracts with either of two antisera: a conformation sensitive, monoclonal antibody Aza2.E8 (Hearing et al, 1985) or a polyclonal human serum (WS). The immunoprecipitated proteins were separated by SDS-PAGE and visualised by Western blotting employing the polyclonal serum WS in conjunction with ^{125}I labelled protein A (also revealing the antiserum light and heavy chains of the immunoprecipitation reaction in all samples). As controls, cell extracts were prepared from the EBV positive, B-cell lines B95-8, IB4 and Raji, and the EBV negative B-cell line Louckes. The B95-8 strain of EBV (used in the transgene construct) generates an EBNA1 serum reactive product migrating at 88 kD and variably a possible break down product of 66 kD, while the Raji strain (deleted in the central repeats of EBNA1 compared to B95-8) generates an EBNA1 serum reactive product of approximately 70 kD. (Hearing et al., 1985).

Mice of line 26 express EBNA1 specifically in spleen and lymph nodes (Fig. 6) without detectable expression in normal liver, kidney, brain, heart or thymus (not shown). Mice of line 59 express EBNA1 to a high level in spleen and lymph nodes (Fig. 6) and expression is also detectable in brain and thymus, but not in normal liver, kidney or heart (not shown). Mice of line 60 do not detectably express the protein in any of these tissues. Using set amounts of tissue extracted protein (see figure legend) in the immunoprecipitation reactions, more EBNA1 steady state protein is detected in line 59 samples (for a given tissue) than in line 26 following immunoprecipitation with Aza2.E8. However, immunoprecipitating with WS serum selectively enhances the signal from line 26 extracts (Fig. 6C: 26.103* and 26.103), indicating that a proportion of EBNA1 in the line 26 samples is in a form not recognised by the conformation dependant antiserum Aza2.E8. Furthermore, from this assay it is not possible to distinguish between differences in the steady state protein level per cell as opposed to differences in the spectrum of cell types and stages within a tissue expressing the protein. Therefore, mice of line 59 may have a higher steady state level of EBNA1 per cell than line 26, and/or express the protein in more cell types and stages (certainly evidenced by the observed brain and thymic expression) and/or harbour a larger fraction of expressed protein in a state selectively recognised by the antiserum Aza2.E8. These differing possibilities are currently being investigated.

A more slowly migrating EBNA1 serum reactive product is also variably detected (Fig. 6B, 26.10 1^{o} spleen sample and Fig. 6C, 26.103*), which may reflect a modified form of the protein. Transplantation of the splenocytes from mouse 26.10 with lymphoma into a syngeneic mouse, resulted in fully malignant spread of the tumour in the recipient. Neoplastic nodules were excised from the liver of this host and either analysed for EBNA1 expression (Fig. 6B 2^{o}) or the cells

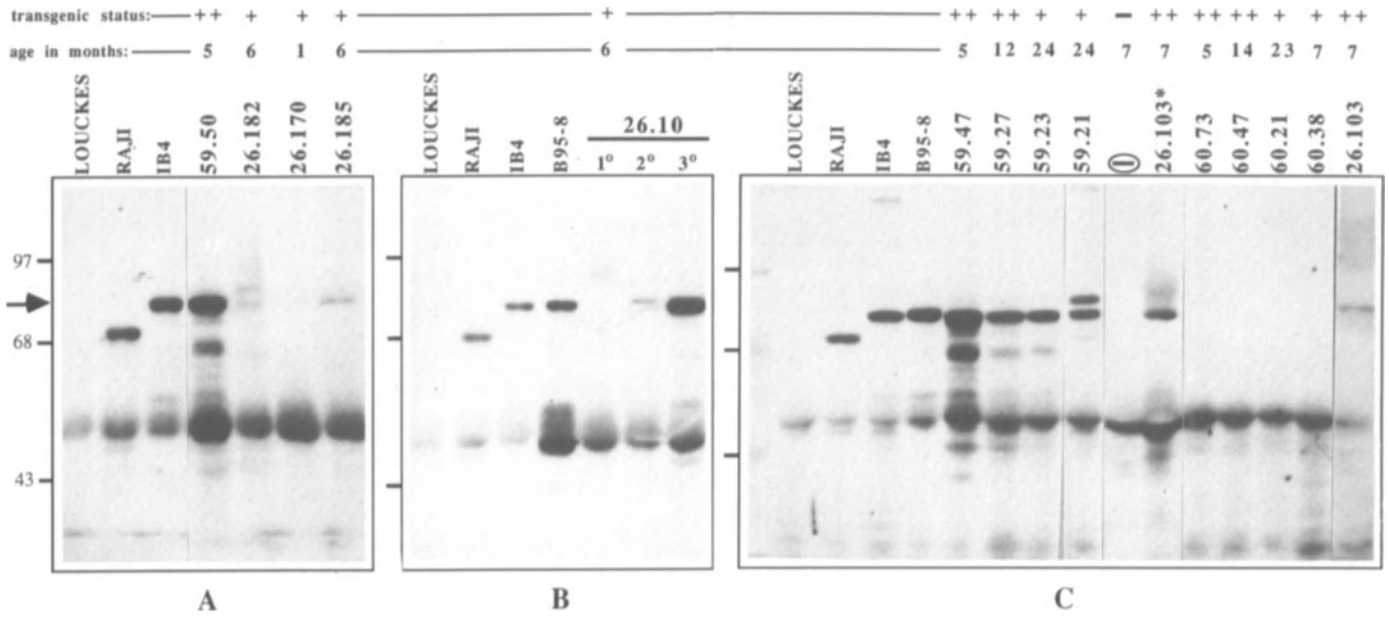


Fig. 6. Expression of EBNA1. Tissue extracts were immunoprecipitated with the anti-EBNA1 monoclonal antibody Aza2.E8, except samples (-) and 26.103* which were immunoprecipitated with the polyclonal human serum WS. Immunoprecipitated proteins were separated on 7.5% denaturing, SDS acrylamide gels, Western blotted and probed with WS/^{125}I-protein A. Aliquots of 5X10^6 Loucke (EBV-), Raji (EBV+), IB4 (EBV+) and B95-8 (EBV+) B-cells were used as controls. Age in months of individual mice is indicated and transgenic status: -=negative, +=hemizygous, ++=homozygous. The 88 kD B95-8 EBNA1 product is indicated with an arrow.

A. 1.5 mg of NP40 extracted protein from peripheral lymph nodes were immunoprecipitated from a line 59 sample, histologically normal (59.50), and three line 26 samples: 26.182-very early stage lymphoma, 26.170-normal and 26.185-early stage lymphoma.

B. 10 mg of NP40 extracted protein from the spleen of mouse 26.10 with malignant lymphoma (1°) and samples taken from syngeneic (transgenic negative) recipients with transplanted 26.10 lymphoma cells (2° and 3°) were immunoprecipitated along with a transgenically negative control spleen sample (-).

C. 20 mg of NP40 extracted protein from spleen (or liver for 59.21) samples were immunoprecipitated from line 59, 60 and 26 mice of varying ages. 59.47 at 5 months old was histologically normal, 59.27 at 12 months showed lymphoid predominance, 59.23 at 24 months had neoplastic lymphoma and 59.21 at 24 months had malignant lymphoma, the sample taken from heavily infiltrated liver. 26.103 had malignant lymphoma and all line 60 samples were histologically normal.

were again *in vivo* passaged and the subsequent liver nodules analysed for expression (Fig. 6B 3°). The level of EBNA1 apparently increased through these sequential passages, either indicating selection for cells expressing a higher level of the protein or perhaps simply reflecting increased cell homogeneity of the tissue sample.

As with line 26, expression of EBNA1 is detectable in line 59 preceding any observable histological change. Immunoprecipitable EBNA1 is readily detected in the three line 59 spleen samples presented in Fig. 6C. The three samples were derived from line 59 mice at 5 months old without phenotype (59.47), 1 year old with white pulp expansion of the spleen (59.27) and 2 years old with neoplastic lymphoma (59.23). Sample 59.21 is a liver sample derived from a second 2 year old line 59 mouse with malignant lymphoma, where the lymphoma had substantially invaded the liver. Normal liver in this line does not express the protein, evidencing that the malignant cells express EBNA1.

Discussion

Two lines of transgenic mice, harbouring the transgene EμEBNA1 have been shown to consistently express the protein EBNA1 in spleen and lymph nodes. One of these lines (line 26) displays a dramatic phenotype of malignant B-cell lymphoma with 100% penetrance between 4 and 12 months of age. The second line (line 59) appears to be predisposed to lymphoma of long latency, for which the degree of penetrance is yet to be determined.

Phenotypic Difference Between Lines 26 and 59

The cause of the difference in extent of the phenotypes between the two lines described here is currently unresolved, however, such phenotypic variation is certainly not an uncommon observation in tumour bearing transgenic mice. There are many possibilities which would explain the disparate phenotypes and further studies are in progress addressing this point.

A potential explanation renders the expression of EBNA1 incidental, whereby the observed lymphomagenesis in line 26 is due to the activation of a potent cellular proto-oncogene by insertional mutagenesis. Although this has never previously been observed in a transgenic context (transgene integration by microinjection is thought to be entirely random and subsequently subject only to the selective pressure of embryonic viability) it remains formally possible. However this would not account for the phenotype, albeit lesser, of line 59. Alternative theories all rest on the premise that the transgene is an oncogene.

Line 59 tissue samples demonstrate a higher level of expression of the protein per cell and/or a broader array of cell types expressing the protein, than line 26 samples (unless the observations can be attributed to differing antigenic states of the protein in the two lines). As such the degree of the tumourigenic phenotype does not parallel the level of steady state EBNA1 protein present in the tissues. Nevertheless, if the difference in the phenotypes is directly due to protein levels, it could be that over-expressed EBNA1 is toxic, and as such a delicate balance between growth promotion and lethality must be achieved to observe proliferative effects. A similar theory has been proposed for the activity of *c-myc* (Evan et al., 1992). Alternatively, while both lines express full length protein, it may be mutationally activated or inactivated in one of the lines as a result of the initial integrative process. An analysis of the modified forms of the protein may prove to be particularly revealing in this context.

Another possibility which could account for the observations is that sequences at the site of insertion of either line 26 or 59 may perturb expression of the transgene in a spatial or temporal way to augment or minimise (respectively) the oncogenicity of the transgene

EBNA1, a Viral Oncogene

Despite the difference in degree of phenotypes between lines 26 and 59 there is a direct correlation between expression of the transgene and predisposition to B-cell lymphoma. These results therefore provide the first evidence that EBNA1 may indeed be a viral oncogene. The expression of the protein in all EBV positive tumours may not simply reflect the viral requirement for persistence, but present a selective growth advantage for the cell simultaneously permitting viral persistence.This idea is particularly applicable to the disease eBL, where only EBNA1 of the viral

latent proteins is detectably expressed in the tumour cells, suggesting that EBNA1 performs an essential tumour maintenance function. Moreover, while other viral proteins may contribute initially to cellular proliferation at the onset of eBL, there clearly can be no tumour maintenance role for these proteins in the disease, since their expression would appear to be selected against. The latent membrane protein (LMP) of EBV has been demonstrated to possess oncogenic properties, both in tissue culture (Wang et al., 1985; Baichwal and Sugden, 1987) and in transgenic mice (Wilson et al., 1990), however this protein, as a potent immunoreactive target, may well be selected against in eBL, similarly perhaps for other viral proteins which induce LMP expression such as EBNA2.

As with other DNA tumour viruses, encoding multiple oncogenes.may permit viral persistence in a variety of cell types and reflect the numerous routes to cell immortality for the different cell types. EBV is associated with tumours of at least two cell types, epithelial and B-cell, for which the viral contribution to malignancy is likely to differ as is the significance of the separate viral oncogenes.

The lymphomas arising in these transgenic mice are monoclonal. This observation conforms to all other findings of oncogenes tested in this manner and serves to reiterate the multi-step path of tumourigenesis. EBNA1 is clearly not sufficient for complete tumourigenic conversion of a B-cell. Translocations involving the *c-myc* locus are a characteristic of BL, but the exact nature of these translocations differ in the sporadic and endemic forms of the disease. The association of EBV with only the endemic form of BL permits the intriguing suggestion that EBV may effect the type of *c-myc* translocation selected for. The transgenic mice described here will allow an analysis of any interactions between EBNA1 and endogenous *c-myc* , as well as interactions with an introduced activated *c-myc* by cross-breeding transgenic lines, thereby approaching a model for BL. Moreover,.the direct activity of EBNA1 *in vivo*, in the absence of other viral proteins, provides a prospective route to dissect the evolution of EBV associated lymphomas.

References

Baichwal VR, Sugden B (1987) Posttranslational processing of an Epstein-Barr virus-encoded membrane protein expressed in cells transformed by Epstein-Barr virus. J Virol 61:866-875

Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD (1985) Factors affecting the efficiency of intoducing foreign DNA into mice by microinjecting eggs. Proc Natl Acad Sci USA 82:4438-4442

Epstein MA, Achong BY (eds) (1986) The Epstein-Barr virus: Recent advances. John Wiley & Sons, New York

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC (1992) Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119-128

Farell PJ, Allen GJ, Shanahan F, Vousden KH, Crook T (1991) p53 is frequently mutated in Burkitt's Lymphoma cell lines. EMBO J 10:2879-2887

Hearing JC, Lewis A, Levine AJ (1985) Structure of the Epstein-Barr virus nuclear antigen as probed with monoclonal antibodies. Virology 142:215-220

Klein G (1983) Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. Cell 32:311-315

Langdon WY, Harris AW, Cory S, Adams JM (1986) The c-myc oncogene peerturbs B lymphocyte developmant in Eμ-myc transgenic mice. Cell 47:11-18

Lenoir GM, Bornkamm GW (1986) In Klein G (ed) Advances in viral oncology. Raven Press, New York 6:173-206

Moss DJ, Burrows SR, Castelino DJ, Kane RG, Pope JH, Rickinson AB, Alpers MP, Heywood PF (1983) A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and malaria-nonendemic regions of Papua-New-Guinea. Int J Cancer 31:727-732

Rawlins DR, Milman G, Hayward SD, Hayward GS (1985) Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell 42:859-868

Reisman D, Sugden B (1986) Transactivation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol Cell Biol 6:3838-3846

Rowe M, Rowe DT, Gregory CD, Young LS, Farrell PJ, Rupani H, Rickinson AB (1987) Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J 6:2743-2751

Tooze J, ed. (1981) DNA tumour viruses, in Molecular Biology of tumor viruses, aprt 2. Cold Spring Harbor Laboratory, New York

Wang D, Liebowitz D, Kieff E (1985) An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell 43:831-840

Wilson JB, Weinberg W, Johnson R, Yuspa S, Levine AJ (1990) Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. Cell 61:1315-1327

Yates J, Warren N, Reisman D, Sugden B (1984) A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc Natl Acad Sci USA 81:3806-3810

Yates JL, Warren N, Sugden B (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammlian cells. Nature 313:812-815

AIDS Associated Non-Hodgkin's Lymphomas Represent a Broad Spectrum of Monoclonal and Polyclonal Lymphoproliferative Processes

B. G. HERNDIER, B. T. SHIRAMIZU and M. S. MCGRATH

Departments of Pathology, Pediatrics, Laboratory Medicine, and Medicine, University of California, San Francisco, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110

Introduction

Lymphoma is a devastating complication of HIV disease occurring at an incidence of approximately 5-15% in AIDS patients, at a 25-100 fold increase as related to the non-HIV-infected population [1-3]. Initial studies emphasized the B-cell nature of these neoplasms analogous, except for the high incidence of Burkitt's lymphoma, to the lymphomas arising in transplant patients [4-6] and patients with congenital immunodeficiencies [7-9]. Previous genotypic surveys of the HIV associated lymphomas found similarities to transplant lymphomas (a subset of transplant "lymphomas" are polyclonal and associated with the EBV) and Burkitt's lymphomas (genotypic analysis of Burkitt's lymphomas reveals chromosomal translocations near immunoglobulin (Ig) gene loci and the proto-oncogene *c-myc*) [10,11]. More recently, phenotypic analysis was initiated to verify the B-cell nature of the neoplasms and this analysis was further expanded to explore the exact cellular milieu of the lymphomas associated with HIV infection [12].

Historically, the definition and classification of lymphomas has been controversial. Despite advances in determining clonality (light chain exclusion, gene rearrangement studies [13]) and elaborate immunophenotypes [14], the mainstay of diagnosis has been the surgical pathologist's impression of a histochemically stained tissue section. Although the histological analysis of lymphoproliferations is subjective, the disease classifications have been a result of a longstanding cumulative experience in the clinical and laboratory presentation of disease, natural course of the disease (prior to chemotherapy) and prognostic information ("response to therapy") [15,16].

Therefore we have defined "lymphoma" in a conservative surgical pathology sense [17], both for ethical reasons and to avoid diagnostic bias from the assumptions inherent in the molecular and immunophenotypic data. This approach was to a large extent justified by the retrospective multisite analysis of several HIV lymphoma autopsies [4]. In this study polyclonal lymphomas as defined by Southern blot analysis and a conservative surgical pathology definition of malignancy were found to cause widespread extranodal parenchymal damage (liver, kidney, lung, etc.) and presumably lead to the death of these patients. To designate such polyclonal proliferations as "benign", analogous to follicular hyperplasias of lymph nodes or peripheral blood lymphocytes which have similar Southern blot patterns, would be naive and we felt would possibly send an irresponsible academic signal to oncologists who ultimately have to manage such patients.

Current Topics in Microbiology and Immunology, Vol. 182

The current study further characterizes the polyclonal lymphomas that occur in HIV-infected individuals. Unfortunately, the concept of an HIV associated polyclonal lymphoma challenges several diagnostic assumptions about lymphomas so readily ported from the early acute leukemia/PBL experience. The assumptions that lymphomas are universally monoclonal (by genotypic or phenotypic analysis) and consist of monomorphic "sheets" of B or T cells will be examined in this study.

Methods

Histochemistry

All the lymphomas in this survey analogous to other studies [2,18] fell into three Working Formulation [19] subclasses: small non-cleaved, large cell, and large cell immunoblastic. The small non-cleaved (SNC) or "Burkitt's" lymphoma is a distinct histological entity. However, we agree with Cossman [20] that there is a continuous spectrum of histology between large cell or "follicular center cell" lymphomas and the various subtypes of "immunoblastic" lymphomas. Furthermore, major studies [21-23] using classifications other than the Working Formulation have shown that this unreproducible distinction among the "large cell" lymphomas has no prognostic significance in non-HIV patients. By analogy, we prefer to group large cell lymphomas and large cell immunoblastic lymphomas into one histological category (LCL) for the purposes of this molecular and phenotypic study. Furthermore, oncologists have not used such a morphological distinction between the large cell lymphomas in the management of HIV-lymphoma patients, preferring to group all diffuse HIV-lymphomas as high grade neoplasms [1,3].

Immunohistochemistry

Biopsy specimens were collected from patients at the time of diagnosis with AIDS-associated non-Hodgkin's lymphoma in accordance with the UCSF Committee on Human Research guidelines. The tissues were quick frozen and stored in airtight sealed plastic tubes at -70°C. An aliquot of sufficient size to obtain accurate morphological diagnosis was submitted for routine formalin-fixation, paraffin embedding and hematoxylin and eosin staining. A selection of cases was reviewed independently by Dr. Ronald Dorfman, Department of Pathology, Stanford University. In each case, a conservative surgical pathology diagnosis of non-Hodgkin's lymphoma was rendered by consensus of several pathologists at UCSF. The -70°C stored specimen was further divided, part for cryostat sections (verified to be predominantly lymphoma cells matching those in both the permanent H & E and frozen sections) and parallel specimens for nucleic acid isolation. Lymphoma tissue not showing a distinct preponderance of tumor cells (>90%) was rejected from the study. Care was taken to insure that the lymphoid tissue submitted for molecular analysis did not contain significant amounts of connective tissue or organ parenchyma.

The cryostat sections were immunohistochemically stained using mouse monoclonal primary antibodies, anti-mouse secondary antibodies conjugated with biotin, and a final avidin-peroxidase color development stage [24]. Positive controls and isotype matched negative controls were included with each run. The following antibody panel was used to determine the specimen phenotypes: CD4, CD8, CD14 (Leu M3) (Becton Dickinson, Mountain View, CA); anti-human IgM (IDEC Pharmaceuticals, Mountain View, CA).

DNA studies

Genomic DNA was isolated from each sample [25]. Ten micrograms of high molecular weight DNA were digested with an appropriate restriction enzyme and separated by electrophoresis on a 0.8% agarose gel for 16 hours. The gel was stained with ethidium bromide, photographed, denatured, neutralized, and transferred to Hybond (Amersham, Arlington Hts., IL). DNA was fixed to the membrane by heating in a vacuum oven at 80°C. After prehybridization, the filter was hybridized with the appropriate probe using standard conditions, and washed to a final stringency of 0.1% SSC with 0.1% SDS. The filter was then exposed to radiographic film.

To analyze rearrangements of the immunoglobulin heavy chain gene, digestions with HindIII and EcoRI were performed and the blots were probed with a human genomic 6.0-kb BamHI-HindIII fragment containing the heavy chain joining region (J_H) [26]. In some samples XbaI, BamHI or HindIII plus EcoRI were also used, in order to discriminate rearranged bands from germ line bands. Samples containing rearranged fragments were defined as monoclonal B-cell tumors. Samples with no detectable rearrangement were designated polyclonal. To test whether a low level of clonal B-cells was present within a biopsy specimen, a control Southern blot lane containing 0.5 micrograms of DNA from an EBV positive clonal B lymphoma cell line (2F7) and 9.5 micrograms of normal lymphocyte DNA was included to represent predicted results of a DNA specimen that contained 5 percent of clonal EBV positive tumor cell DNA. Light chain gene rearrangements were performed to confirm polyclonality [12].

Polyclonal lymphomas may contain a high proportion of T-cells, such that a clonal population of T-cells exists. To test whether a clonal T-cell population might be present within the tumor, a T-cell beta (TCß) rearrangement analysis was performed on DNA digested with either BamHI or EcoRI. The TCß probe, a 400-bp BglII cDNA fragment containing the human Cß2 region, was used as a probe in specimens where DNA was available for analysis [27].

The analysis of Epstein-Barr virus (EBV) was performed on DNA digested with BamHI enzyme. The presence of EBV genomes was assessed by probing digested DNA with the EBV terminal repeat probe of the virus [28]. To test whether a low level of EBV infection was present, a Southern blot lane containing 0.5 micrograms of DNA from an EBV positive lymphoma cell line (2F7) and 9.5 micrograms of normal lymphocyte DNA was included to approximate predicted results of a 5 percent EBV infection.

To analyze the *c-myc* gene, all samples were digested with HindIII and EcoRI and hybridized with both the first and third exon probe of *c-myc* [29]. *C-myc* translocations or rearrangements would be identified if bands other than the germ line *c-myc* fragment were present. To test whether a low level of cells bearing a *c-myc* translocation might be present, a Southern blot lane containing 0.5 micrograms of DNA from a cell line containing a rearranged *c-myc* gene and 9.5 micrograms of normal lymphocyte DNA was included. The *c-myc* gene was defined as germ line if no band of greater intensity than the 5 percent control was identified as rearranged.

All procedures were performed in accord with the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules.

Results

Forty diagnostic biopsy specimens obtained from AIDS-associated lymphoma patients were analyzed for clonality, the presence of EBV genomes, and the presence of *c-myc* rearrangement by Southern blot. The results of this analysis are shown in Table 1. Three discrete categories of lymphoma were identified by this Southern blot analysis. The

primary CNS lymphoma specimens were all large cell in morphology, monoclonal by J_H rearrangement studies, and the majority (6/7) showed evidence of EBV infection. There was no evidence for *c-myc* rearrangement in this class of tumor. The peripheral lymphomas were equally divided into two categories based on clonality studies. The monoclonal tumors contained the majority of small non-cleaved cell lymphomas (8/17) and over half of these tumors showed evidence for *c-myc* rearrangement (9/17). EBV genomes were found in approximately one-third of the monoclonal tumors (6/17). Of interest, only two tumors in the entire study showed evidence for both EBV infection and *c-myc* rearrangement. Virtually all of the polyclonal tumors showed large cell morphology (15/16) and in contrast to polyclonal tumors arising in immunosuppressed transplant patients, only a minority of AIDS-associated polyclonal lymphomas showed evidence for EBV infection (3/16). There was no evidence for *c-myc* rearrangement in this group of tumors.

Table 1. Molecular Analysis of the AIDS Associated Lymphomas

	Peripheral		CNS
Clonality	Monoclonal	Polyclonal	Monoclonal
Morphology	8/17 SNC	15/16 LCL	7/7 LCL
EBV+	6/17	3/16	6/7
c-myc abnormality	9/17 (2EBV+)	0/16	0/7

SNC - small non-cleaved cell lymphoma
LCL - large cell lymphoma
Polyclonal: <5% control rearranged JH band on Southern blot
Monoclonal: >5% control rearranged JH band
EBV+: >5% control EBV hybridization
c-myc: >5% rearranged c-myc gene

Fig. 1 shows representative results of J_H rearrangement studies on six peripheral AIDS-associated lymphoma specimens. Lane 1 contains the 95% PBL/5% monoclonal tumor DNA specimen. In the control lane 1, the prominent band at 13.7 kb represents the germ line J_H configuration, whereas the higher molecular weight band represents the rearranged J_H band of the 5% monoclonal tumor DNA. The hybridization intensity of the rearranged band in Lane 1 represents the result one would see if a tumor specimen contained only a 5% population of monoclonal B cells. Operationally, any tumor specimen that had an equal or greater intensity rearranged band was designated as monoclonal, and any specimen that contained no rearranged band or a band of lesser intensity than the band shown in Lane 1 was designated polyclonal. Lanes 2, 4 and 7 represent monoclonal tumor specimens, whereas Lanes 3, 5 and 6 represent polyclonal tumors. The faint rearranged bands seen in Lane 4 with the faint germ line band suggest that a small subpopulation of monoclonal B cells are present within an overall polyclonal B cell population.

In contrast to the monoclonal tumors in Lanes 2, 4 and 7, no rearranged bands were seen in the polyclonal tumor specimens. The very faint germ line bands in Lanes 3 and 6 suggest that the vast majority of cells present within those biopsy specimens must have been B cells, having rearranged their variable regions to uniquely sized DNA fragments so that no single rearranged band was noted. The faint germ line band in comparison to the dominant germ line band in Lane 1, suggests that these polyclonal tumors must have been predominantly B cells. Immunophenotypic analysis of these tumors confirmed that they contained a preponderance of lymphoma cells and that those lymphoma cells were IgM expressing B cells (not shown).

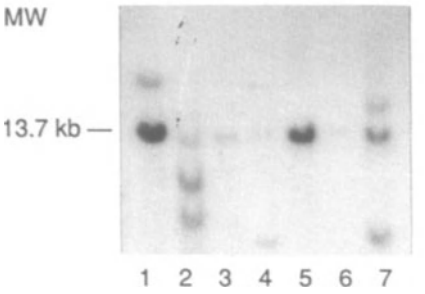


Fig. 1. J_H analysis of AIDS NHL DNA.
DNA extracted from six different AIDS-NHL's was digested with Eco-R1 and probed with a ^{32}P-labelled J_H gene probe as described in Methods. Lane 1: Control DNA (95% PBL, 5% 2F7); lanes 2-7: AIDS NHL's

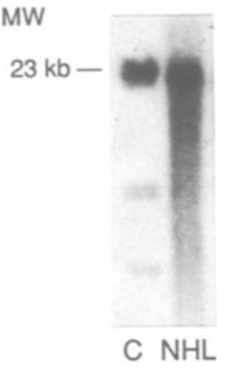


Fig. 3. T cell receptor gene rearrangement analysis of polyclonal NHL.
DNA extracted from a monoclonal T cell lymphoma (lane 1) or from AIDS-NHL DNA from lane 5, Fig. 1 (NHL) were digested with Bam H1 and probed with a ^{32}P-labelled T_C beta gene probe as described in Methods.

In comparison with Lanes 3 and 6, Lane 5 had a prominent germ line band, although of lesser intensity that the PBL control lane (Lane 1). To determine what type of cell was contributing this germ line band, an in depth immunophenotypic analysis was performed on this tumor specimen. Fig. 2a shows that this tumor showed a characteristic large cell lymphoma morphology (polymorphous immunoblastic variant) with many cells staining with anti-IgM. However in Fig. 2b-d many cells were found to stain with anti-CD4, anti-CD8, as well as anti-CD14. The CD14 positive macrophages were not apparently phagocytic as are the "tingible-body" macrophages in Burkitt's lymphomas. In order to test whether the lymphoma shown in Lane 5 was a T cell rather than B cell lymphoma, T cell receptor beta chain rearrangement analysis was performed. Fig. 3 shows that in comparison to a germ line C_T beta band (23kb) as well as clonally rearranged C_T beta bands in a control monoclonal T cell lymphoma (Lane C), the tumor specimen from Lane 5 in Fig. 1 above showed only a germ line representation of the C_T beta genes (NHL). Therefore it appears that the polyclonal lymphomas in this molecular analysis represent a broad spectrum of disease processes, ranging from predominant polyclonal B cell proliferations as shown in Lanes 3 and 6, all the way to polyclonal B and T cell proliferations with the presence of prominent macrophages (Lane 5).

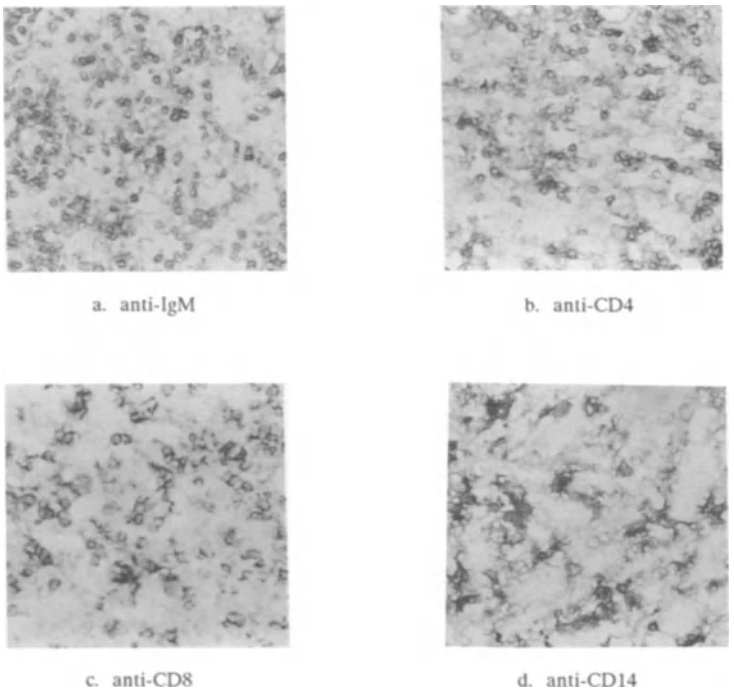


Fig. 2. Immunophenotypic analysis of polyclonal NHL (Fig. 1, lane 5)

Discussion

In this study we found that the AIDS-associated lymphomas could be broadly divided into three discrete molecular categories:

1. Primary CNS lymphomas were monoclonal, with the majority showing evidence for EBV infection, but no *c-myc* rearrangements.
2. Peripheral monoclonal lymphomas, a category which contained the majority of small non-cleaved cell morphology, in which half showed evidence for *c-myc* rearrangement and one-third showed evidence of EBV infection. Of note, only rarely did we find evidence for both EBV infection and *c-myc* rearrangement in contrast to earlier reports that this was a frequent observation in AIDS-associated lymphomas [30,31].
3. Peripheral polyclonal lymphomas predominantly of large cell morphology with only rare evidence for EBV infection and no evidence for *c-myc* rearrangement.

The peripheral polyclonal lymphomas and some of the monoclonal B-cell subtypes have been shown to be a complex mixture of B cells, T cells and even macrophages. This admixture is reminiscent of the recently appreciated T cell rich B cell lymphomas and appears similar to some of the animal models of lymphoma - particularly the retroviral-induced MAIDS and BLV associated lymphomas [32]. The presence of all three major cells of the immune system, regardless of whether one defines these tumors as lymphomas or as "benign" prelymphomatous conditions, has important pathogenetic implications. A

dysfunctional immune response directed against currently undefined antigens may be an important first step in lymphoma development. Another possibility is that a dysfunctional or a clonal macrophage population may be the actual neoplastic cell analogous to Hodgkin's disease - a polyclonal mixture of T cells, B cells and the neoplastic Reed-Sternberg cells.

Southern blot analysis of lymphoproliferations must be interpreted with caution. Verification of tissue is paramount - the surgical pathologist and the molecular biologist must be examining the same tissue. In the current study we verified specimens by frozen section histochemistry and used frozen section immunohistochemistry to phenotype the tumors. Although B cells (surface IgM+) comprised the majority of cells within the tumors analyzed, there was no emergence of a monoclonal band at 5% sensitivity in a substantial fraction (16/40). Multiple restriction digest strategies precluded the possibility of a monoclonal rearranged band "hiding behind" the germline band. We feel that by establishing internal standards for rearranged bands we prevented overinterpretation of lines on overexposed gels as oligoclonal or monoclonal bands. Lane 3 in Fig. 1 represents a standard type of polyclonal B cell proliferation. In this case the tumor was predominantly comprised of B cells. Upon polyclonal rearrangement of the IgH alleles the germline band significantly diminished in intensity and a faint polyclonal "smear" appeared in the lower portion of the gel. A classical monoclonal rearrangement in an HIV lymphoma as described by Dalla Favera and colleagues [18,30] is shown in lane 2. In keeping with the Burkitt's histology, the rearrangement of the *c-myc* proto-oncogene was also demonstrated (not shown). Some HIV lymphomas appeared to be complex admixtures of B cells, T cells and macrophages. The Southern blot pattern (J_H probe, Bam-H1 digest) seen in Fig. 1, lane 5 is characteristic of a T cell rich B cell lymphoma with significant numbers of macrophages and stromal cells (immunophenotype, Fig. 2).

Corroborative evidence for establishing clonality was provided by similar light chain rearrangement patterns, compatible clonality patterns for EBV circulization as measured by terminal repeat probing of Southern blots, absence or presence of the *c-myc* rearrangement, and demonstration that there was not a concomitant monoclonal T-cell proliferation [12]. In each case of genotypic polyclonality histological analysis confirmed the difficult conceptual diagnosis of polyclonal lymphoma.

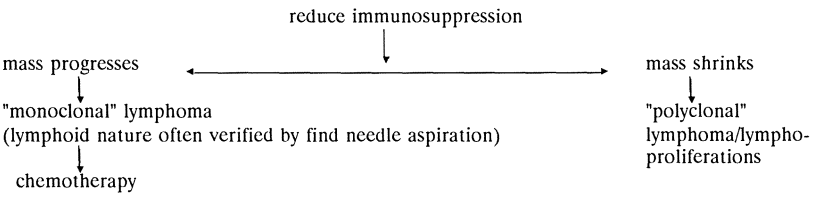
The definition of a hematolymphoid malignancy can be problematic. For example, textbook definitions comparing benign and malignant epithelial tumors emphasize rapid and infiltrating modes of growth and metastasis. However, in the multicentric immune system criteria for malignancy such as "metastasis" and "rapid growth" are mimicked in the normal immune system's response to antigen. Thus the surgical pathologist uses a careful historical definition of lymphoma [16]. If the cytology is not unique (Burkitt's lymphoma or severe lymphoid anaplasia) the diagnosis of high grade lymphomas, to a large extent, depends on observing a diffuse monomorphic growth pattern of large lymphoid cells.

The concept of a high grade lymphoma diffuse monomorphic growth pattern has a comforting diagnostic simplicity - it quickly allows the pathologist to distinguish a hyperplasia which often feature mixtures of large lymphoid cells, smaller lymphocytes, macrophages and plasma cells from a malignant lymphoma. Furthermore, analogous to the acute lymphoid leukemias, many high grade lymphomas when phenotyped, show either Ig light chain exclusion, are either of B cell or T cell origin and/or feature monoclonal Ig gene or T cell receptor rearrangement patterns by Southern analysis [13,14,33]. Thus the diagnostic assumption, borrowing heavily from the acute leukemia/peripheral blood lymphocyte experience, was born - monoclonal equals malignant, polyclonal equals benign and thus the corollary that all the cells in a malignant tumor are derived from the same clone. The non-morphological tests of clonality such as light chain exclusion, B/T phenotyping and gene rearrangement studies, especially in early studies with a narrow

selection of benign controls (classic follicular hyperplasias) and classic malignant morphological subtypes strengthened the diagnostic assumptions. However, several key types of observations illustrate the complexity of the phenotype and genotype of the prelymphomatous condition and lymphomas and have challenged or "ignored" the above narrow diagnostic assumptions:

1) transplant lymphomas - polyclonal lymphoma, polymorphic immunoblastic lymphoma [5,6]
2) congenital immunodeficiency lymphomas - polyclonal lymphoma [7-9]
3) T cell rich B cell lymphomas [34]
4) pseudolymphoma versus lymphoma controversy (lung, orbit) - prognosis similar; genotypic analysis doesn't correlate with morphology [35-38]
5) prelymphomatous autoimmune conditions such as Sjögrens [39]
6) phenotypic heterogeneity of lymphomas of the gastrointestinal tract [40-42]

The observations with the transplant and congenital immunodeficiency associated lymphomas are particularly germane to the HIV-lymphomas. Clearly the pathogenesis, natural history and clinical course of lymphomas is partially dependent on the immune status of the host. It is pointless to designate a histologically defined lymphoma "benign" on the basis of a clonality measurement when without intervention (either diminution of immunosuppression or cancer chemotherapy) the lymphoproliferation leads to the rapid demise of the patient. This is precisely what we found in a retrospective multisite analysis of several HIV lymphoma autopsies [4]. Furthermore, a clinical algorithm that has evolved in transplant medicine completely ignores the surgical pathologist and laboratory "clonist" and uses a strict managerial definition of lymphoma in the context of post transplant lymphoproliferative disease:



The transplant lymphoma algorithm is useful because neither morphology nor clonality are not particularly predictive in determining the response to immunosuppression or chemotherapy. Unfortunately, the current status of HIV therapy does not allow the clinician to modify the immune status of the patient in a straightforward manner.

We concede that polyclonal lymphomas are clinically rare outside of the context of immunosuppression, thus incorporating monoclonality in the definition of lymphoma might indeed be expedient. However, if one considers lymphomas as outgrowths of the immune system, presumably a dysregulated immune system, a polyclonal stage of a lymphoma/lymphoproliferation is theoretically possible. A severe degree of immunosuppression, immune dysregulation or a chronic antigen stimulus could propel such a process to clinically mimic classical lymphomas and lead to the death of the patient. Currently, from an oncologist's viewpoint the distinction between polyclonal and monoclonal lymphomas is not clinically relevant. However, in the future, analogous to the transplant lymphomas, the clonality of a lymphoma may be an important diagnostic tool as physicians become more adept in manipulating the immune system and B cell proliferation in the setting of HIV disease.

Acknowledgements

We thank Sandra Santulli Marotto and Jane Marsh for technical assistance, and John Flickinger for preparation of this manuscript. This work was supported by the NIH PO1 AI24286 (McGrath), NIH RO1 CA54743 (McGrath), the Campini Foundation (Shiramizu), and the San Francisco Center for AIDS Research, CFAR (Shiramizu). Address correspondence to Michael McGrath, San Francisco General Hospital, Bldg 80, Ward 84, 1001 Potrero Avenue, San Francisco, CA 94110.

References

1. Kaplan CD, Abrams D, Feigal E et al (1989) AIDS associated non-Hodgkin's lymphoma in San Francisco. J of the Am Med Assoc 261:719-724
2. Ziegler J, Beckstead J, Volberding P, et al (1984) Non-Hodgkin's lymphoma in 90 homosexual men. Relation to generalized lymphadenopathy and the acquired immunodeficiency syndrome. N Eng J Med 311:565-570
3. Levine AM (1987) Non-hodgkin's lymphomas and other malignancies in AIDS. Seminars in Oncology 14:34-39
4. McGrath MS, Shiramizu B, Meeker T, Kaplan L, Herndier B (1991) AIDS-associated polyclonal lymphoma: Identification of a new HIV-associated disease process. JAIDS 4:408-415
5. Frizzera G, Hanto DW, Gajl-Peczalska KJ et al (1981) polymorphic diffuse B cell hyperplasias and lymphomas in renal transplant recipients. Cancer Research 41:4262-4279
6. Hanto DW, Gajl-Peczalska KJ, Frizzera G et al (1983) Epstein-Barr virus (EBV) induced polyclonal and monoclonal B cell lymphoproliferative diseases occurring after renal transplantation. Clinical, pathologic, and virologic findings and implications for therapy. Annals of Surgery 198:356-368
7. Frizzera G, Rosai J, Dehner LP, Spector BD, Kersey JH (1980) Lymphoreticular disorders in primary immunodeficiencies: new findings based on an up-to-date histological classification of 35 cases. Cancer 46:692-699
8. Penn I (1986) The occurrence of malignant tumors in immunosuppressed states, In: Klein E (ed) Progress in Allergy. Karger, Basel, pp 259-300 (AIDS, vol 37)
9. Purtilo DT (1981) Immune deficiency predisposing to Epstein-Barr virus-induced lymphoproliferative diseases: the X-linked lymphoproliferative syndrome as a model. Advances in Cancer Res 34:279-312
10. Meeker TC, Shiramizu B, Kaplan L, Herndier B, Sanchez H, Grimaldi JC, Baumgartner J, Rachlin J, Feigal E, Rosenblum M, McGrath MS (1991) Evidence for molecular subtypes of HIV-associated lymphoma: Division into peripheral monoclonal lymphoma, peripheral polyclonal lymphoma, and central nervous system lymphoma. AIDS 5:669-674
11. Dalla-Favera R, Matinotti S, Gallo RC, Erickson J, Croce CM (1983) Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B cell lymphomas. Science 219:963-967
12. Shiramizu B, Herndier B, Meeker T, McGrath MS (1992) Molecular and immunophenotypic characterization of AIDS-associated EBV-negative polyclonal lymphoma. J Clin Oncol 10:1-8
13. Cossman J, Uppenkamp M, Sundeen J, Coupland R, Raffeld M (1988) Molecular genetics and the diagnosis of lymphoma. Arch Pathol Lab Med 112:117-127
14. Picker LJ, Weiss LM, Medeiros LJ, Wood GS, Warnke RA (1987) Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. American Journal of Pathology 128:181-201
15. Dorfman RF and Warnke R (1974) Lymphadenopathy stimulating the malignant lymphomas. Human Pathology 5:519-550
16. Berard CW, Dorfman RF (1974) Histopathology of malignant lymphomas. Clin Hematology 3:39-76
17. Herndier BG (1991) Surgical pathology of HIV associated lymphoproliferations. In: Beral V, Jaffe HW, Weiss RA (eds) Cancer, HIV, and AIDS. Cold Spring Harbor Laboratory Press, New York, pp 135-149 (vol 10)
18. Knowles DM, Chamulak GA, Subar M, et al (1988) Lymphoid neoplasia associated with the Acquired Immunodeficiency Syndrome (AIDS). Ann Intern Med 108:744-753

19. Cossman J (1985) Diffuse, aggressive non-Hodgkin's lymphomas. In: Jaffe ES (ed) Surgical Pathology of the lymph nodes and related Organs. WB Saunders, Philadelphia, pp 203-217
20. Fisher RI, Hubbard SM, De-Vita VT, et al (1981) Factors predicting long-term survival in diffuse mixed, histiocytic or undifferentiated lymphoma. Blood 58:45-51
21. Nathwani BN, Dixon DO, Jones SE, et al (1982) The clinical significance of the morphological subdivision of diffuse "histiocytic" lymphoma: A study of 162 patients treated by the Southwest Oncology Group. Blood 60:1068-1074
22. Dorfman RF, Burke JS, Berard CW (1982) A working formulation of non-Hodgkin's lymphomas background, recommendations, histological criteria, and relationship to other classifications. In: Kaplan H, Rosenberg SA (eds) Malignant Lymphomas, Academic Press Inc, New York, pp 351-368
23. Simon R, Durrleman S, Hoppe RT, Bonadonna G, Bloomfield CD, Rudders RA, Cheson BD, Berard CW (1988) The non-Hodgkin lymphoma pathologic classification project. Annals of Internal Medicine 109:939-945
24. Wood GS, Warnke R (1981) Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. J Histochem Cytochem 29:1196-1204
25. Maniatis T, Fritsch EF, Sambrook J (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor
26. Ravetch JV, Siebenlist U, Korsmeyer S, et al (1981) Structure of the human immunoglobulin mu locus: characterization of embryonic and rearranged J and D genes. Cell 27:583-91
27. Felix CA, Reaman GH, Korsmeyer SJ, et al (1987) Immunoglobulin and T cell receptor gene configuration in acute lymphoblastic leukemia in infancy. Blood 70:536-541
28. Raab-Traub N, Flynn K (1986) The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell 47:883-89
29. Dalla-Favera R, Matinotti S, Gallo RC, et al (1983) Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219:963-67
30. Pelicci P-G, Knowles DM, Arlin ZA, Wieczorek R, Luciw P, Dina D, Basilico C, Dalla-Favera R. (1986) Multiple monoclonal B cell expansions and c-myc oncogene rearrangements in AIDS-related lymphoproliferative disorders. Implications for lymphomagenesis. J Exp Med 164:2049-2076
31. Neri A, Barriga F, Inghirami G, Knowles DM, Neequaye J, Magrath IT, Dalla-Favera R (1991) Epstein-Barr virus infection precedes clonal expansion in Burkitt's and AIDS-associated lymphoma. Blood 77:1092-1095
32. Salzman LA (ed) (1986) Animal models of retrovirus infection and their relationship to AIDS. Academic Press, New York
33. Kamat D, Laszewski MJ, Kemp JD, Goeken JA, Lutz CT, Platz CE, Dick FR (1990) The diagnostic utility of immunophenotyping and immunogenotyping in the pathologic evaluation of lymphoid proliferations. Modern Pathology 3:105-112
34. Ramsay AD, Smith WJ, Isaacson PG (1988) T-cell-rich B-cell lymphoma. Am J Surg Pathol 12:433-443
35. Knowles DM, Jakobiec FA, McNally L, Burke JS (1990) Lymphoid hyperplasia and malignant lymphoma occurring in the ocular adnexa (orbit, conjunctiva, and eyelids): A prospective multiparametric analysis of 108 cases during 1977 to 1987. Human Pathology 21, 959-973
36. Kennedy JL, Nathwani BN, Burke JS et al (1985) Pulmonary lymphomas and other lymphoid lesions: A clinicopathologic and immunologic study of 64 patients. Cancer 56:539-552
37. Burke JS, Sheibani K, Nathwani BN et al (1987) Monoclonal small (well-differentiated) lymphocytic proliferations of the gastrointestinal tract resembling lymphoid hyperplasia: A neoplasm of uncertain malignant potential. Hum Pathol 18:1238-1245
38. Jakobiec FA, Neri A, Knowles DM (1987) Genotypic monoclonality in immunophenotypically polyclonal orbital lymphoid tumors. A model of tumor progression in the lymphoid system. Ophthal 94:980-994
39. Schmid U, Helbon D, Lennert K (1982) Development of malignant lymphoma in myoepithelial sialadenitis (Sjögren's Syndrome). Virchows Arch 395:11-43
40. Hall PA, Levison DA (1991) Malignant lymphoma in the gastrointestinal tract. Seminars in Diagnostic Pathology 8:163-177
41. Laszewski MJ, Kamat D, Kemp JD, Goeken JA, Mitros FA, Platz CE, Dick FR (1990) Immunophenotypic and genotypic characterization of primary non-Hodgkin's lymphoma of the gastrointestinal tract. Modern Pathology 3:432-428
42. Knowles DM, Athan E, Ubriaco A et al (1989) Extranodal non-cutaneous lymphoid hyperplasias represent a continuous spectrum of B-cell neoplasia: Demonstration by molecular genetic analysis. Blood 73:1635-1645

Lymphomas in Mice with Retrovirus-Induced Immunodeficiency

Y. TANG, T. N. FREDRICKSON, S. K. CHATTOPADHYAY, J. W. HARTLEY and H. C. MORSE, III

Laboratory of Immunopathology, NIAID, NIH, Bethesda, MD

Introduction

Severe impairments in immune function - genetically determined, iatrogenic or acquired as a consequence of infection - are associated with increased frequencies of lymphomas, most often of B cell origin. The mechanisms responsible for development of these tumors are poorly understood although roles for EBV and alterations in the structure of the myc protooncogene have been suggested for human lymphomas appearing in the post-transplant setting or during the course of AIDS. We previously reported that mice with a retrovirus-induced immunodeficiency syndrome, termed mouse AIDS (MAIDS), develop B cell lineage lymphomas at a low frequency at 20 or more weeks after infection (1). These lymphomas appeared to pass through several stages during their evolution - an initial phase of polyclonal B cell activation and differentiation to Ig secretion (1,2) followed by oligoclonal expansion of cells with preferred growth advantage and eventually, a monoclonal neoplastic population. We suggested previously (3) this progression might be attributed to chronic stimulation of B cells by T cells responding to the virus etiologic for MAIDS (4) but more recent studies (5) demonstrated that the tumors recovered in tissue culture contained multiple integrated copies of this virus.

To develop a better understanding of the lymphomas developing in this setting, we have utilized a potentially more sensitive technique to determine the time after infection at which malignant tumors arise - transplantation of cells from infected animals to mice bearing the scid mutation. We have also examined in greater depth the possible direct contribution of retroviruses to the development of these tumors.

Adoptive transfer of tumors to scid mice

Weanling mice sensitive (B6 and BALB.B), moderately resistant [(B6xCBA/J)F_1], or resistant (BALB/c) to induction of MAIDS were inoculated with LP-BM5 murine leukemia viruses (MuLV) - a virus mixture (BM5mix) containing nonpathogenic helper viruses, both ecotropic and polytropic, and an etiologic replication defective virus (BM5def) - or with LP-BM5 ecotropic virus (BM5eco) alone. At various times after infection, spleen cells or lymph node (LN) cells from infected mice were transferred i.p. to adult scid recipients that were observed for 3 months for development of disease (splenomegaly, lymphadenopathy, hepatomegaly).

None of the scids inoculated with cells from B6 mice infected with BM5mix for 4 wk developed tumors but cells for 1/4 mice infected for 9 wk and from 2/5 mice infected for 12 wk produced tumors in scid recipients. Studies of B6 donors infected for 118 to 223 days revealed that spleens from 5/6 animals induced tumors. The ability to transfer cells expanding rapidly in scid recipients was found not to be restricted to infected B6 mice as cells from 2/2 BALB.B, 1/1 BALB/c and 3/3 (B6xCBA/J)F_1 mice infected for 70 to 411 days also transferred disease.

To examine the contributions of the different viruses in BM5mix to transfer of tumors, we used spleens from B6 mice infected for 1 year with BM5eco alone. None of the scid recipients developed disease, indicating a requirement for BM5def and/or polytropic viruses for the induction of transferrable populations of cells.

Finally, we asked if the cells expanded in first generation (G1) recipients of cells from infected mice could be serially transferred in scid mice. All G1 tumors examined but one underwent repeated transfer, permitting additional phenotypic and molecular characterization.

Phenotypic characterization of transferred cells

Histopathologic studies of tissues from scid mice with enlarged spleen and lymph nodes revealed that these tissues contained large populations of homogeneous cells with characteristics of lymphoblastic or immunoblastic lymphomas. To evaluate the lineage relationships of these presumed lymphoid tumors, cells from enlarged spleens of scid recipients were examined by flow cytometry for expression of surface antigens characteristic of different hematopoietic lineages. For individual recipient animals, the expanded population of transferred cells was found to consist predominantly of B cells or T cells and, in some mice, a mixture of T cells and B cells. In each case, the cells were considered not to be of scid origin, based on homogeneous increase in size and spectrum of cell surface antigen expression different from that of normal scid spleen cells. Because of their serial transplantability, the uniformity of their lymphoid phenotypes and their histopathologic characteristics, we considered these cells to be T and B cell lineage lymphomas.

The detection of T cell lineage tumors was unexpected as lymphomas of this lineage had not been recovered in tissue culture in a previous study (1) and clonal rearrangements of TCR genes were observed infrequently in DNA from spleens of mice infected long term with LP-BM5 MuLV (1). It was of further interest that the T cell lymphomas involved were uniformly $CD4^+$, $CD8^-$ raising questions regarding the potential contributions of transformed T cells to disease progression.

Molecular characteristics of transferred cells

The lymphomas that develop in some human organ transplant recipients and AIDS patients can be subdivided into those with monoclonal and polyclonal origins. The clonality of the MAIDS lymphomas during serial transfer to scid was evaluated with probes for IgJ_H, TCRβ, BM5def and BM5eco. Each of the

transferred lines was found to have clonal rearrangements of T or B cell receptor genes and in almost every instance, the rearrangement observed in the transplant could be detected in the donor cell population. Furthermore, the rearrangement detected, either Ig or TCR, was found to be appropriate for the lymphoma lineage designation determined by flow cytometry.

Two lymphomas in a single mouse

In the studies noted above, phenotypic studies of transferred cells suggested that some populations contained mixtures of B and T cells. To examine this question, spleen cells from a scid mouse with an apparent mixed cell population was separated into T and B cell subsets by plate adherence techniques using an anti-B cell antibody and anti-Ig coated plates. The adherent B cells and nonadherent T cells were transferred individually to scids. Tumors developed in both recipients. By flow cytometric and molecular analyses, the recipient of the separated B cells had a B cell lineage tumor with a pattern of Ig J_H rearrangements and proviral integrations representing some of those observed in the unseparated cell population. Using the same techniques, a tumor developing in the recipient of the separated T cells was shown to have a T cell lymphoma with a distinct set of T cell receptor gene rearrangements and proviral integrations. Although similar attempts to separate tumors of apparently separate lineages have not been performed, it would appear that 4 other tumors of B6 origin contained mixed T and B cell populations. These results demonstrate that T and B cell tumors can develop independently in a single mouse and are not interdependent for their growth.

Conclusions

Our studies indicate that lymphomas of the T cell and B cell lineages develop early and at high frequency in mice susceptible to MAIDS. The tumors seem to differ from many of those in AIDS in several ways. First, a high proportion of the MAIDS tumors are of T cell origin whereas T cell lymphomas have been observed only rarely in AIDS. It may be important, however, that the T cell lymphomas were recovered only from one of the MAIDS susceptible strains, B6. Second, all the MAIDS tumor have experienced one or more proviral integrations while almost all AIDS tumors are HIV negative. Furthermore, histopathologic studies of the donor mice and many other mice with advanced MAIDS showed that the lymphomas that develop in this disease are not as aggressive as those characteristic of AIDS. In spite of these differences, the MAIDS system should prove to be helpful for determining how immunodeficiency contributes to the development of lymphomas.

References

1. Klinken SP, Fredrickson TN, Hartley JW, Yetter RA, Morse HC III (1988) Evolution of B cell lineage lymphomas in mice with a retrovirus-induced immunodeficiency syndrome, MAIDS. J. Immunol 140:1123-1131.
2. Klinman DM, Morse HC III (1989) Characteristics of B cell proliferation and activation in murine AIDS J Immunol 142:1144-1149.

3. Morse HC III (1987) in Mechanisms of B cell neoplasia. Editiones Roche, Basel, Switzerland, p 375.
4. Hügin AW, Vacchio MS, Morse HC III (1991) A virus-encoded "superantigen" in a retrovirus-induced immunodeficiency syndrome of mice. Science 252:424-427.
5. Chattopadhyay SK, SenGupta DN, Fredrickson, TN, Morse HC III, Hartley JW (1991) Characteristics and contributions of defective ecotropic and MCF viruses involved in a retrovirus-induced immunodeficiency syndrome of mice. J Virol 65:4232-4241.

Does HIV Infection of B Lymphocytes Initiate AIDS Lymphoma? Detection by PCR of Viral Sequences in Lymphoma Tissue

S. M. ASTRIN[1], E. SCHATTNER[2], J. LAURENCE[2], R. I. LEBMAN[1] and C. RODRIGUEZ-ALFAGEME[1]

[1]Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia PA 19111
and
[2]Cornell University Medical College, New York NY 10021

Abstract

Individuals infected with HIV (Human Immunodeficiency Virus) frequently develop B cell non-Hodgkins lymphoma. Although previous studies have failed to document the presence of HIV sequences in these tumors, the recent demonstration of malignant transformation of primary B lymphocytes by HIV-1 has prompted us to reinvestigate this issue. We have examined DNA extracted from 7 lymphomas and 5 lymphadenopathy specimens for HIV LTR (long terminal repeat), *gag*, and *tat* sequences using the polymerase chain reaction (PCR). All samples produced products of the expected size with primers for these regions, indicating the presence of HIV proviral sequences in these tissues. The amount of provirus in the tissue was estimated by normalizing the amount of HIV product to the amount of product for the cellular *myc* gene or β globin gene. Products were quantitated during the exponential phase of DNA accumulation. These studies indicated that provirus was present at approximately one copy per cell in the 7 lymphoma samples and in 4 of the 5 lymphadenopathy samples. These results are consistent with a direct role for virus in the initiation of lymphoma. Studies to determine whether provirus resides in the lymphoma cells *per se* will be necessary to further substantiate this hypothesis.

Introduction

HIV infection is associated with a wide range of lymphoproliferative disorders including lymphoid hyperplasia and B-cell non-Hodgkins lymphoma (NHL). As the HIV epidemic progresses and patients live longer with the disease, the incidence of NHL has increased. The risk of developing lymphoma in the presence of advanced HIV infection has been estimated at 10% per year. In addition, the occurrence of NHL as a presenting condition, in the absence of overt compromise of immunity, is increasingly common. This situation has led some investigators to postulate a direct role for virus in the disease. Although failure to detect HIV sequences in lymphoma tissue has led to the assumption that HIV is not directly involved in the development of malignancy [1,2], the observation that HIV can induce malignant transformation in normal B lymphocytes *in vitro* [3,4] has prompted us to reinvestigate the role of virus in lymphomagenesis.

HIV Transformation of B Lymphocytes *In Vitro*

The potential for induction of malignant transformation of B cells by HIV has been demonstrated in an *in vitro* system [3,4]. Our studies have shown that non-immortalized peripheral B lymphocytes from EBV-seropositive, HIV-seronegative donors can be infected by HIV and that a subset of these lymphocytes become malignantly transformed [3,4]. Transformation was documented by the following criteria: HIV transformed B cells (B-HIV) display altered growth properties, propagating in 1% serum and cloning in soft agar; in addition, they form invasive tumors of Burkitt lymphoma phenotype after subcutaneous injection into severe combined immunodeficiency (SCID) mice. Levels of EBV DNA and

RNA are highly elevated in B-HIV relative to levels in B cells immortalized by EBV alone. Levels of c-*myc* RNA and protein are also markedly elevated relative to levels in normal B cells. HIV infection of immortalized B cell lines leads to a similar upregulation of EBV and c-*myc* transcripts. These studies document the malignant potential of HIV infected B lymphocytes and demonstrate that HIV mediates two events linked to B cell neoplasia: deregulation of c-*myc* and activation of EBV. They also raise the possibility of a role for HIV, apart from induction of immune suppression, in the pathogenesis of B cell lymphoma in the acquired immune deficiency syndrome.

The transformation of B cells by HIV appears to require continuous expression of an HIV gene product. The transformed cells contain one copy of HIV provirus as measured by PCR, virus is inducible by phorbol ester, and clones lacking inducible virus no longer display the properties of transformed cells [Laurence, Grimison, and Astrin, unpublished]. These findings, in conjunction with the observation that the transformed cells display a normal karyotype [4], suggest that HIV sequences are necessary for maintenance of the transformed phenotype. All genes necessary for virus production are expressed in these cells as evidenced by the appearance of budding viral particles in electron micrographs (Fig. 1).

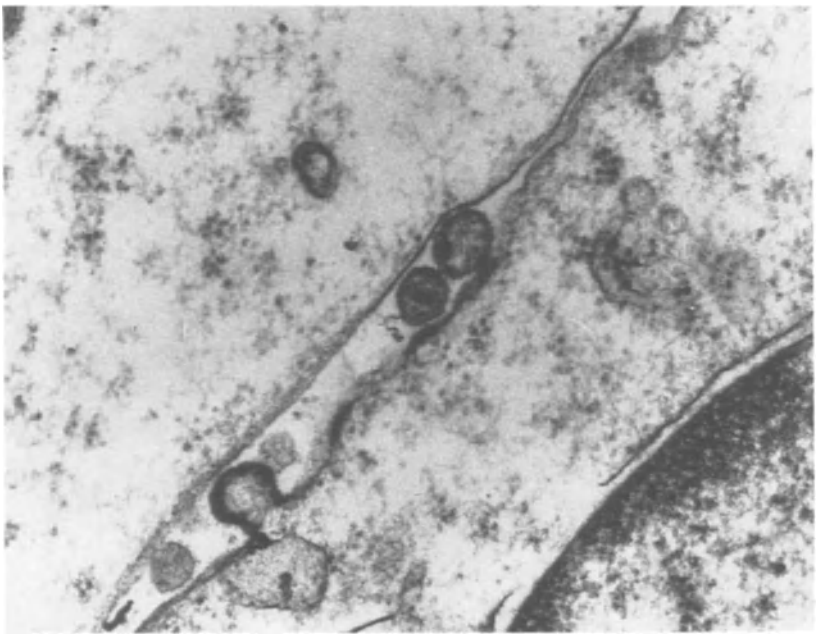


Fig. 1. Electron micrograph of HIV transformed B cells (B-HIV cell line). B-HIV was fixed in 2.0% glutaraldehyde after treatment with PMA for 1 week. Cells were then postfixed in 1% OsO_4, and embedded in plastic. After being sectioned with uranyl acetate-lead citrate, the cells were examined by transmission electron microscopy. HIV virions are shown in various states of maturation, including a budding virion (top), and a mature virion adjacent to an area of membrane thickening (middle) (110,000 x).

Investigation of the mechanism of HIV activation of c-*myc* has focused on TAT, an HIV gene product that functions as a trans-activator of HIV transcription. TAT protein binds to a "stemloop" structure in the first 50 nucleotides of the HIV transcript [5]; it also appears to bind to proteins associated with the HIV LTR (long terminal repeat), which contains promoter and enhancer elements. We have directly tested the ability of TAT to activate *myc* transcription in a cell line that, when infected with HIV, shows a consequent 10 to 20 fold elevation of *myc* transcripts[3]. A similar elevation of *myc* transcript levels was seen when these cells were exposed to media containing 40 μg/ml TAT peptide for 6 hours [Astrin and Laurence, unpublished] These results are consistent with the conclusion that TAT alone can act to upregulate c-*myc* transcription.

Deregulation of c-*myc* has been shown to be an oncogenic event for B cells in a number of systems [reviewed in 6]. It is therefore possible that HIV infection of appropriate B cell targets and consequent deregulation of c-*myc* expression may be an initiating event for B cell lymphoma in HIV infected individuals. If such is the case, one would expect to find HIV*tat* gene sequences along with LTR sequences (which contain the HIV transcriptional promoter) in B cell lymphoma tissue of infected patients. The experiments described below were designed to test this hypothesis.

PCR Detection of HIV Sequences in Lymphoma Tissue

Our PCR strategy was devised by taking into account available HIV sequence information for different isolates of HIV, some of which have as little as 48% nucleic acid homology with the lab strain of HIV-1 (HTLV-IIIB). Sequences for 12 isolates were aligned, and only regions with sequence homology of 95% or greater among the various isolates were chosen for primers. Cognizant of the reported failures to detect viral sequences by Southern blots of lymphoma DNA [1,2], we initially chose to assay for those regions that might be indispensable for transformation, i.e. the *tat* gene and the viral LTR. The LTR sequences are very highly conserved as they serve as the site for sequence specific binding of transcription factors and RNA polymerase. We reasoned that previous failures to detect provirus could be due to inadequate sequence homology of the probes when compared with viral sequences in the lymphoma, and/or to the presence of a highly defective provirus in the tumors. Therefore, we also designed multiple pairs of primers for each region. In all, 10 primer pairs have been synthesized and used to detect viral sequences: 4 primer pairs detect *tat*, 3 pairs detect LTR, and 3 pairs detect *gag*.

Clinical specimens consisted of paraffin sections of fixed tissue from 7 lymphomas and 5 lymphadenopathy specimens. Tissue was deparaffinized using xylene and dehydrated with ethanol. DNA was prepared by digestion with proteinase K [7]. DNA was amplified, and a portion of each reaction subjected to electrophoresis in a 2% agarose gel and stained with ethidium bromide to detect the amplification products. This procedure consistently yields amplifiable DNA from pathologic specimens.

The PCR primers consist of pairs of oligonucleotides of approximately 20 base pairs each, designed to amplify a highly conserved 150-200 base pair segment of the HIV genome. Separate primer pairs were used for each of several segments of the HIV genome, e.g. the long terminal repeat sequence (LTR), *gag*, and *tat* genes. DNA extracted from the clonal T cell line 8E5, which contains 1 integrated HIV genome per cell, or from the clonal monocyte cell line U-1, containing 2 intact copies of HIV per cell, was used as a positive control in the PCR reactions. DNAs from uninfected human cells and from EBV immortalized cell lines were used as negative controls. A panel of more than 30 DNAs from tissues of different uninfected individuals has been tested in PCR reactions with each primer pair to rule out detection of endogenous retroviral sequences by the primers. No products were detected with any uninfected cell DNA tested or with DNA from EBV immortalized B lymphocytes. In addition, each primer pair produces a band of the expected size with the control 8E5 and U-1 DNAs; only a single band is detected with each primer pair. Primers for the c-*myc* gene, used as a control for the detection of cellular genes, always produce a band of the expected size with each human DNA sample as do primers for the globin gene.

HIV, *myc* , and globin sequences were detected in DNA from all 7 lymphomas and 5 lymphadenopathy specimens. Typical results are shown in Fig. 2, 3, and 4. The lymphoma

samples all give products of the expected size with LTR, *gag*, and *tat* primers as well as with *myc* and globin primers. The lymphadenopathy specimens also give the expected products with HIV, *myc*, and globin primers. Results similar to those shown were obtained with the remaining lymphoma and lymphadenopathy samples. These data indicate that both lymphoma and hyperplastic lymph node tissues harbor HIV proviral sequences.

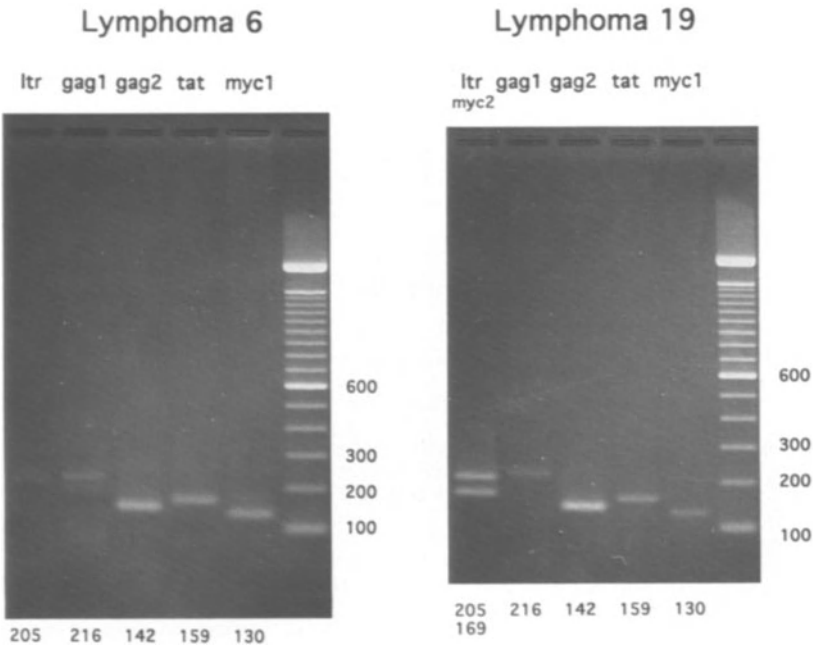


Fig 2. Detection of PCR products for the HIV LTR, *gag*, and *tat* genes and for the cellular *myc* gene in reactions with DNA from lymphoma tissue. 125 ng of DNA was added to 30µl of buffered reaction mix containing 1.5mM $MgCl_2$, 25µM each deoxynucleoside triphosphate and 0.2µM concentrations of each oligonucleotide primer. The sample was heated to 98°C for 5 minutes to denature the DNA, Taq polymerase (0.025 U/µl) added and the mixture subjected to 35 cycles of amplification: annealing at 55° for 1 minute, extension at 72° for 1 minute, denaturation at 95° for 1 minute. Following amplification, 10µl of each reaction was subjected to electrophoresis in a 2% agarose gel and stained with ethidium bromide to detect the amplification products. The expected size in base pairs for each product is indicated below the gel lane; size markers are in the right hand lane with numbers of base pairs in individual fragments indicated. Each product was seen to co-migrate with product produced with the 8E5 control DNA containing 1 provirus per cell. Each reaction was run in parallel with a sample of the reaction mix lacking input DNA. No product was seen in these controls.

Quantitation of HIV Proviral Sequences in Lymphoma Tissue

In order to estimate the amount of HIV gene sequence present in the tissue samples (one copy per cell, one per 10 cells, one per 100 cells, etc.), we co-amplified the HIV sequences and the *myc* exon 3 sequences in the same reaction tube for each DNA sample. In this way we have an internal control for the efficiency of amplification of each DNA, and can also eliminate anomalies due to running the control and experimental sample in different tubes.

Reaction conditions were optimized such that both the *myc* and HIV primers were maximally efficient. Product was sampled at cycle numbers where the reaction was in the exponential accumulation phase. Products were detected by densitometric scans of photographic negatives of ethidium bromide stained agarose gels which had been photographed under UV illumination. With this method it was possible to estimate the relative ratio of HIV sequence to *myc* sequence in each sample.

Reconstruction experiments in which 8E5 DNA (containing one provirus per cell) was mixed in various ratios with DNA from uninfected cells indicated that the ratio of HIV product to *myc* product is reflective of the ratio of these sequences in the input DNA. Data showing quantitation of *myc* and HIV LTR products produced in PCR reactions with DNA from 3 lymphomas and one lymphadenopathy sample are shown in Fig. 5 and Fig 6.

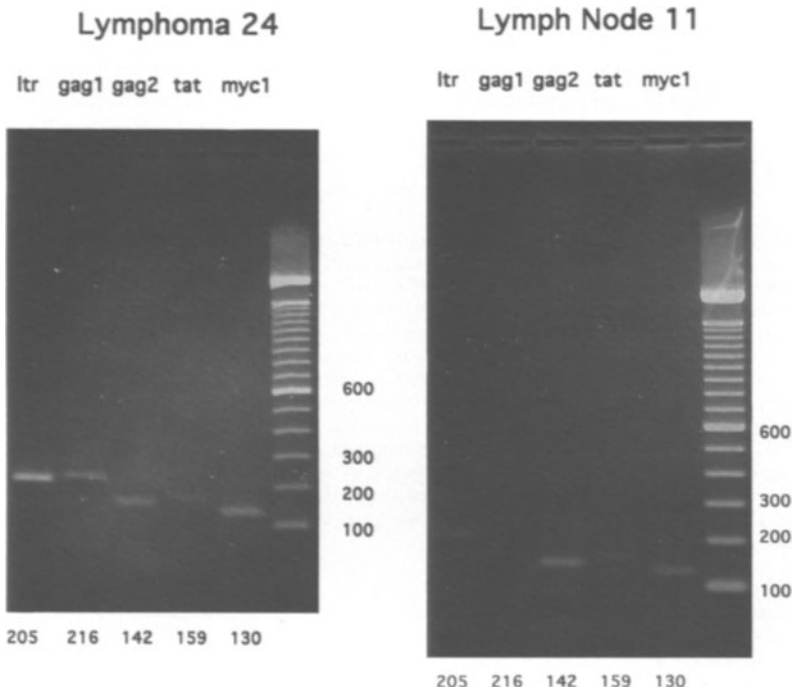


Fig. 3 Detection of PCR amplification products for lymphoma and hyperplastic node tissue. See Fig.2 for details.

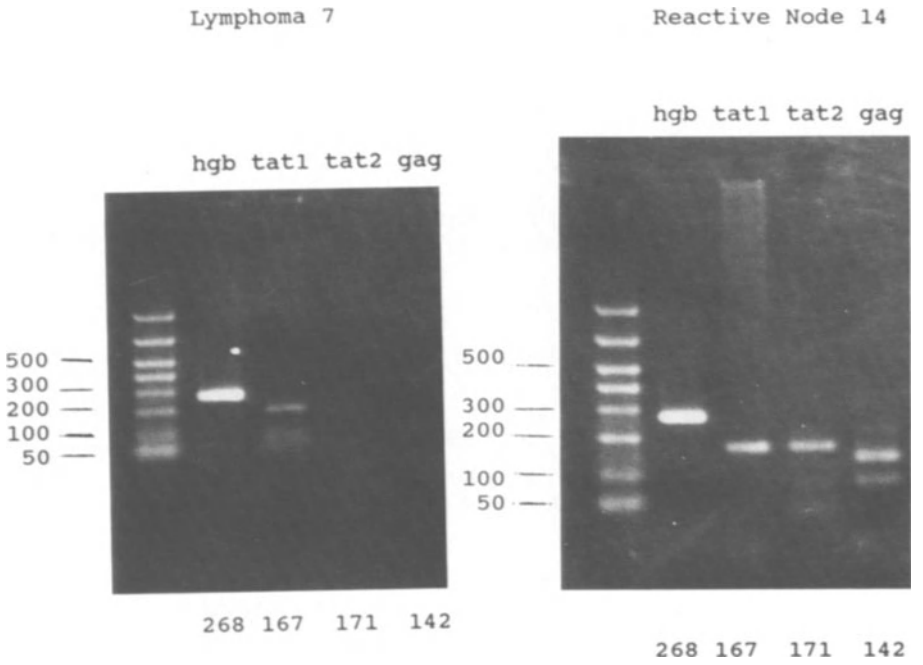


Fig. 4 Detection of PCR amplification products for lymphoma and hyperplastic lymph node tissue. One μg of DNA was added to 50 μl of buffered reaction mix containing 0.75mM $MgCl_2$, 200nM of each primer, and 2.5 U of Taq polymerase. Each sample was heated to 80° C for 8 min and then to 98° C for 45 sec, deoxynucleoside triphosphates (200μM each final concentration) were added and the mixture subjected to 35 cycles of amplification: denaturation at 94° C for 1 min, annealing at 55° C for 2 min, and extension at 72° C for 2 min. Following amplification, 10μl of each reaction was subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide to detect the amplification products. Gels were photographed and densitometrically scanned. Copies per cell of HIV sequences calculated by normalization to the signal for β globin were as follows: For case 7, β globin = 2, *tat* 1 = 0.7, *tat* 2 = 0.2, *gag* = 0.4; for case 14, β globin = 2, *tat* 1 = 1.7, *tat* 2 = 0.9, *gag* = 1.6. The expected size in base pairs for each product is indicated below the gel lane; size markers are in the left hand lane with numbers of base pairs in individual fragments indicated. Each product was seen to co-migrate with product produced with the U-1 control DNA containing 2 HIV proviruses per cell. Each reaction was run in parallel with a sample of the reaction mix lacking input DNA. No product was seen in these controls.

These data indicate that the amount of HIV LTR is comparable to the amount of *myc* in each of the 4 samples, i.e. there appears to be at least one copy of HIV provirus on average per cell in the lymphoma and lymphadenopathy tissue.

A second method for estimating the proviral copy number was also employed. For each sample, multiple reactions were run under the conditions described in the legend to Fig. 4. The amount of product was determined by densitometric scans of photographic negatives of ethidium bromide stained gels which had been photographed under UV illumination. Relative copy number for HIV sequences was determined by normalizing the PCR product for each viral sequence to the β globin product obtained from each DNA. Copies of HIV per cell calculated by this method agreed with the above estimates; i.e. values were close to 1 with ranges in general of 0.5 to 2.0 for amounts of individual products.

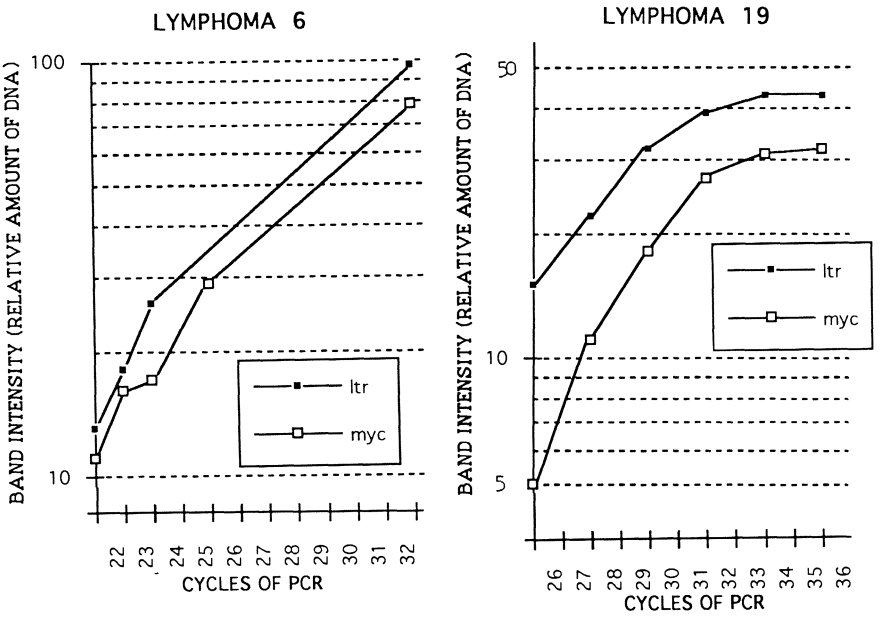


Fig. 5. Quantitation of *myc* and HIV LTR products in PCR of DNA from lymphoma tissue. See text for details.

Data similar to those shown in Fig. 5 and Fig. 6 were obtained with 3 other lymphomas and 3 additional lymphadenopathy specimens. For these samples, both methods for estimating proviral copy number indicated an average of about one provirus per cell. One lymphoma DNA sample has not been completely analyzed and one lymphadenopathy sample gives results in which the amount of LTR product is considerably less than that for *myc*. Thus both lymphoma and hyperplastic lymph node tissue from HIV infected individuals appear to harbor a significant level of HIV provirus. Further experimentation will be required to determine the cellular distribution of this HIV DNA.

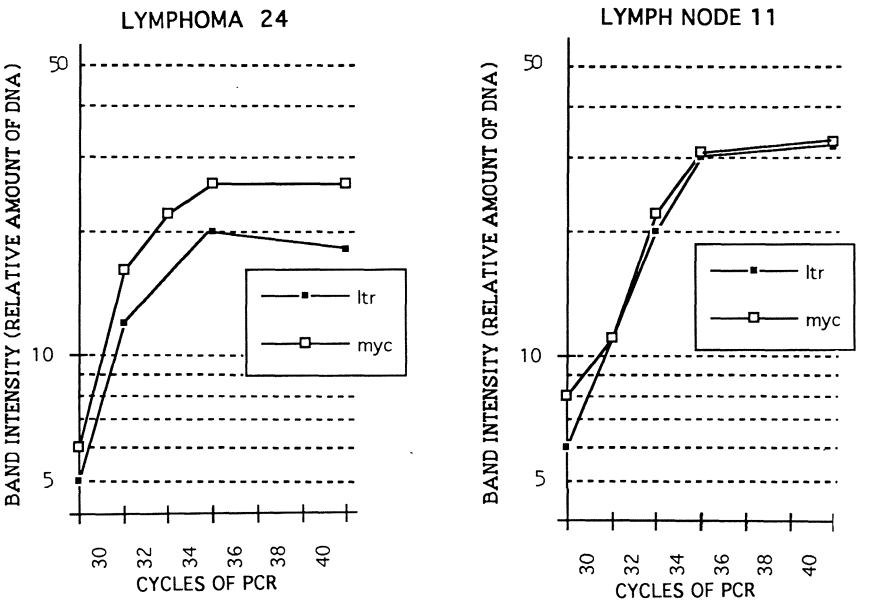


Fig. 6. Quantitation of *myc* and HIV LTR sequences in PCR of DNA from lymphoma and lymph node tissue.

Conclusions

The data presented here are consistent with a model in which HIV infection of B lymphocytes is an initiating event for AIDS lymphoma. However, it is also possible that the proviral sequences detected in the tissues analyzed reside not in the B lymphoma cells or proliferating B cells of the hyperplastic nodes, but rather in infiltrating T lymphocytes, macrophages, endothelial cells, or other non-B lineage cell types. This question can be answered with *in situ* PCR experiments.

Data from *in situ* PCR experiments with lymphoma 24 (Rodriguez-Alfageme, unpublished) confirm the presence of HIV provirus at the level of one provirus per cell and show that the HIV sequences are present exclusively in the lymphoma cells. If such is the case for the remaining lymphoma and lymphadenopathy samples, it would seem a distinct possibility that HIV infection is an initiating event for AIDS lymphoma. What role might virus play in lymphomagenesis? The answer to this question may come from our *in vitro* studies.

We have shown that HIV infection of primary B lymphocytes results in a tenfold upregulation of c-*myc* expression. Studies in Eμ *myc* transgenic mice show that deregulated expression of c-*myc* in the B cell compartment causes B cell hyperplasia and the eventual outgrowth of clonal tumors [8]. In this system, a second event is required for the formation of tumors. Studies on B cell lymphomagenesis in the chicken also indicate the need for a second event, in addition to deregulation of c-*myc*, for the induction of malignant tumors [9]. Thus, HIV infection of a small fraction of B cells in a patient may produce a persistent B cell hyperplasia by upregulating c-*myc* in the infected cells, but further genetic events would be

required for a lymphoma to arise. In this model, both the hyperplastic nodes and the lymphoma would be expected to contain HIV.

In addition to the above model, it is possible that lymphadenopathy may ensue from the ability of HIV to activate Epstein-Barr Virus. Spread of EBV is thought to be a significant factor in the induction of transplant lymphomas in immunosuppressed patients [10]. In this model, the hyperplastic lymph nodes may not harbor HIV provirus, but infection of a proliferating B lymphocyte with HIV could supply a second event necessary for lymphomagenesis. In this case only the lymphoma cells would be found to harbor HIV.

Acknowledgement

We acknowledge the excellent technical assistance of Chen Zheng. This work was supported by National Institutes of Health Grants AI 29119 (J.L.) and CA 40636 and CA 06927 (S.M.A.); U.S. Army Medical Research Acquisition Activity Contract DAMD 17-90-Z0049 (J.L.); and Council for Tobacco Research Grant 2480 (S.M.A.); and an appropriation from the Commonwealth of Pennsylvania.

References

1. Pelicci PG, Knowles DM, Arlin ZA, Wieczorek R, Luciw P, Dina D, Basilico C, Dalla-Favera R (1984) Multiple monoclonal B cell expansions and c-myc oncogene rearrangements in acquired immune deficiency syndrome-related lymphoproliferative disorders. J Exp Med 164:2049-2076
2. Groopman JE, Sullivan JL, Mulder C, Ginsburg D, Orkin SH, O'Hara CJ, Falchuk K, Wong-Staal F, Gallo RC (1986) Pathogenesis of B cell lymphoma in a patient with AIDS. Blood 67:612-616
3. Laurence J, Astrin SM (1991) Human immunodeficiency virus induction of malignant transformation in human B lymphocytes. Proc Natl Acad Sci USA 88:7635-7639
4. Astrin SM, Laurence J (1992) Human immunodeficiency virus activates c-myc and Epstein-Barr virus in human B lymphocytes. Annals NY Acad Sci 651:422-431
5. Roy S, Delling U, Chen CH, Rosen CA, Sonenberg N (1990) A bulge structure in HIV-1 RNA is required for Tat binding and Tat-mediated trans-activation. Genes and Dev 4:1365-1373
6. Cole MD (1986) The myc oncogene: its role in transformation and differentiation. Ann Rev Genet 20:361-384
7. Wright DK, Manos MM (1990) Sample preparation from paraffin-embedded tissues. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. Academic Press Inc., San Diego, pp 153-158
8. Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL (1985) The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature 318:533-538
9. Clurman RE, Hayward WS (1989) Multiple proto-oncogene activations in avian leukosis virus-induced lymphomas: evidence for stage-specific events. Mol Cell Biol 9:2657-2664
10. Hanto DW, Gajl-Peczalska KJ, Frizzeria G, Arthur DC, Balfour HH, McClain K, Simmons RL, Najarian JS (1983) Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occurring after renal transplantation. Annals Surg198:356-369

Oncogenes and Transcriptional Factors

Lymphocyte Activation and the Family of NF-κB Transcription Factor Complexes

V. Bours, G. Franzoso, K. Brown, S. Park, V. Azarenko, M. Tomita-Yamaguchi, K. Kelly#, and U. Siebenlist

Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases and #Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20892

NF-κB, an Activation-Sensitive Transcription Factor Complex with Great Significance For Immune Responsiveness

NF-κB is a transcription factor complex known for some time to play a pivotal role in the regulated expression of a large number of genes which are activated during an immune response. Antigen in the context of the appropriate antigen presenting cell (APC) leads to the proliferation of T cells and the expression of factors (predominantly cytokines) from the competent T cells. These factors in turn stimulate various cells involved in an immune response. Both the signal emanating from the initial antigenic encounter and the secondary factor-mediated responses in other cells involve NF-κB, at least in part. For example, NF-κB has been shown to be essential for the regulated expression of the immunoglobulin k light chain (whence the name NF-κB, a nuclear factor binding to the kappa light chain B element), the tumor necrosis factor alpha (TNF-α) and beta (TNF-β), interferon-β, the IL-2 receptor, the IL-6 cytokine, the IL-2 growth factor, GM-CSF, G-CSF, and MHC-Class I, just to name a few immunomodulatory gene products. NF-κB is implicated also in the induction of various acute phase proteins (e.g. angiotensinogen), some transcription factors/oncogenes (e.g. IRF-1 and c-myc) and, most importantly, in the induction of various viruses, including the human immunodeficiency virus (HIV), cytomegalovirus, adenovirus and SV40. NF-κB mediates its effects through so-called κB elements; the consensus sequence for a κB site reads GGGRNNYYCC, but additional variants exist to which NF-κB binds. A variety of agents as well as factors produced by stimulated cells activate NF-κB in the respective target cells; included among these are a large array of T cell mitogens (e.g. lectins, anti CD3 and anti CD2 antibodies, in addition to antigen/APC), the factors TNF-α and TNF-β, IL-1, double-stranded RNA, chemical agents which cause PKC activation, DNA damaging agents and treatments which produce oxygen radicals. In addition, several viruses have been shown to cause NF-κB activation like the HTLV-I and -II viruses, the HSV-1 virus, the HHV-6 virus, the Hepatitis B virus and the adenovirus. These viruses apparently subvert the cellular mechanisms to induce an activation phenotype in cells. The listing given here for agents which activate NF-κB and for genes regulated by NF-κB is only a partial one and is only meant to convey the central role this transcription factor plays during cellular activation and, in particular, during immune activation (for a complete listing and the appropriate references see recent reviews [1, 2]).

What exactly is NF-κB and how is it activated? The paradigm of NF-κB was established several years ago prior to gene clonings and it is still true today, at least in its general form (Figure 1). An inhibitory molecule, IκB, prevents nuclear entry of a heterodimeric DNA binding complex [3 - 8]. IκB associates with the p65 subunit of the most common heterodimeric form of NF-κB (see below), namely p50/p65 [9]. Upon receiving the appropriate signal IκB is likely to be modified in a way which leads to dissociation from p65, thus allowing the heterodimer to translocate to the nucleus and bind to κB elements, activating gene expression. IκB has been reported to be directly phosphorylated during activation and oxygen radicals have been implicated in signalling [10 - 12]. The relatively simple picture of NF-κB has grown much more complex, largely because of the recent clonings of NF-κB by a number of laboratories. It has become evident that NF-κB-like activity is generated from a family of

Current Topics in Microbiology and Immunology, Vol. 182

proteins, each member presumably with distinctive properties; similarly there exists a family of inhibitory proteins. In the following we will describe some salient features of the various proteins known to determine NF-κB activity in cells. We will also point out the relevance of some of these genes in B cell neoplasia.

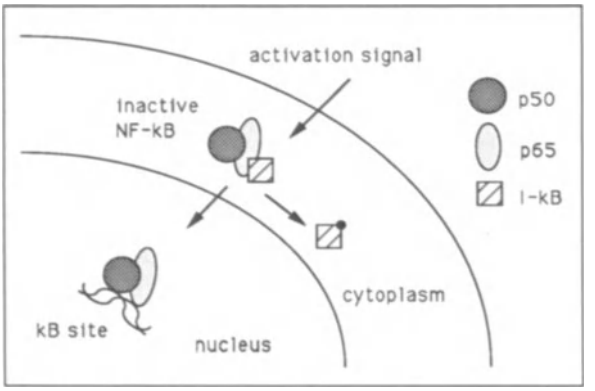


Fig. 1. The NF-κB complex. A number of different activation signals initiate a cascade of events inside the cell which presumably lead to a modification of IκB and the release of the transcription factor complex. The NF-κB complex, depicted here as a p50/p65 heterodimer, then translocates to the nucleus to bind κB DNA sites.

A Family of Interacting Rel-Related Genes Determines NF-κB Activity in Cells.

The identification of a gene encoding p50 represented the first cloning of a known component of NF-κB. We cloned this gene by a subtractive approach aimed at isolating genes which are rapidly induced upon activation of primary human T cells [13]. Others cloned it by way of nucleic acid probes based on partial peptide sequence obtained from purified NF-κB [14 - 16]. p50 is part of a larger precursor protein of 105 kDa (Figure 2). The precursor consists of two domains, an amino-terminal region of about 320 amino acids related to the amino-terminal part of the c-Rel protein, the so-called Rel-homology domain (see below) and a carboxy-terminal portion containing so-called cell cycle or ankyrin repeat structures. An ever-growing number of interesting regulatory proteins have been found to contain such repeats [2], including several yeast cell cycle proteins (SWI4, SWI6, cdc10) [17 - 19], the human ankyrin

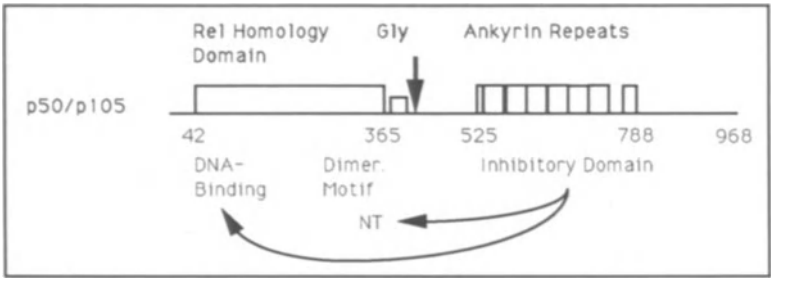


Fig. 2. Structure of the precursor (p105) for the p50 subunit of NF-κB. The precursor protein contains a Rel homology domain, a glycine-rich linker (Gly) and a domain containing ankyrin repeats. There are six full repeats which are preceded and followed by two partial repeats (before and after the six full repeats). The numbers indicate amino acid positions in the full protein. The large vertical arrow at the carboxy-end of the glycine-rich area denotes the approximate position for processing of the precursor into the p50 molecule (the amino-terminal part). In the precursor molecule the ankyrin repeat region acts as an inhibitor of nuclear translocation and DNA-binding. The end of the Rel homology domain contains a nuclear translocation signal (NT). The inhibitory domain may also interfere with homodimerization, which is mediated by the dimerization motif in the Rel homology domain (see text).

protein (whence the name for the repeat motif) [20] and, more recently, IκB (see below) [21]. To date no unifying theme exists which relates these proteins functionally. The repeats, partially conserved and 33 amino acids in length, may simply specify protein-protein interaction motifs which are used by functionally very disparate proteins in order to interact with distinct targets. The Rel-homology domain and the repeat domain of the p105 precursor are separated by a glycine-rich stretch which is thought to function as a flexible hinge. Processing of the precursor [22, 23] gives rise to the p50 molecule which corresponds approximately to the Rel-homology domain; this domain specifies κB-binding activity and dimerization [24, unpublished observations]. Dimerization with another Rel-homology domain, for example with itself, is necessary for DNA-binding. The ankyrin repeat domain appears to be rapidly degraded upon processing and is usually not found in cells [22] (an exception to this is IκB-γ discussed below). Within the precursor molecule the repeat domain is responsible for inhibiting κB-binding by the amino-terminal part and for retention in the cytoplasm. The repeat domains are thought to 'cover up', by close contact, a nuclear translocation signal located at the end of the Rel-homology domain [23, 25]. This could explain the cytoplasmic localization of the precursor. Less clear is how DNA binding is inhibited in vitro. One report [25] suggests that this may be accomplished by inhibition of dimerization, although other data suggest inhibition of already formed dimers as a mechanism [23, 26].

In addition to p50 we cloned another member of the NF-κB family of proteins by way of isolating mitogen-inducible genes. We called the encoded protein p50B because it is structurally and functionally related to p50 [27]. p50B has also been independently cloned by others (called p49 or lyt-10) [28,29]. Like p50, p50B is synthesized as a precursor protein. The precursor is a 97 kDa protein which contains a Rel-homology domain and a carboxy-terminal ankyrin repeat domain which inhibits binding of the unprocessed protein to the κB site (Figure 3).

p50 and p50B constitute one class of NF-κB- or Rel-related proteins. p65 [30, 31], c-Rel [32] and RelB [33] as well as the drosophila protein Dorsal [34] constitute the second class: these proteins do contain Rel-homology domains as well but they are not known to be processed and they do not harbor ankyrin repeats (Figure 3). Dorsal is the essential morphogen which regulates dorsal-ventral polarity in drosophila embryos [35 - 37]. To date it is the only Rel-related protein known in this species. c-Rel is the homolog of v-Rel, the oncogene of the acutely tumorigenic avian virus Rev-T [38]. c-Rel, v-Rel and Dorsal have been studied for some time, but it was not known until recently that these proteins are part of the NF-κB family,

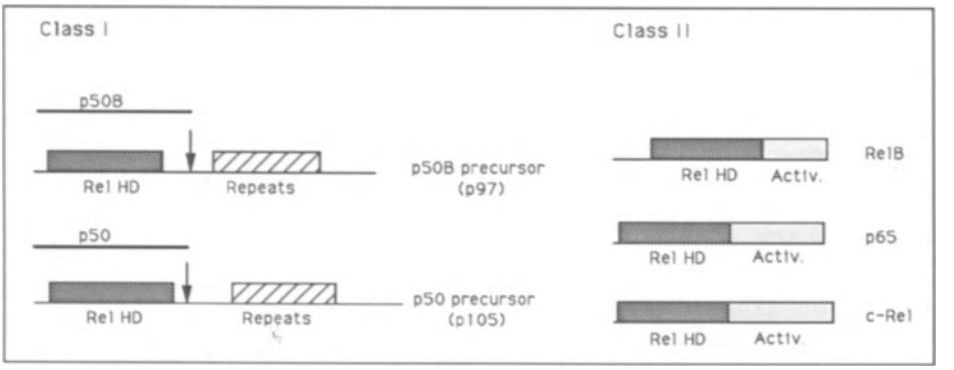


Fig.3. Two classes of Rel-related proteins. All proteins share similar but non-identical Rel homology domains (Rel HD). Class I proteins are processed (processing site denoted by the arrow), giving rise to p50 and to p50B. The precursors contain ankyrin repeats in their carboxy-terminal half. Class II proteins are not processed and harbor sequences in their carboxy-terminal portions which are important for transactivation (Activ.). The drosophila protein Dorsal also belongs to this second class.

nor was it realized that they can bind DNA sequence-specifically. p65 [39, 40], c-Rel [41, 42], RelB [33] and Dorsal [43, 44] all have been demonstrated to contain transcriptional activation domains in their carboxy-terminal parts. The activation domains of this class of Rel-related proteins appear to be very dissimilar, suggesting distinct functions. The human homolog of the mouse RelB gene, called I-Rel, has been reported not to contain an activation domain, rather, I-Rel inhibited transactivation [45]. The reason for this apparent discrepancy vis-a-vis RelB has yet to be resolved. A distinct feature of the RelB/I-Rel protein is a short domain amino-terminal to the Rel-domain which resembles a leucine zipper. It is possible that this domain controls function by interacting with unknown proteins which may vary between cells. Another unique feature of RelB/I-Rel is that it is unable to homodimerize. p65 [30, 31], c-Rel [41, 46 - 48], p50 [13 - 16] and p50B [27 - 29] (as well as Dorsal [43]) homodimerize readily and bind κB sites, although binding by p65 homodimers is relatively weak [31, 49].

Both p50 and p50B can form heterodimers in vitro with any member of the second class of Rel-related proteins (p65, c-Rel and RelB) [15, 27 - 31, 33]. The members of this latter group are not known to heterodimerize among themselves, with the exception of a p65/c-Rel complex found associated with the urokinase κB site [50]. The homo- and heterodimeric complexes which can form have distinct binding preferences with respect to the exact nucleotide sequence of the κB site; in general, the heterodimers are less discriminating than the homodimers, thus able to bind a wider variety of κB sites [27, 51, 52, unpublished observations]. One exception is the IL-2 κB site which strongly prefers p50 homodimers over p50/p65 heterodimers [53, unpublished observation]. The heterodimers involving either p50 or p50B and a member of the transactivating class of proteins, and the p65 and c-Rel homodimers can transactivate a κB-dependent reporter in vivo [27, 28, 39, 41, 49]. However, it has been reported also that c-Rel can inhibit endogenous NF-κB in some cells [54]. The precise functions and mechanisms of action of the various complexes in different cells and within the context of different promoters/enhancers remains to be elucidated. Although p50 and p65 are generally more highly and more universally expressed, there are nonetheless many cells in which various different κB-binding complexes coexist [27, unpublished observations]. The structural complexity inherent in this family of Rel-related proteins suggests an equally complex regulation of genes through cis-acting κB elements.

NF-κB: Activation of Preformed Complexes and Induction of New Complexes

The mRNAs for the p50 and p50B precursors are mitogenically induced in T and B cells [13, 16, 27]; we could demonstrate also that the corresponding proteins are induced as are the resulting processed forms (p50 migrates as a 49 kDa protein and p50B as a 52 kDa protein on SDS-PAGE (unpublished observations)). In addition, c-Rel and RelB are mitogenically induced [55, 56, 33]. NF-κB must therefore exist in two distinct forms, one form defined by the preformed complexes present in resting cells and thus directly responsive to activation signals and another form dependent on newly synthesized Rel-related proteins induced during activation. In this scenario regulation of a given gene by a κB element may change during the course of activation, due to a change in complexes present. For example, it has been suggested that newly synthesized c-Rel may modulate κB-dependent transcription [40, 55]. We have observed significant changes in the relative amounts of various Rel-related proteins in cells upon activation (unpublished observations). The preformed complexes present in resting cells may be the result of very low level transcripts made constitutively and/or may be due to long-lived complexes from a previous activation. In any case, the newly synthesized proteins are likely to influence κB-dependent transcription during the course of activation.

p50 Homodimers Act Like Competitive Inhibitors

As noted above the p65 homodimers are potent transactivators of κB-dependent transcription in vivo, judged by transient transfections into mammalian cells (Figure 4A) [28, 39, 40, 49]. This raises the question as to why p50 is needed, since it could not transactivate a κB reporter by itself. However, physiological levels of p65 appear to be fairly low in most cells (although there may be exceptions) and binding affinities are weaker than they are for heterodimers [30, 31, 49]. Consequently, p50 becomes a necessary partner for good transactivation. This is revealed clearly when small amounts of p65 are transfected together with p50, leading to synergistic transactivation (Figure 4B). The surprising result of this experiment was that excess levels of p50, levels well above those of p65, led to an apparent decrease of transactivation [49]. Under these conditions p50 homodimers were much more abundant than p50/p65 heterodimers. The p50 homodimers apparently competed effectively for the κB elements, thus preventing transactivation through p65. p50 homodimers acted like repressors.

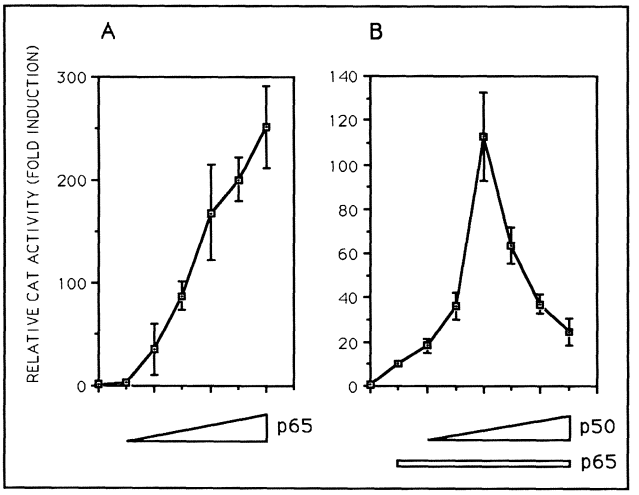


Fig.4. (A) p65 is a potent transactivator by itself and (B) p50 can act as a repressor of transactivation. NTera-2 cells were co-transfected with an HIV-κB CAT reporter plasmid and (A) increasing amounts of p65 (0.01, 0.03, 0.1, 0.3, 1, 3 ug) or (B) a constant amount of p65 (0.03 ug) together with increasing amounts of p50 (0.01, 0.03, 0.1, 0.3, 1, 3 ug). CAT activities were recorded as Fold Inductions over the reporter vector alone.

Do p50 homodimers occur naturally? Analysis of nuclear extracts from resting peripheral blood T cells revealed a large excess of p50 homodimers in the nucleus of unstimulated peripheral blood T cells when compared to the heterodimeric form (unpublished observations). This implies a functional role for p50 homodimers and our data would suggest that they act as competitive inhibitors of the heterodimer, a role not previously recognized. The repressive effects of p50 may be important for certain genes. For example, the IL-2 κB element has very strong affinity for the p50 homodimer [53, unpublished observation] and a recent report [53] suggests that the expression of the IL-2 growth factor gene is repressed by p50 homodimers. Furthermore, a reduction in the binding of the p50 homodimer correlates with activation of IL-2. Although these data provide evidence for a repressive role of p50 homodimers, other functions can not be ruled out. For example, in vitro transcription experiments revealed a stimulating activity of the p50 homodimers, implying that p50 homodimers have the ability to transactivate [57, 58]. However, this has not been observed by transfections into cells.

The IκB Family of Proteins Includes the Oncoprotein Bcl-3 and the C-Terminal Halves of the p50 and p50B Precursor Molecules

Just as NF-κB is a family of related complexes, so is IκB-like activity determined by a family of genes. To begin with, partial biochemical purification of IκB suggested two forms, IκB-α and -β [59]. Cloning of an IκB-like molecule (as an inducible gene [21]) revealed that it contained ankyrin or cell cycle repeats, similar to the C-terminal ends of p50 or p50B. We could demonstrate in transfection experiments that this IκB-like molecule could inhibit p65 as well as c-Rel and RelB, i.e. all transactivating subunits of NF-κB (unpublished observations). At this time it is unclear whether the cloned protein corresponds to the original alpha or beta fraction. The recently cloned chicken protein pp40 may be a homolog of the cloned human IκB [60, 61, 62]; it was originally discovered in complexes with c-Rel and v-Rel [63, 64]. Another probable but not yet reported member of this family of IκB-like molecules is the drosophila protein Cactus which inhibits Dorsal from entering nuclei [65]. The C-terminal halves of the p50 and p50B precursors may also function as IκB-like molecules; they are structurally and functionally related to IκB (Figure 5). The ankyrin repeat domain of the p50 precursor inhibited the Rel-homology domain not only as part of the precursor, but also when expressed separately [26]. We showed that the ankyrin domain of the p50B precursor acted similarly, targeting primarily the p50B Rel homology domain (unpublished observations). Although there is currently no evidence that processing of the precursor gives rise to stable C-terminal peptides, the p50 precursor derived ankyrin repeat domain may be expressed as a stable molecule from a separate mRNA present in certain mouse cell lines; this domain was consequently referred to as IκB-γ [26]. A similar phenomenon may exist for the p50B precursor derived ankyrin domain (tentatively designated IκB-δ in Fig. 5), although the overall physiologic significance of these observations remains to be determined. It is important to note that the p50 and p50B precursor molecules may function as inhibitory molecules by themselves since they can heterodimerize with the second class of Rel-related proteins in the cytoplasm, thereby preventing nuclear translocation (unpublished observations).

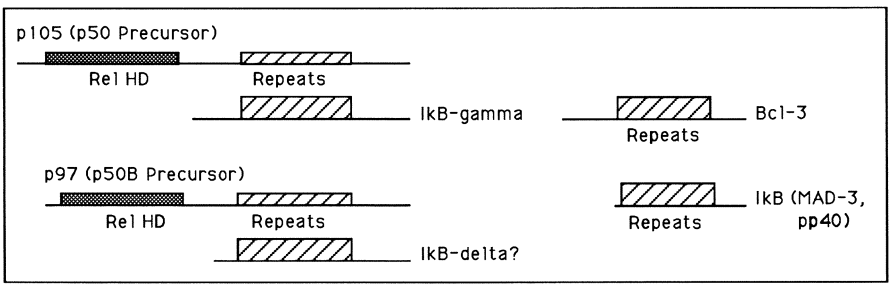


Fig. 5. The family of IκB-related proteins. The proteins shown all have between 5 and 7 similar but non-identical ankyrin repeats, but contain little homology elsewhere. IκB-γ and IκB-δ are derived from the precursor genes for p50 and p50B, respectively. IκB-δ is a genetically engineered protein only and thus is shown with a question mark. The cloned MAD-3 protein is listed as IκB here; pp40 appears to be the chicken homolog of MAD-3. The p105 and p97 precursor proteins can act like IκB proteins as well (see text).

Bcl-3 constitutes another member of the IκB-like family of proteins. Its ankyrin repeat structure is most similar to that of the ankyrin domains in the p50 and p50B precursors [66, 67]. To test whether Bcl-3 could function like IκB we cotransfected Bcl-3 together with p50 and p65 into recipient cells devoid of NF-κB activity (Figure 6A). Although Bcl-3 inhibited transactivation, it could do so only at concentrations significantly higher than those required to observe strong inhibition by IκB. Bcl-3 appears to target the p50 molecule as suggested by recently demonstrated inhibition of binding by p50 homodimers [67] and we could demonstrate

a physical association between the two molecules (unpublished data). We therefore tested Bcl-3 under repressed conditions of transactivation, i.e. when the p50 homodimers existed in excess over p50/p65 heterodimers (Figure 6B). Under these conditions Bcl-3 effectively facilitated transactivation, presumably by binding to and inhibiting the p50 homodimers and allowing the heterodimers to function. Bcl-3 acted like an anti-repressor or a pseudo-activator. At much higher concentrations Bcl-3 eventually also targeted the heterodimers, inhibiting their function, as suggested by Figure 6A. Bcl-3 is inducible in cells upon activation [66]. We speculate that Bcl-3 may function during cellular activation by relieving p50 homodimer-mediated repression of certain genes. The IL-2 gene may be such an example, as discussed above.

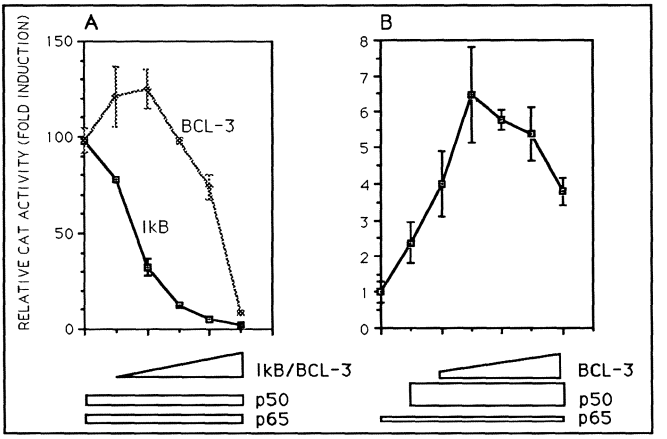


Fig. 6. Bcl-3, an antagonist of p50-mediated repression. (A) IκB is a potent inhibitor and Bcl-3 is a weak inhibitor of p50/p65-mediated transactivation of a co-transfected HIV-κB CAT reporter. Transfections were performed as for Fig. 4. Amounts of transfected constructs: p50, 0.3 ug; p65, 0.15 ug; IκB or Bcl-3, 0.03, 0.1, 0.3., 1, 3 ug. (B) Bcl-3, a facilitator of transactivation. Transfections as in (A) and the amounts used were: p65, 0.03 ug; p50, 5 ug; Bcl-3, 1.5, 2.5, 3.5, 4.5, 5 ug. For (B) the CAT activity was relative to p65 transfected alone; transactivation with Bcl-3 approaches levels seen with optimal p50/p65 synergy (Fig. 4B).

NF-κB and B Cell Neoplasias

The avian Rev-T virus which carries the v-Rel oncogene is one of the most acutely transforming viruses known. It is unclear how overexpression of v-Rel causes tumors. Experimental evidence suggests that v-Rel homodimers can inhibit κB-dependent transcription by acting as a competitive inhibitor of true transactivating complexes [41, 48, 54, 68]. v-Rel differs most significantly from c-Rel by deletion of a carboxy-terminal domain important for transactivation and cytoplasmic retention [38, 42]. On the other hand, v-Rel may transform also by complexing IκB-like molecules in the cytoplasm [69]. v-Rel can be found in cells associated with c-Rel, pp40 (the apparent chicken homolog of the cloned human IκB) and two larger molecules, one of which may be the p50 precursor protein [63, 64, 70]. In any case, κB-dependent transcription is likely to be modified by the presence of v-Rel. c-Rel has also been reported to be involved in human tumors. Amplifications and rearrangements of the c-Rel locus have been observed in follicular lymphomas and in diffuse large cell lymphomas [71].

The second gene of the greater family of NF-κB genes to be implicated in B-cell tumors is p50B. This gene was found to be translocated to the immunoglobulin alpha locus in a non-Hodgkin's B cell lymphoma [29]. The translocation truncated the gene in half, presumably leading to overexpression of the p50B protein and alleviating the need for processing from the precursor. The absence of the C-terminal ankyrin domain may be relevant as well. Even

though the precise mechanism for the presumed role in transformation remains elusive, p50B overexpression is likely to influence κB-dependent gene expression.

The third gene of importance to B cell neoplasias is Bcl-3. It was originally cloned by way of identifying a gene locus which translocated to the immunoglobulin alpha gene in several chronic lymphocytic leukemias [66]. A rearrangement of Bcl-3 has also been detected in a case of diffuse large cell lymphoma [72]. The resulting overexpression of Bcl-3, presumed to be due to nearby immunoglobulin control elements, is likely to modify NF-κB activity in cells. As discussed above, Bcl-3 may function as a pseudo-activator of κB-dependent gene expression, i.e. as an antagonist of p50-mediated repression. Because of the deregulation of Bcl-3 in these tumor cells other genes may be expressed aberrantly in non-activated or partially activated cells.

In addition to the three proteins discussed other members of the NF-κB family may have the potential to contribute to tumorigenesis as well. For example, a naturally occurring alternatively spliced version of p65 can transform transfected rat embryo fibroblasts to tumorigenic growth in vitro and in vivo [73].

References

1. Baeuerle PA (1991) The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochimica et Biophysica Acta 1072:63-80.
2. Grilli M, Chiu JJ-S, Lenardo MJ (1992) NF-κB and Rel: participants in a multiform transcriptional regulatory system. Int Rev Cytology, in press.
3. Sen R, Baltimore D (1986) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46:705-716.
4. Sen R, Baltimore D. (1986) Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell 47:921-928.
5. Baeuerle PA, Baltimore D (1988) IκB: a specific inhibitor of the NF-κB transcription factor. Science 242:540-546.
6. Baeuerle PA, Baltimore D (1988) Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell 53:211-217.
7. Lenardo MJ, Kuang A, Gifford A, Baltimore D (1988) NF-κB purification from bovine spleen: nucleotide stimulation and binding site specificity. Proc Natl Acad Sci USA 85:8825-8829.
8. Lenardo MJ, Baltimore D (1989) NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell 58:227-229.
9. Baeuerle PA, Baltimore D (1989) A 65-kD Subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev 3:1689-1698.
10. Shirakawa F, Mizel SB (1989) In vitro activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol Cell Biol 9:2424-2430.
11. Ghosh S, Baltimore D (1990) Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature 344:678-682.
12. Schreck R, Rieber P, Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 10:2247-2258.
13. Bours V, Villalobos J, Burd PR, Kelly K, Siebenlist U (1990) Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. Nature 348:76-80.
14. Ghosh S, Gifford AM, Riviere LR, Tempst P, Nolan GP, Baltimore D (1990) Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell 62:1019-1029.
15. Kieran M, Blank V, Logeat F, Vandekerckhove J, Lottspeich F, Bail OL, Urban MB, Kourilsky P, Baeuerle PA, Israël A (1990) The DNA Binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell 62:1007-1018.
16. Meyer R, Hatada EN, Hohmann H-P, Haiker M, Bartsch C, Röthlisberger U, Lahm H-W, Schlaeger JE, Van Loon APGM, Scheidereit C (1991) Cloning of the DNA-binding subunit of human nuclear factor κB: The level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. Proc Natl Acad Sci USA 88:966-970.
17. Andrews BJ, Herskowitz I (1989) The yeast SW14 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. Nature 342:830-833.
18. Breeden L, Nasmyth K (1987) Similarity between cell-cycle genes of budding yeast and fission yeast and the *notch* gene of *Drosophila*. Nature 329:651-654.
19. Aves SJ, Durkacz BW, Carr A, Nurse P (1985) Cloning, sequences and transcriptionalcontrol of the *Schizosaccharomyces pombe cdc10* 'start' gene. EMBO J 4:457-463.

20. Lux SE, John KM, Bennett V (1990) Analysis of cDNA for human erythrocyte ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. Nature 344:36-42.
21. Haskill S, Beg AA, Tompkins SM, Morris JS, Yurochko AD, Sampson-Johannes A, Mondal K, Ralph P, Baldwin AS, Jr (1991) Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell 65:1281-1289.
22. Fan C-M, Maniatis T (1991) Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature 354:395-398.
23. Blank V, Kourilsky P, Israël A (1991) Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J 10:4159-4167.
24. Logeat F, Israël N, Ten R, Blank V, Bail OL, Kourilsky P, Israël A (1991) Inhibition of transcription factors belonging to the rel/NF-κB family by a transdominant negative mutant. EMBO J 10:1827-1832.
25. Henkel T, Zabel U, van Zee K, Müller JM, Fanning E, Baeuerle PA (1992) Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell 68:1121-1133.
26. Inoue J-I, Kerr LD, Kakizuka A, Verma IM (1992) IκBγ, a 70 kd protein identical to the c-terminal half of p110 NF-κB: a new member of the IκB family. Cell 68:1109-1120.
27. Bours V, Burd PR, Brown K, Villalobos J, Park S, Ryseck R-P, Bravo R, Kelly K, Siebenlist U (1992) A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. Mol Cell Biol 12:685-695.
28. Schmid RM, Perkins ND, Duckett CS, Andrews PC, Nabel GJ (1991) Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature 352:733-736.
29. Neri A, Chang C-C, Lombardi L, Salina M, Corradini P, Maiolo AT, Chaganti RSK, Dalla-Favera RSK (1991) B cell lymphoma-associated chromosomal translocation involves candidate oncogene *lyt*-10, homologous to NF-κB p50. Cell 67:1075-1087.
30. Ruben SM, Dillon PJ, Schreck R, Henkel T, Chen C-H, Maher M, Baeuerle PA, Rosen CA (1991) Isolation of a *rel*-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science 251:1490-1493.
31. Nolan GP, Ghosh S, Liou H-C, Tempst P, Baltimore D (1991) DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell 64:1-10.
32. Brownell E, Mittereder N, Rice NR (1989) A human *rel* proto-oncogene cDNA containing an *Alu* fragment as a potential coding exon. Oncogene 4:935-942.
33. Ryseck R-P, Bull P, Takamiya M, Bours V, Siebenlist U, Dobrzanski P, Bravo R (1992) RelB, a new rel family transcription activator that can interact with p50-NF-κB. Mol Cell Biol 12:674-684.
34. Steward R (1987) *Dorsal*, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, c-*rel*. Science 238:692-694.
35. Rushlow CA, Han K, Manley JL, Levine M (1989) The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in Drosophila. Cell 59:1165-1177.
36. Steward R (1989) Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. Cell 59:1179-1188.
37. Roth S, Stein D, Nüsslein-Volhard C (1989) A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the Drosophila embryo. Cell 59:1189-1202.
38. Wilhelmsen KC, Eggleton K, Temin HM (1984) Nucleic acid sequences of the oncogene v-*rel* in reticuloendotheliosis virus strain t and its cellular homolog, the proto-oncogene c-*rel*. J Virol 52:172-182.
39. Ruben SM, Narayanan R, Klement JF, Chen C-H, Rosen CA (1992) Functional characterization of the NF-κB p65 transcriptional activator and an alternatively spliced derivative. Mol Cell Biol 12:444-454.
40. Ballard DW, Dixon EP, Peffer NJ, Bogerd H, Doerre S, Stein B, Green WC (1992) The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc Natl Acad Sci USA 89:1875-1879.
41. Inoue J-I, Kerr LD, Ransone LJ, Bengal E, Hunter T, Verma IM (1991) c-rel activates but v-rel suppresses transcription from κB sites. Proc Natl Acad Sci USA 88:3715-3719.
42. Kamens J, Richardson P, Mosialos G, Brent R, Gilmore T (1990) Oncogenic transformation by vRel requires and amino-terminal activation domain. Mol Cell Biol 10:2840-2847.
43. Ip YT, Kraut R, Levine M, Rushlow CA (1991) The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in Drosophila. Cell 64:439-446.
44. Thisse C, Perrin-Schmitt F, Stoetzel C, Thisse B (1991) Sequence-specific transactivation of the Drosophila *twist* gene by the *dorsal* gene product. Cell 65:1-20.
45. Ruben SM, Klement JF, Coleman TA, Maher M, Chen C-H, Rosen CA (1992) I-Rel: a novel *rel*-related protein that inhibits NF-κB transcriptional activity. Genes Dev 6:745-760.
46. Sica A, Tan T-H, Rice N, Kretzschmar M, Ghosh P, Young HA (1992) The c-*rel* protooncogene product c-Rel but not NF-κB binds to the intronic region of the human interferon-γ gene at a site related to an interferon-stimulable response element. Proc Natl Acad Sci USA 89:1740-1744.

47. Kochel T, Rice NR (1992) v-*rel*- and c-*rel*- protein complexes bind to the NF-κB site *in vitro*. Oncogene 7:567-572.
48. Ballard DW, Walker WH, Doerre S, Sista P, Molitor JA, Dixon EP, Peffer NJ, Hannink M, Greene WC (1990) The v-*rel* encodes a κB enhancer binding protein that inhibits NF-κB function. Cell 63:803-814.
49. Schmitz ML, Baeuerle PA (1991) The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J 10:3805-3817.
50. Hansen SK, Nerlov C, Zabel U Verde P, Johnsen M, Baeuerle PA, Blasi F (1992) A novel complex between the p65 subunit of NF-κB and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. EMBO J 11:205-213.
51. Urban MB, Baeuerle PA (1990) The 65-kD subunit of NF-κB is a receptor for IκB and a modulator of DNA-binding specificity. Genes Dev 4:1975-1984.
52. Urban MB, Baeuerle PA (1991) The role of the p50 and p65 subunits of NF-κB in the recognition of cognate sequences. New Biologist 3:279-288.
53. Kang S-M, Tran A-C, Grilli M, Lenardo MJ (1992) NF-κB subunit regulation in nontransformed $CD4^+$ T lymphocytes. Science 256:1452-1456.
54. McDonnell PC, Kumar S, Rabson AB, Gelinas C (1992) Trasncriptional activity of *rel* family proteins. Oncogene 7:163-170.
55. Molitor JA, Walker WH, Doerre S, Ballard DW, Green W (1990) NF-κB: a family of inducible and differentially expressed enhancer-binding proteins in human T cells. Proc Natl Acad Sci USA 87:10028-10032.
56. Bull P, Hunter T, Verma IM (1989) Transcriptional induction of the murine c-*rel* gene with serum and phorbol-12-myristate-13-acetate in fibroblasts. Mol Cell Biol 9:5239-5243.
57. Fujita T, Nolan GP, Ghosh S, Baltimore D (1992) Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev 6:775-787.
58. Kretzschmar M, Meisterernst M, Scheidereit C, Li G, Roeder RG (1992) Transcriptional regulation of the HIV-1 promoter by NF-κB in vitro. Genes Dev 6:761-774.
59. Zabel U, Baeuerle PA (1990) Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell 61:255-265.
60. Davis N, Ghosh S, Simmons DL, Tempst P, Liou H-C, Baltimore D, Bose HR Jr. (1992) Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science 253-1268-1271.
61. Kerr LD, Inoue J-i, Davis N, Link E, Baeuerle PA, Bose HR Jr, Verma IM (1991) The Rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev 5:1464-1476.
62. Inoue J-I, Kerr LD, Rashid D, Davis N, Bose HR Jr, Verma IM (1992) Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc Natl Acad Sci USA 89:4333-4337.
63. Tung HYM, Bargmann WJ, Lim MY, Bose HR Jr (1988) The v-*rel* oncogene product is complexed to a 40-kDa phosphoprotein in transformed lymphoid cells. Proc Natl Acad Sci USA 85:2479-2483.
64. Kochel T, Mushinski JF, Rice NR (1991) The v-*rel* and c-*rel* proteins exist in high molecular weight complexes in avian and murine cells. Oncogene 6:615-626.
65. Isoda K, Roth S, Nusslein-Volhard C (1992) The functional domains of the *Drosophila* morphogen *dorsal*: evidence from the analysis of mutants. Genes Dev 6:619-630.
66. Ohno H, Takimoto G, McKeithan TW (1990) The candidate proto-oncogene *bcl*3 is related to gene implicated in cell lineage determination and cell cycle control. Cell 60:991-997.
67. Hatada EN, Nieters A, Wulczyn FG, Naumann M, Meyer R, Nucifora G, McKeithan TW, Scheidereit C (1992) The ankyrin repeat domains of the NF-κB precursor p105 and the protooncogene *bcl*-3 act as specific inhibitors of NF-κB DNA binding. Proc Natl Acad Sci USA 89:2489-2493.
68. Richardson PM, Gilmore TD (1991) vRel is an inactive member of the Rel family of transcriptional activating proteins. J Virol 65:3122-3130.
69. Gilmore TD (1991) Malignant transformation by mutant Rel proteins. Trends Gen 7:318-322.
70. Capobianco AJ, Chang D, Mosialos G, Gilmore TD (1992) p105, the NF-κB p50 precursor protein, is one of the cellular proteis complexed with the v-rel oncoprotein in transformed chicken cells. J Virol 66:3758-3767.
71. Lu D, Thompson JD, Gorski GK, Rice NR, Mayer MG, Yunis JJ (1991) Alterations at the *rel* locus in human lymphoma. Oncogene 6:1235-1241.
72. van Krieken JHJM, McKeithan TW, Raghoebier S, Medeiros LJ, Kluin PM, Raffeld M (1990) Chromosomal translocation t(4:19) as indicated by bcl-3 rearrangment is a rare phenomenon in non-Hodgkin's lymphoma and chronic lymphocytic leukemia: A molecular genetic analysis of 176 cases. Leukemia 4:811-812.
73. Narayanan R, Klement JF, Ruben SM, Higgins KA, Rosen CA (1992) Identification of a naturally occurring transforming variant of the p65 subunit of NF-κB. Science 256:367-370.

Transactivation of the c-*myc* Gene by HTLV-1 *tax* is Mediated by NFkB

M. P. DUYAO, D. J. KESSLER, D. B. SPICER, and G. E. SONENSHEIN

Biochemistry Department, Boston University School of Medicine, Boston, MA 02118

INTRODUCTION

The human T cell leukemia virus type I is the causative agent of adult T cell leukemia (Hinuma et al. 1981) and B cell chronic lymphocytic leukemia (Mann et al. 1987). Its genome encodes a 40 kD protein called *tax* (Lee et al. 1984) that is important for the immortalization of cells (Siekevitz et al. 1987a). Stimulation of genes by *tax* protein is mediated by indirect mechanisms involving the activation of several cellular transcription factors, including NFkB and CREB. We have recently demonstrated that the murine c-*myc* gene has two elements that bind and are transactivated by the transcription factor NFkB (Duyao et al. 1990; Kessler et al., 1990). They are located -1101/-1081 bp and +440/+459 bp with respect to the P1 promoter and thus were called upstream regulatory element or URE and internal regulatory element or IRE, respectively. Expression of the c-*myc* gene has been implicated in both induction of proliferation of quiescent mammalian cells and in immortalization and transformation of cells. Thus here we have tested whether *tax* can transactivate the c-*myc* gene through induction of NFkB. The studies were performed in collaboration with Miriam Siekevitz, Christopher Bartholomew and John Cleveland.

RESULTS

Transactivation of the URE and IRE Elements by *tax*

To test for activation by *tax* of transcription through the c-*myc* NFkB elements, transient co-transfection analysis of Jurkat T cells was performed using *tax* expression vectors (Rosen et al. 1987) and chloramphenicol acetyl transferase (CAT) reporter gene constructs containing multimerized copies of wildtype URE or IRE driving the Herpes virus thymidine kinase (TK) promoter. These constructs were prepared as described previously (Kessler et al., 1990), and included the URE element sequence: 5'-AAGTCCGGGTTTTCCCCAACC-3' or the IRE sequence: 5'-TCCGGGGAGGGAATTTTTGT-3' (where the core NFkB binding sequence is underlined). We found, in three separate experiments, that *tax* can transactivate the URE (2 copy)- and IRE (4 copy)-TK-CAT constructs giving induction values between 8.7- and 12- fold compared to vectors expressing antisense *tax*. In contrast, versions with mutated URE or IRE elements, in which the two internal guanine residues were altered to two cytosine residues to prevent binding of NFkB, were not activated. Similar results were obtained with co-tranfection of these vectors into HeLa cells. Thus c-*myc* NFkB elements can be transactivated by expression of *tax*.

Transactivation of the c-*myc* Promoter by *tax*

We next tested whether *tax* expression could induce transcription of the murine c-*myc* promoter. Constructs bearing murine c-*myc* upstream sequences of various lengths and between 513 to 519 bp of exon 1 sequences were prepared linked to CAT, and tested in co-transfection analysis. All constructs that contained both elements were induced by expression of *tax* (Table 1). Values of induction in Jurkat and HeLa cells ranged from 3.2- to 5.5-fold. Furthermore, a human c-*myc*-CAT construct, containing sequences -2238

Table 1. Transactivation of murine c-*myc* CAT constructs by *tax*

Construct[a]	HeLa[b]	Jurkat[b]
pKpn/Xho	5.5 +/-0.95 (3)	5.1 +/-2.2 (7)
p1.9Xba	4.9 +/-1.9 (2)	3.2 +/-0.7 (3)
1.6Bgl	5.0 (1)	3.2 +/-0.8 (4)

[a]Constructs include the following c-*myc* sequences: pKpn, -3660 to +519bp; p1.9Xba, -1437 to +513bp; 1.6Bgl, -1141 to +513bp.
[b]Values for fold-induction represent the mean of the indicated number of experiments () +/-SD.

to +936 of the human c-*myc* gene (Avigan et al. 1990), was similarly induced upon co-transfection with the *tax* expression vector (data not shown). These findings indicate that expression of *tax* can transactivate the c-*myc* promoter.

Role of NFkB in the *tax*-Mediated Induction of c-*myc* Transcription

To test the specific role of NFkB in this induction, we co-transfected mutants of *tax* that were capable of activating either only NFkB or only CREB elements (Smith and Greene, 1990) with the URE/IRE-TK-CAT and 1.6Bgl constructs (Fig. 1). The mutant p*tax*M22 (m22), incapable of NFkB activation, failed to induce URE-TK-CAT, IRE-TK-CAT, and 1.6 Bgl c-*myc*-promoter CAT transcription; the human c-*myc*-CAT construct also failed to

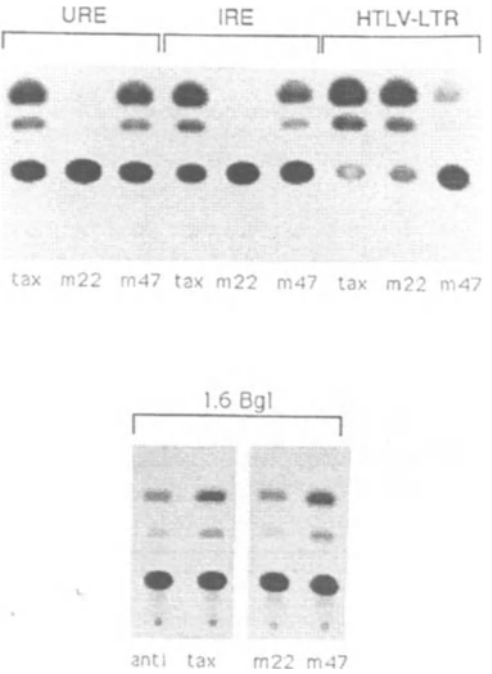


Fig. 1. Effects of mutant *tax* expression vectors on the activity of the c-*myc* promoter.

be induced by p*tax*M22 (data not shown). As expected, this mutant was still able to induce the CREB element within the HTLV-1 LTR (Smith and Green, 1990). In contrast, the mutant p*tax*M47 (m47), incapable of CREB activation, successfully induced the URE/IRE-TK-CAT and 1.6 Bgl c-*myc*-CAT constructs. The induction of c-*myc* transcription by *tax*, therefore, appears to be mediated specifically though activation of NFkB.

NFkB Binding to the c-*myc* Promoter Mediates *tax* Transcriptional Induction
To address whether c-*myc* transactivation by *tax* was due to the direct effect of the binding of NFkB to the URE and IRE, site-directed mutations were made within the URE and IRE in the context of the murine c-*myc* promoter. These mutations, which converted two guanine to cytosine residues within the URE and IRE, were introduced into the 1.6 Bgl c-*myc*-CAT construct. Co-transfection into Jurkat cells of the 1.6 Bgl c-*myc* construct mutated at both sites (m1.6 Bgl) resulted in significantly decreased ability of *tax* to induce CAT activity (1.3-fold), compared to the wildtype construct (5-fold). Similarly, mutation of either element individually severely reduced the ability of *tax* to transactivate the c-*myc* promoter. As seen in Fig. 2, the parent construct was induced 3.2-fold whereas the mutant constructs gave values of 1.2-fold for 1.6 Bgl-mURE and 1.4-fold for 1.6 Bgl-mIRE. These results suggest that the interaction of NFkB with both the URE and IRE elements plays a significant role in transactivation of the c-*myc* gene by *tax*.

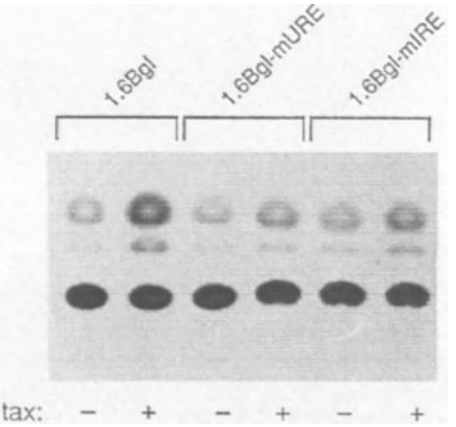


Fig. 2. Effects of mutated URE and IRE elements within the context of the c-*myc* gene on transactivation by *tax*.

DISCUSSION

We have demonstrated that activation of NFkB via *tax* gene expression can induce transcription of the murine and human c-*myc* genes. In the case of the murine gene this activation is mediated directly through the URE and IRE NFkB elements. We have noted putative NFkB elements within the human c-*myc* gene, and are in the process of confirming their role in transactivation by *tax*. Recent studies have shown that *tax* induces continuous proliferation of primary T cells (Grassman et al. 1989). Previously NFkB has been shown to mediate the direct stimulation, by *tax*, of other cellular genes that play a role in regulation of T cell proliferation (Sodroski et al. 1984; Gazzolo and Dodon, 1987;

Siekevitz et al. 1987). Thus it was proposed that the stimulatory effects of *tax* on these growth-related genes plays a role in the immortalization of T cells by the HTLV-1 virus. Given the role of the c-*myc* oncogene in cell growth, stimulation of c-*myc* gene transcription via induction of NFkB is likely to play a role in activation of T cell proliferation resulting from *tax* expression. Subsequent events can then lead to immortalization of these cells.

Our finding that *tax* can transactivate the c-*myc* promoter is one of the first cases demonstrating modulation of activity of a transfected c-*myc* promoter. Futhermore, NFkB represents one of the first identified family of factors mediating regulation of the c-*myc* gene whose expression is influenced by agents known to activate c-*myc* expression. Specifically, in non-B cells, NFkB can be activated by IL-1, TNF-α, phorbol ester, and serum (Sen and Baltimore, 1986; Osborn et al. 1989; Baldwin et al. 1991). All of these agents can induce c-*myc* gene transcription. For example, IL-1 treatment of human FS-4 diploid fibroblasts induces c-*myc* expression and cell proliferation (Lin and Vilcek, 1987). In more recent studies in our laboratory, we have now shown that NFkB plays a role in the activation of the c-*myc* promoter by IL-I in these cells (manuscript submitted). These results indicate an important role for NFkB in control of c-*myc* expression. The ability of the various members of the NFkB family of *rel*-related proteins to transactivate the specific c-*myc* URE and IRE elements is currently under investigation.

ACKNOWLEDGMENTS

We thank C. Rosen and W. Greene for generously providing *tax* expression constructs, and D. Levens for the human c-*myc*-CAT construct. This research was supported by N.I.H. grant CA36355 and training grants AG00115, AI07309, and HL07429.

REFERENCES

Avigan MI, Strober B, Levens D (1990) J Biol Chem 265: 18538-18545
Baldwin AS, Azizkhan JC, Jensen DE, Beg AA, Coodly LR (1991) Mol Cell Biol 11: 4943-4951
Duyao MP, Buckler AJ, Sonenshein GE (1990) Proc Natl Acad Sci USA 87: 4727-4731
Gazzolo L, Dodon MD (1987) Nature (London) 326: 714-717
Grassmann R, Dengler C, Muller-Fleckenstein I, Fleckenstein B, McGuire K, Dokhelar M, Sodroski JG, Haseltine WA (1989) Proc Natl Acad Sci USA 86: 3351-3355
Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, Shirakawa S, Miyoshi I (1981) Proc Natl Acad Sci USA 78: 6476-6480
Kessler DJ, Duyao MP, Spicer DB, Sonenshein GE, (1990) Current Topics in Microbiology and Immunology 166: 211-220
Lee T, Coligan JE, Sodroski JG, Haseltine WA, Salahudddin SZ, Wong-Staal F, Gallo RC Essex M (1984) Science 226: 57-61
Lin J-X, Vilcek J (1987) J Biol Chem 262: 11908-11911
Mann DL, DeSantis P, Mark G, Pfeifer A, Newman M, Gibbs N, Popovic M, Sarngaharan MG, Gallo RC, Clark J, Blattner W (1987) Science 236L: 1103-1106
Osborn L, Kunkel S, Nabel GJ (1989) Proc Natl Acad Sci USA 86: 2336-2340
Rosen CA, Park R, Sodroski J, Haseltine WA (1987) Proc Natl Acad Sci USA 84: 4919-4923
Sen R, Baltimore D (1986) Cell 47: 921-928
Siekevitz M, Feinberg MB, Holbrook N, Wong-Staal F, Greene WC (1987a) Proc Natl Acad Sci USA 84: 5389-5393
Siekevitz M, Josephs SF, Dukovich M, Peffer N, Wong-Staal F, Greene WC (1987b) Science 238: 1575-1578
Smith MR, Greene WC (1990) Genes Dev 4: 1875-1885
Sodroski J, Rosen C, Haseltine W (1984) Science 225: 381-385

Cloning and Characterization of DNA Binding Factors Which Bind Sequences Required for Proper *c-myc* Initiation

S. A. Bossone[1], A. J. Patel[2], C. Asselin[3] and K. B. Marcu[1,2,4]

Departments of Pathology[1], Biochemistry and Cell Biology[2], and Microbiology[4], State University of New York at Stony Brook, Stony Brook, New York; Department of Anatomy and Cell Biology[3], University of Sherbrooke, Sherbrooke, Canada.

Introduction

The c-MYC proto-oncogene encodes a nuclear phosphoprotein with a lengthy history in the genesis of a variety of malignancies (reviewed in Marcu et al. 1992). The definition of normal c-MYC control pathways should reveal initiating events leading to normal and abnormal cellular growth. Numerous positive and negative regulatory elements have been reported for the c-MYC gene which regulate transcription from two initiation sites specified by the P_1 and/or P_2 promoters (Marcu et al. 1992). ME1a1, a nuclear factor site at +97 to +118 with respect to P_1, has been identified by deletional mutagenesis as being necessary for efficient P_2 initiation (Asselin et al. 1989). In addition to a 180 nucleotide (nt) sequence within the gene's first exon, ME1a1 was also required for transcriptional blockage of P_2 initiated transcripts (Miller et al. 1989). Furthermore, attenuation of P_1 initiated transcripts was shown to be dependent upon sequences residing between P_1 and P_2, inclusive of the ME1a1 site (Wright et al. 1991). The 180 nt P_2 termination region and the sequences residing between P_1 and P_2 can functionally substitute for one another as attenuators (Wright et al. 1991) A close comparison of the sequences required for P_1 and P_2 blockage has revealed a region in the P_2 attenuator region homologous to the ME1a1 site. In addition, another ME1a1-like sequence has been reported in the termination region of the human C2 gene (Ashfield et al. 1991). The ME1a2 nuclear factor site, residing between +57 to +82 has also been implicated in P_2 initiation (Asselin et al. 1989; Moberg et al. 1992).

We have extended our earlier studies (Asselin et al. 1989) on the sequence requirements for P_2 usage by employing site-directed mutagenesis, and also report the cloning and partial characterization of DNA binding factors which interact with functionally significant sequences in the c-MYC promoter and attenuation region, and to the C2 gene termination region.

Requirement of the ME1a1 and ME1a2 Sites for Proper P_1:P_2 Promoter Usage

A murine c-MYC promoter-CAT fusion construct, pMBgCAT (Yang et al. 1986), containing either wild type or site-directed mutations engineered into the ME1a1 and ME1a2 sites was stably transfected into CV-1 monkey fibroblast cells. Total cellular

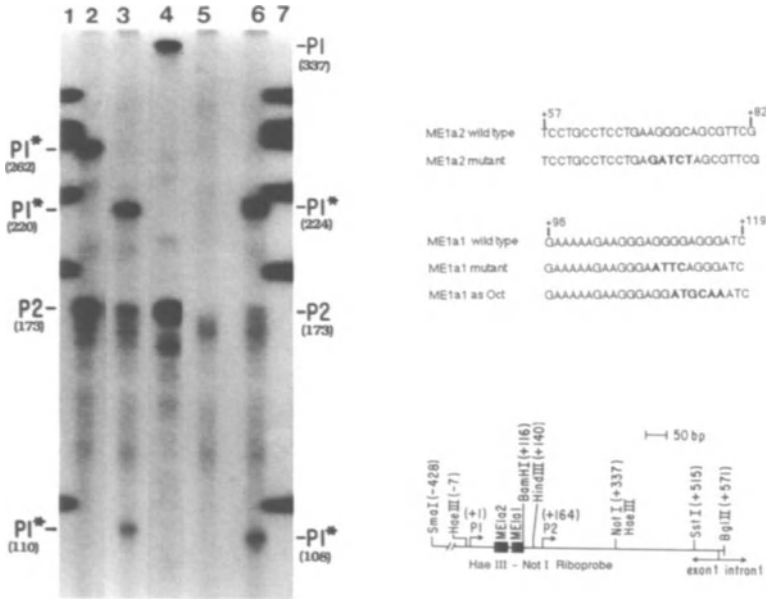


Fig. 1. RNase protection assay. Transcripts initiating from P_1 and P_2 are indicated. The asterisk indicates the digestion of message initiating at P_1 which is cleaved into two fragments by RNase because a wild type antisense riboprobe was used. A schematic of the murine C-MYC gene is also shown. Lanes 1 and 7) ϕX174 Hae III molecular weight markers of 310, 281, 271, 234, 194 and 118 bp in descending order; Lane 2) ME1a2 mutant; Lane 3) ME1a1 as oct; Lane 4) pMBgCAT (wild type); Lane 5) CV-1 nontransfected control; Lane 6) ME1a1 mutant.

RNA from large populations of transfectants was prepared and subjected to RNase protection using a uniformly labeled RNA probe of the c-MYC promoter region and 3' flanking exon one sequences (-7 to +337) (Fig. 1). A normal P_1:P_2 of 1:5 for the wild type construct (Fig. 1, Lane 4) was obtained by densitometric analysis. When the P_2 promoter was mutated, converting a GGGG sequence in the core of the ME1a1 nuclear factor binding site to ATTC, the P_1:P_2 ratio shifted to 10:1 with P_2 activity virtually abolished (Fig. 1, Lane 6). When the ME1a1 site was replaced by an octamer binding site (ME1a1 as oct), the normal P_1:P_2 was not restored (Fig. 1, Lane 3). When the ME1a2 site was mutated and tested as above, the P_1:P_2 ratio shifted to 1:2 (Fig. 1, Lane 2) indicating that the ME1a2 site contributes to P_2 usage but is not as critical as ME1a1 in these fibroblastic cell lines.

Sequence-Specific Binding of cDNA Clones to the ME1a1 and ME1a2 Sites

Previous studies have shown that the ME1a1 and ME1a2 sites bound nuclear factors from both human and mouse cell lines (Asselin et al. 1989). In light of the above data demonstrating that the ME1a1 and to a lesser extent the ME1a2 sites are involved in P_2

usage, we sought to obtain cDNA clones encoding polypeptides which bound specifically to each site. A HeLa cell λgt11 expression library was screened using a DNA probe (Vinson et al. 1988) consisting of tandem repeats of either the ME1a1 or ME1a2 sequence. One million bacteriophage plaques were screened with each probe (Me1a1 and ME1a2) and a single positive clone was obtained for each probe (#19-2-3-1 and 15-3-1, respectively). To determine their individual binding specificity, a series of wild type and mutated probes were used in a filter binding assay for each clone. Table 1 summarizes the results. Clone #19-2-3-1, obtained by screening with the ME1a1 probe, bound to the wild type ME1a1 probe, as expected, and failed to bind a mutated ME1a1 probe which contained the same mutation as that in Fig. 1. In addition, it was able to bind other mutated sequences (Table 1, M-1 to M-4). It failed to bind to the ME1a2 probes (both wild type and mutant) and to a high affinity SP_1 probe. These data, combined with methylation interference analysis (data not shown) revealed an eight bp sequence (GGGAGGGG) as clone #19-2-3-1's binding site. Clone #15-3-1, obtained with the ME1a2 probe, bound in addition to the wild type ME1a2 probe, the ME1a1 wild type and mutant probes. It failed to bind the ME1a2 mutant (containing the same mutation as in Figure 1), the SP_1 probe and M-1 to M-3. It did exhibit partial binding to M-4. These data indicate that the core binding sequence for 15-3-1 is GAAGGG, a sequence present in the ME1a1 and ME1a2 probes. Therefore, the ME1a1 binding site is able to bind two distinct polypeptide factors encoded by clones 19-2-3-1 and 15-3-1. It has yet to be determined whether the factors can bind simultaneously or if the binding of one factor precludes binding of the other.

Table 1. Binding specificity of clone #19-2-3-1 and #15-3-1

		Binding ability	
Probe sequence		19-2-3-1	15-3-1
GAAAAAGAAGGGAGGGGAGGGATC	(wt ME1a1)	+	+
GAAAAAGAAGGGAATTCAGGGATC	(ME1A1 MUT)	-	+
GAAAAACTTGGGAGGGGAGGGATC	(M-1)	+	-
GAAAATCTAGGGAGGGGAGGGATC	(M-2)	+	-
GAAATTCAAGGGAGGGGAGGGATC	(M-3)	+	-
GAATTTGAAGGGAGGGGAGGGATC	(M-4)	+	+/-
TCCTGCCTCCTGAAGGGCAGCGTTCG	(wt ME1a2)	-	+
TCCTGCCTCCTGAGATCCAGCGTTCG	(ME1A2 MUT)	ND	-
TCGACGGGGCGGGGCTTACTGC	(Sp1)	-	-

In each case the mutated sequence is underlined
+, binds; -, fails to bind; +/-, partial binding; ND, not determined; wt, wild type; MUT, mutant.

Clone 19-2-3-1 Encodes a Zinc Finger Protein

The 19-2-3-1 cDNA insert was subcloned into pBluescript II KS- (Stratagene) and its nucleic acid sequence was determined. A λZap II HeLa library (Stratagene) was screened with the 19-2-3-1 cDNA as a probe to obtain a larger clone of 2,422 bp. An open reading frame of 477 amino acids was identified which potentially encoded a polypeptide with a predicted M_r of 52.5 kDa. *In vitro* transcription and translation of the full length clone revealed a polypeptide product with an apparent M_r of approximately 58.5 kDa which generated a complex with an ME1a1 probe in a gel mobility shift assay (data not shown).

Upon analysis of the amino acid sequence, six potential zinc fingers were found of the C_2-H_2 type (Fig. 2). Zinc finger containing proteins have been shown to bind DNA in a sequence-specific manner (Miller et al. 1985) with examples found among transcription factors(Kadonaga et al. 1987; Sukhatme et al. 1988), developmental control genes(Rosenberg et al. 1986) and in at least one gene associated with neoplastic disease (Morris et al. 1991). The implication is that all are involved in gene regulation. A proline-rich region resides at the amino terminal portion of the polyeptide consisting of 22% proline in the first 150 amino acids (Fig.2). Proline-rich regions can function as transcriptional activation domains as exemplified by AP_2 (Williams et al. 1988) and CCAAT transcription factor (CTF) (Mermod et al. 1989). Another striking feature of the primary amino acid sequence is the presence of several polyalanine tracts (Fig. 2). These domains are thought to form α-helices

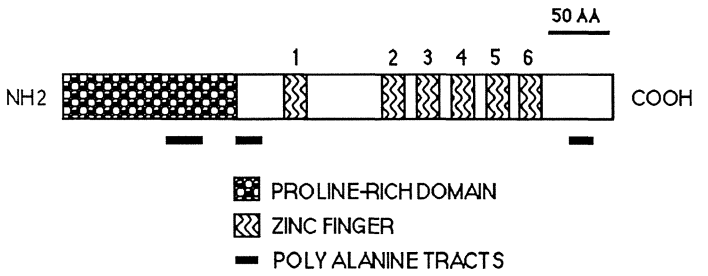


Fig. 2. Schematic showing the organization of the protein product of MAZ. Proline-rich region, zinc fingers and polyalanine tracts are as indicated.

and have been found in a number of other proteins involved in regulating drosophila development including runt (Kania et al. 1990), engrailed (McDonald et al. 1986) and even-skipped (Poole et al. 1985). Related sequences are found at the amino terminus of the kruppel gene product which line up with polyalanine tracts of even-skipped and engrailed (Licht et al. 1990). This region of kruppel was shown to facilitate the repression of a reporter gene with multiple kruppel binding sites in mammalian cells (Licht et al. 1990), while the polyalanine tract and adjacent sequences functioned at low concentration as a transcriptional activator in Drosophila cells (Sauer and Jackle, 1991). The fact that a single polypeptide domain could have two opposing functions indifferent systems indicates that it may be interacting with other species-specific or cell-type specific factors to either repress or activate transcription.

Searches of the GenBank and EMBL databases revealed this to be a novel sequence which we designate MAZ for myc-associated-zinc finger protein.

MAZ is Differentially Expressed in Human Tissues

Various human tissues were examined for MAZ expression by Northern blot as shown in Figure 3. MAZ was predominantly expressed as a broad band of approximately 2.4 - 3.0 kb (Fig. 3A), which was resolved into two species of 2.5 and 2.7 kb in a shorter exposure (Fig. 3B). The 2.5 and 2.7 kb transcripts were present at comparable levels in all tissues tested except for kidney where it was nearly absent (Fig. 3A). Prolonged exposure revealed three minor species, one of 1.9 kb expressed mainly in brain, placenta and lung, and to a lesser extent in heart, a second of 2.1 kb in pancreas and a third of approximately 500 bp in brain and pancreas (Fig. 3A). We have obtained a second cDNA

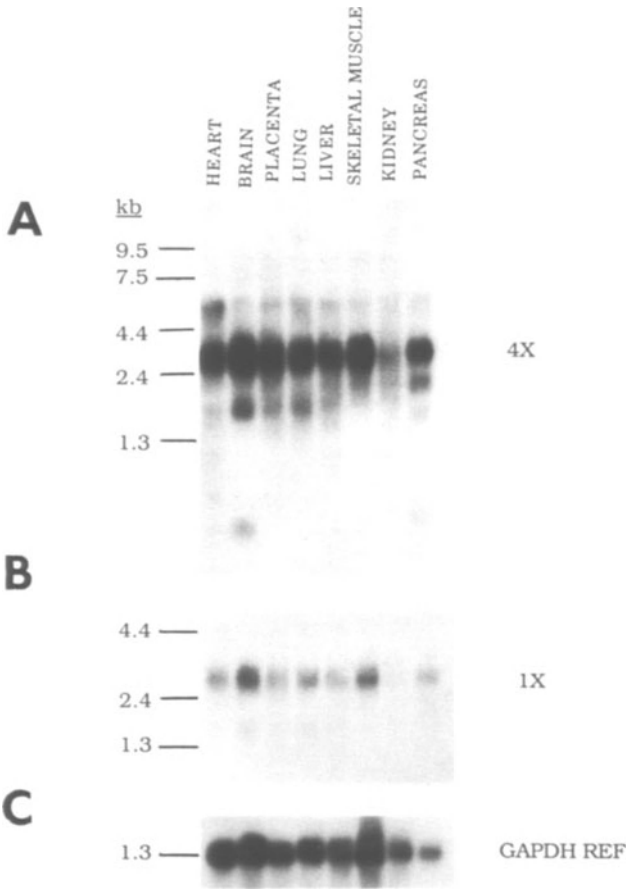


Fig. 3. Northern analysis of MAZ with various human tissue mRNAs. A) Northern blot showing tissue distribution of MAZ mRNA, 16 hour exposure; B) Same as A with a four hour exposure; C) Control using GAPDH probe.

of MAZ which caries an insertion which alters the coding region and removes one of the polyalanine tracts (unpublished observation). The function of this alternative form of MAZ as well as the significance of multiple transcripts in different tissues remains to be elucidated. It is interesting to note that murine c-MYC is expressed at a very low level in kidney (Asselin and Marcu, 1989), and that during kidney regeneration following tissue injury c-MYC message is dramatically induced as is the block to elongation. Studies are currently underway to determine if MAZ is induced concomitantly with c-MYC following kidney injury which may indicate that MAZ plays a role in c-MYC initiation and/or termination.

MAZ-Maltose Binding Protein Chimera Binds to Two Sites in the C-MYC Gene and to the Terminator of the C2 Gene

Recent reports have identified sequences involved in transcriptional termination which bear homology to the ME1a1 site (Wright et al. 1991; Ashfield et al. 1991). The region which contains the ME1a1 site was found to participate in premature termination of MYC P_1 initiated transcripts (Wright et al. 1991). A second ME1a1 like sequence, located toward the end of the C-MYC first exon, plays a role in the premature termination of P_2 initiated transcripts (C-MYC att, Fig. 4B) (Wright et al. 1991). A third sequence with homology to ME1a1 was found in the C2 gene's terminator region and similar, if not identical, proteins were found to be present in HeLa cell nuclear extracts which bound to the C2 termination and ME1a1 sites (Ashfield et al. 1991). To further characterize the DNA binding properties of MAZ, the original cDNA clone obtained from λgt11 was subcloned into the pMAL-c fusion protein vector (pMAZ-MBP). This fuses MAZ with the maltose binding protein and places its expression under the control of an IPTG inducible promoter. MAZ-MBP fusion protein was isolated from bacterial lysates by heparin-sepharose chromatography and the binding of MAZ-MBP fusion protein to the above sites was tested by employing the GMSA. Synthetic oligonucleotides corresponding to the ME1a1, C2 and C-MYC att sites were endlabeled and incubated with MAZ-MBP. A specific co-migrating band appeared with all three probes which disappeared upon the addition of a 100-fold molar excess of a specific oligonucleotide competitor, but was unaffected by the presence of a 100-fold molar excess of an unrelated competitor (Fig. 4A). Each of the MAZ-MBP-DNA complexes were more retarded than the native complex obtained with the HeLa crude nuclear extract (Fig. 4A). The maltose binding protein contributes 42 kDa to the recombinant MAZ-MBP fusion protein. Therefore, the migration of the MAZ-MBP DNA complex could be more retarded than the DNA-protein complex obtained with crude nuclear extract. Furthermore, since the fusion protein was obtained using a prokaryotic system, postranslational modifications which could alter the mobility of the shifted complex would not be present. No binding was detected with the maltose binding protein alone (data not shown). The three binding sites contain an identical GGGAGGG minimal core motif (Fig. 4B) which agrees with the methylation interference pattern obtained with MAZ-MBP (data not shown). The differences in the intensities of the bound complexes could reflect the contributions of different flanking sequences.

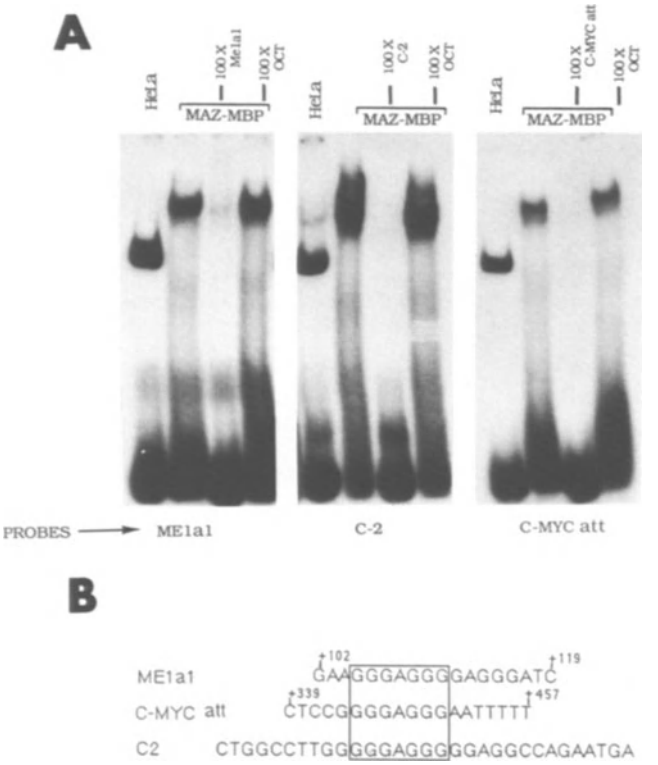


Fig. 4 Gel mobility shift assay of MAZ-MBP with various oligonucleotide probes. A) Each probe was incubated with either 5 µg HeLa nuclear extract; or MAZ-MBP in the presence or absence of 100-fold molar excess of homologous competitor DNA or an unrelated competitor (100 X OCT). B) Sequence of probes used with consensus binding site boxed. Numbers are relative to P_1.

Significance of MAZ Binding Properties

In vivo transfections indicated that the ME1a1 site participates in transcription initiation, but surprisingly it may also have a dual role in transcription termination considering that MAZ-MBP binds not only to ME1a1 but to a site in the c-MYC P_2 blockage region and to the C2 gene's termination region. The poly(A) addition site of C2 is only 421 bp 5' of the cap site of the Factor B gene (Ashfield et al. 1991). C2 transcripts are terminated within 400 bp of this poly(A) signal and this termination is dependent upon a sequence which has homology to the ME1a1 site (Fig. 4B)(Ashfield et al. 1991). The dual promoter of c-MYC may be analogous to two independently regulated, closely linked cellular genes, such as C2 and Factor B. The ME1a1 site could facilitate initiation from the downstream start site (P_2 and Factor B) while effectively blocking readthrough from an upstream start site (P_1 and C2). It remains to be determined if the ME1a1-like sequences within the C2 terminator region positively regulates Factor B expression.

In the P_2 attenuator region an ME1a1-like sequence seems to be playing a single role as a termination factor contrasted with its dual contributions to initiation and termination when it lies between P_1 and P_2. Close inspection of our RNase protection experiment (Fig. 1) reveals that P_1 initiated transcripts are slightly elevated upon mutation of the ME1a1 site, while P_2 is virtually abrogated. This observation suggests that the ME1a1 mutation might have increased P_1 activity by relieving its transcriptional block, thereby allowing for enhanced polymerase readthrough. Different sequences flanking the MAZ binding site may help to dictate MAZ properties by facilitating its interactions with different accessory factors. Indeed, the clone #15-3-1 which binds to the ME1a1 and ME1a2 oligos may in fact interact with MAZ and may serve to modify the biological activity of MAZ. The binding of MAZ may also cause a perturbation in the local region of DNA possibly generating a bend which could bring other cis-acting elements into position to exert a regulatory effect on transcription.

The involvement of a single DNA target sequence in the regulation of transcription initiation and termination is not without precedent. McStay and Reeder (1990) have shown that in two RNA pol I ribosomal genes a terminator of one gene is present 60 bp upstream of the promoter of a second gene. Deletion of this terminator not only abrogated termination but also negatively affected initiation. It was shown that the binding of the terminator not only protects the initiation complex from readthrough by the upstream gene's transcriptional machinery, but this site also directly interacted with the promoter region. This situation is analogous to the c-MYC and possibly the C2-Factor B scenarios. ME1a1-like sequences may bind more than one protein to accomplish dual functions in transcription and MAZ may represent one of these factors. It remains to be tested directly whether MAZ has the properties of a transcription factor which can either effect termination and/or initiation.

Acknowledgements

We wish to thank the members of the Marcu laboratory for their invaluable suggestions; Ms. Margo Reyes for assistance with artwork and in typing this manuscript; Ms.Rebecca Ashfield and Dr. Nick Proudfoot for helpful discussions concerning C2 termination and for supplying information prior to publication. C.A. was supported by a grant from the Medical Research Council of Canada. This work was supported by National Institutes of Health grand CA36246 awarded to K.B.M.

References

Ashfield R, Enriquez-Harris P, Proudfoot NJ (1991) Transcriptional termination between the closely linked human complement genes C2 and Factor B: Common termination factor for C2 and c-myc. EMBO j 10:4197-4207

Asselin C, Marcu KB (1989) Mode of c-myc gene regulation in folic acid-induced kidney regeneration. Oncogene Res 5:67-72
Asselin C, Nepveu A, Marcu, KB (1989) Molecular requirements for transcriptional initiation of the murine c-myc gene. Oncogene 4:549-558
Kadonaga JT, Carner KR, Masiarz FR, Tjian R (1987) Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51:1079-1090
Kania MA, Bonner AS, Duffy JB, Gergen JP (1990) The drosophila segmentation gene runt encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes & Dev 4:1701-1713
Licht JD, Grossel MJ, Figge J, Hansen UM (1990) Drosophila kruppel protein is a transcriptional repressor. Nature 346:76-79
Macdonald PM, Ingham P, Struhl G (1986) Isolation, structure, and expression of even-skipped: A second pair-rule gene of drosophila containing a homeo box. Cell 47:721-734
Marcu KB, Bossone SA, Patel AJ (1992) Myc function and regulation. Ann Rev Biochem, in press
McStay B, Reeder RH (1990) A DNA-binding protein is required for termination of transcription by RNA polymerase I in Xenopus laevis. Mol Cell Biol 10:2793-2800
Mermod N, O'Neill EA, Kelly TJ, Tjian R (1989) The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. Cell 58:741-753
Miller H, Asselin C, Dufort D, Yang J-Q, Gupta K, Marcu KB, Nepveu A (1989) A cis-acting element in the promoter region of the murine c-myc gene is necessary for transcription block. 9:5340-5349
Miller J, McLachlan AD, Klug A (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J 4:1609-1614
Moberg KH, Logan TJ, Tyndall LW, Hall DJ (1992) Three distinct elements within the murine c-myc promoter are required for transcription. Oncogene 7:411-421
Morris JF, Madden SL, Tournay OE, Cook DM, Sukhatme VP, Rauscher III FJ (1991) Characterization of the zinc finger protein encoded by the WT1 Wilm's tumor locus. Oncogene 6:2339-2348
Poole SJ, Kauvar LM, Drees B, Kornberg T (1985) The engrailed locus of drosophila: structural analysis of an embryonic transcript. Cell 40:37-43
Rosenberg UB, Schröder C, Preiss A, Kienlin A, Côté S, Riede I, Jäckle H (1986) Structural homology of the product of the drosophila kruppel gene with Xenopus transcription factor IIIA. Nature 319:336-339
Sauer F, Jäckle H (1991) Concentration-dependent transcriptional activation or repression by kruppel from a single binding site. Nature 353:563-566
Sukhatme VP, Cao X, Chang LC, Tsai-Morris C-H, Stamenkovich D, Ferreira PC, Cohen DR, Edwards SA, Shows TB, Curran T, Le Beau MM, Adamson ED (1988) A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell 53:37-43
Vinson CR, LaMarco KL, Johnson PF, Landschulz WH, McKnight SL (1988) In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes & Dev 2:801-806
Williams T, Admon A, Lüscher B, Tjian R (1988) Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. Genes & Dev 2:1557-1569
Wright S, Mirels LF, Calayag CB, Bishop JM (1991) Premature termination of transcription from the P1 promoter of the mouse c-myc gene. Proc Natl Acad Sci USA 88:11383-11387
Yang J-Q, Remmers E, Marcu KB (1986) The first exon of the c-myc proto-oncogene contains a novel positive control element. EMBO J 5:3553-3562

Transcriptional Activities of the Myc and Max Proteins in Mammalian Cells

L. KRETZNER[1], E. M. BLACKWOOD[1,2], and R. N. EISENMAN[1]

[1]Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle WA 98104, USA;
[2]Department of Pathology, University of Washington School of Medicine, Seattle WA 98195.

The *myc* family of oncogenes exhibit deregulated expression in a host of neoplasias. Though the molecular function of the Myc protein in both normal and tumorigenic cells has remained uncertain, it has been postulated to play a role in gene transcription on the basis of amino acid homologies with known transcription factors such as MyoD (Lüscher & Eisenman, 1990). We report here the direct testing of full-length Myc and its dimerization partner, Max, on the transcriptional activity of reporter genes bearing Myc/Max binding sites. Such reporter constructs display an endogenous level of activity in transient transfections which is dependant on the presence of the CACGTG sequence. Exogenous expression of *myc* results in modest activation of reporter gene transcription. Similar overexpression of *max* results in a repression of reporter gene activity, an effect which is reversed by co-expression with c-*myc*. Max repression is dependant on an intact DNA binding region, while Myc activation depends on both the N-terminal activation and the C-terminal dimerization domains. These results suggest a model in which Max homodimers can act as as repressors, and Myc-Max heterodimers as activators, of potential target genes.

INTRODUCTION

The *myc* oncogene was first discovered as the transforming gene (v-*myc*) of a group of avian acute leukemia retroviruses (Bister and Jansen, 1986, and references therein). Subsequently, the cellular homolog c-*myc* and other members of the *myc* family, particularly L- and N-myc (Alt et al, 1986), were identified and also found to be perturbed in a variety of neoplasias. For example, retroviral insertions near c- and N-*myc* have been associated with avian and mammalian B- and T-cell lymphomas (Bister and

Current Topics in Microbiology and Immunology, Vol. 182

Jansen, 1986; van Lohuizen et al, 1989), and chromosomal translocations involving c-*myc* and immunoglobulin loci have been found in human Burkitt's lymphomas and murine plasmacytomas (Magrath, 1990). These abnormalities, as well as amplification of c-, L-, or N-*myc* genes found in some tumors (Alitalo and Schwab, 1985), all result in deregulated *myc* expression. Consequently, Myc protein levels are generally higher in tumorigenic cells, whereas normal *myc* expression is tightly regulated at virtually every level of control of gene expression: transcriptional initiation and elongation, message turnover, translation initiation, and protein stability (Spencer and Groudine, 1990). Furthermore, overexpression of Myc protein is known to lead to changes in cell growth rates, heightened sensitivity to growth factors, inhibition of differentiation, and the ability to cooperate with other activated oncogenes in cotransformation assays (Weinberg, 1989; Lüscher & Eisenman, 1990).

While this body of research clearly implicates *myc* overexpression in deregulated cell growth, the molecular function of Myc proteins in the cell has remained unclear. This impasse in understanding Myc's function was broken with the recent demonstrations of sequence-specific DNA binding by the Myc protein (Blackwell et al, 1990; Fisher et al, 1991; Halazonetis and Kandil, 1991; Kerkhoff et al, 1991; Prendergast and Ziff, 1991), and by the discovery of a Myc dimerization partner, Max (Blackwood and Eisenman, 1991; Prendergast et al, 1991). Max proteins, p21 and p22, apparent products of alternative splicing, can homodimerize or heterodimerize specifically with Myc family proteins, and Myc-Max heterodimers have enhanced specific DNA binding relative to homodimers (Blackwood and Eisenman, 1991). Myc and Max associate both *in vitro* and *in vivo*, and unlike Myc, Max proteins are very stable in cells (Blackwood et al, 1992). Myc and Max are both members of the basic-helix-loop-helix-zipper (bHLHzip) class of proteins (Landschulz et al., 1988; Murre et al., 1989), and have been postulated to modulate transcriptional activity because of their homology with other bHLHzip transcription factors. Furthermore, studies with chimeric proteins have shown that the amino terminus of c-Myc can act as a transcriptional activator when fused to a heterologous DNA binding domain (Kato et al, 1990). The discoveries of Max and a Myc/Max DNA binding sequence present the opportunity to show directly that Myc and Max possess distinct transcriptional activities.

RESULTS

Since cellular targets of Myc or Max regulation remain speculative at this time, we constructed reporter plasmids pM4TKCAT and pM4MinCAT (Fig. 1) bearing four Myc/Max binding sites (M4). Reporter plasmids were cotransfected with *myc* and/or *max* expression vectors (Fig. 1), as well as with an autonomous β-galactosidase vector to assess and correct for varying transfection efficiencies. This system produced negligible CAT activity in the absence of the M4 sequence (not shown).

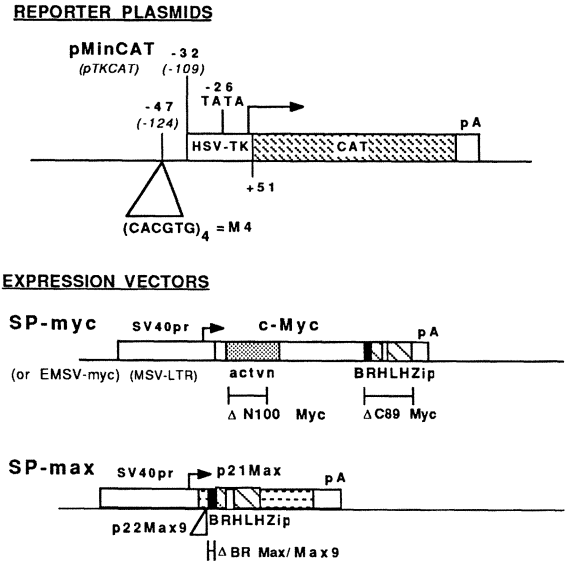


Fig. 1 Summary of reporter and expression constructs. The pMinCAT reporter gene (pGLCAT4 in Nyborg et al, 1990) fuses chloramphenicol acetyltransferase (CAT) coding sequences with the TATA box and transcription start site of the HSV-TK promoter, nucleotides (nts) -32 to +51. The pTKCAT reporter is identical except it contains 77 additional nts of the HSV-TK promoter, from -109 to +51. Both reporters contain a four-fold repeat (M4) of the Myc/Max binding site, CACGTG, inserted 15 nts 5' of the minimal promoter, and both gave similar results. The M4 sequence was synthesized by T. K. Blackwell as a pair of complementary oligonucleotides. The complete sequence of one repeating unit is: 5'CCCACCACGTGGTGCCT3'.

Expression vectors were derived from either pEMSV (expression driven by the Murine Sarcoma Virus LTR; Harland and Weintraub, 1985) or pSP271 (expression driven by the SV40 origin promoter; a gift of M. Emmerman). The human c-*myc* cDNA is the 0/1 clone of Watt et al (1983). ΔN100-Myc lacks the first 100 amino acids of Myc, while ΔC89-Myc lacks the 89 C-terminal amino acids. The *max* gene encodes the protein p21, while the *max9* gene contains a 27 base pair insert and encodes the protein p22. The ΔBRmax mutants lack amino acids 24-36 of Max and the corresponding residues of Max9. Key structural features encoded by each gene are indicated: actvn = apparent activation domain of Myc; BR= basic region; HLHZip = helix-loop-helix-zipper domain; pA = polyadenylation signal.

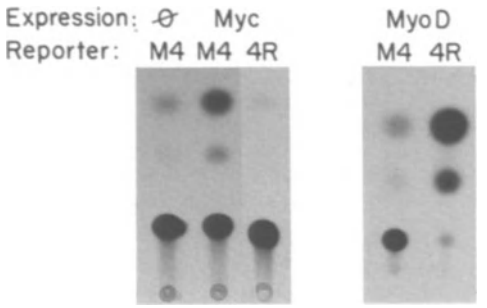


Fig. 2 Sequence-specific transcriptional activation by c-Myc. CAT assay results from CV-1 fibroblasts transfected with 10 μg of pEMSV alone, pEMSV-Myc, or pEMSV-MyoD, and 3 μg pTKCAT reporter constructs bearing the indicated binding sequences (M4 = 4x Myc/Max sequence; 4R = 4x MyoD sequence; see text). Cells were transfected by the $CaPO_4$ method and harvested ~36 hours after transfection. CAT assays were performed according to Gorman et al (1982).

Figure 2 demonstrates the effect of exogenous *myc* expression on the pM4TKCAT reporter, as well as the sequence-specificity of this response. The empty expression vector lane shows there is an endogenous level of reporter activity in these cells when the M4 sequence is present. This is expected since these cells contain Myc, Max and probably other CACGTG-binding factors (Blackwood et al, 1992; Beckmann et al, 1990; Gregor et al, 1990). Myc overexpression results in an approximately five-fold activation of the pM4TKCAT reporter in this experiment. The effect of *myc* expression on M4-containing reporters is somewhat variable (Table 1), but reproducible in several cell types and also concentration dependent (not shown). Figure 1 also shows that Myc fails to activate a reporter gene fused to a four-fold repeat of a closely related binding sequence (4R), that targeted by the myogenic differentiation factor, MyoD (CACGTG for Myc versus CACCTG for MyoD). MyoD likewise fails to activate the Myc target sequence, while stimulating its cognate reporter considerably (Weintraub et al., 1990). Although c-Myc stimulation of transcription is modest, it is comparable to *in vivo* activation with Fos and Jun (Chiu et al, 1988; Boyle et al, 1991).

The repressive effect of *max* overexpression is shown in Figure 3. In this experiment, using the pM4minCAT reporter, there is again an appreciable level of endogenous reporter activity, which was set to 1x (see Fig. 3 legend). Introduction of *max* or *max9* expression vectors depresses this basal level of

transcription by four and ten fold, respectively (Fig. 3). These values are typical of multiple experiments (Table 1), and we note in particular that Max9 (p22) reproducibly represses transcription to a greater extent than Max (p21). The effect of c-*myc* cotransfection with *max* or *max9* is a striking elevation of reporter gene activity, typically more pronounced with Myc+Max than with Myc+Max9 (Fig. 3). Max and Max9 repression is also titratable, with clear suppressive effects seen even at submicrogram amounts of either *max* expression plasmid added (not shown).

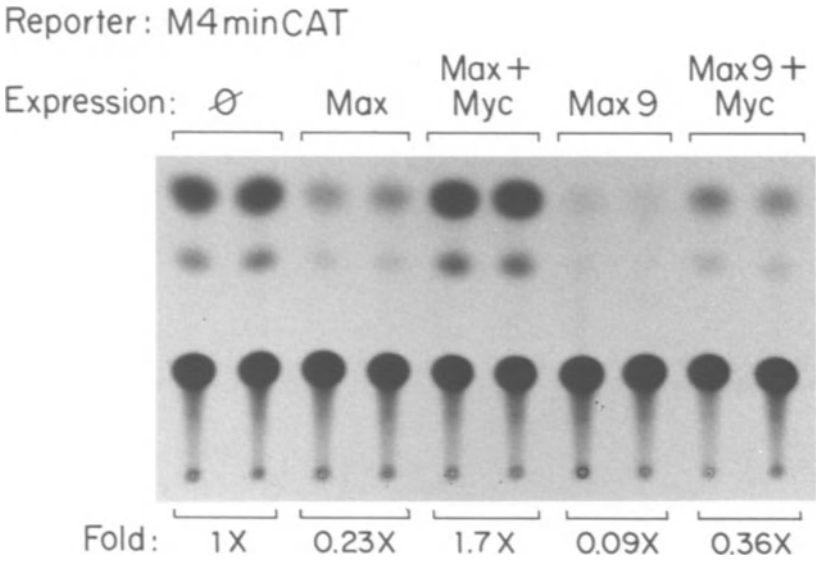


Fig. 3 Max overexpression represses transcriptional activity while coexpression of Myc reverses this effect. Shown are CAT assay results from duplicate plates of NIH3T3 cells transfected with pSPMax/Max9 -/+ pSPMyc and the pM4MinCAT reporter. Endogenous reporter activity is shown first (empty vector), with *max* -/+ *myc* additions following as indicated. All plates received 3 μg of the reporter plasmid, 10 μg of each cDNA, and the same total amount of expression vector DNA (20 μg). Methods were identical to those in Figure 2. Fold inductions given beneath data are based on the % of chloramphenicol acetylated (c.p.m.s determined directly from TLC plates). The amount of CAT activity in the absence of exogenous Myc or Max is set to 1x.

Table I. Averaged responses of pM4minCAT in six experiments.*

Gene(s)	Fold Induction	Standard Deviation
0	1x	----
myc	3.35x	1.5
max	0.37x	0.2
max + myc	1.5x	0.8
max9	0.14x	0.07
max9 + myc	0.46x	0.2

* Each performed in duplicate; i.e., N = 12.

Mutant forms of Max were also constructed which lacked the basic region required for DNA binding (Fig. 1). These ΔBRMax mutants are stable in cells but fail to bind DNA in gel-shift assays and fail to repress the M4 reporter genes (not shown), arguing that Max and Max9 do not exert repressive effects simply though non-specific squelching behavior (Ptashne, 1988). Similarly, c-Myc mutants lacking either the proposed N-terminal activation domain or the C-terminal dimerization motifs were constructed (Fig. 1). Both N-terminal and C-terminal c-Myc deletions fail to activate transcription or relieve Max repression (not shown), showing that the activation domain and dimerization capability of c-Myc are required for its proper function.

DISCUSSION

We believe our data are consistent with a model in which Myc-Max heterodimers act as transcriptional activators, while Max homodimers act as transcriptional repressors (Figure 4). The endogenous levels of activity from the M4 reporter genes seen in our experiments presumably reflect the net effects of all repressive and stimulatory transacting factors in these cells. This includes Myc, Max and other CACGTG-binding factors, (eg., Gregor et al, 1990; Beckmann et al, 1990) Evidence suggests that intracellular Myc homodimerizes poorly if at all (Dang et al, 1991), though short C-terminal forms of Myc can both dimerize and bind DNA (Blackwell et al, 1990). While Max readily homodimerizes and binds DNA *in vitro*, both of these behaviors appear to be energetically favored with Myc-Max heterodimers. Therefore we assume that both endogenous as well as exogenously expressed Myc must act by forming transcriptionally active Myc-Max dimers. On the other hand, overexpression of p21 or p22 in our experiments would push the association equilibrium toward Max homodimers, and transcriptional repression is seen.

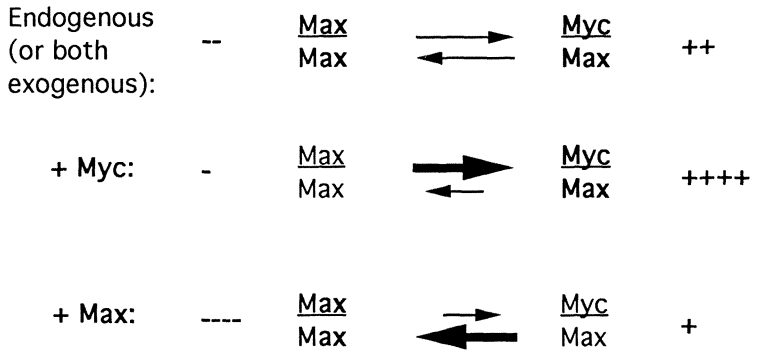


Fig. 4 Myc/Max heterodimers activate transcription of target genes while Max homodimers repress it. The relative balance of activation versus repression of reporter genes is a reflection of the equilibrium between Myc-Max and Max-Max dimers, respectively. Transient increases in Myc levels, such as occur in the G_0 to G_1 transition of the cell cycle, would result in increased formation of Myc-Max heterodimers, relieving Max repression and stimulating transcription of target genes.

Whether Max overexpression ever occurs naturally in the course of cell growth or differentiation is not known, but in fact *max* appears to be expressed at constant levels in excess of Myc in the cells studied thus far, including those used here (Blackwood et al, 1992; and unpublished data). It is therefore significant that transient Myc overexpression both stimulates reporter gene transcription when transfected alone (Figure 2), and relieves the repressive effects of exogenous Max (Figure 3). Intracellular Max/Max9 in excess of Myc would appear to form transcriptionally non-active dimers, and transient overexpression of Myc - either experimentally, as here, or conceivably as seen in many tumorigenic cells - is able to reverse this repressive effect.

It is not clear yet whether Max acts as a bona fide repressor of transcription or if it is merely transcriptionally inactive and exerts a repressive effect by competing with activating Myc-Max heterodimers (and/or other factors) for binding sites. It is also conceivable that Max forms repressive dimers with cellular factors other than itself. In any case, previous data show that *max* products are constitutively expressed and very stable (Blackwood et al, 1992), in contrast to *myc*, whose encoded mRNA and protein products have extraordinarily short half-lives (Lüscher and Eisenman, 1990; Spencer and Groudine, 1990). The model therefore presents a plausible mechanism by which cells could parsimoniously regulate transcription of Myc/Max target genes. Such genes would be kept constitutively silent by Max

homodimers until tightly regulated changes in Myc levels drive the formation of transcriptionally active Myc-Max heterodimers. Such active heterodimers are likely to be transient in nature, as association of Myc with Max does not appear to lengthen the half-life of Myc (Blackwood et al, 1992). The actual target genes for Myc and Max, as well as the full range of stimuli which may influence their association and subsequent activities, are the subject of active experimentation.

REFERENCES

Alitalo K, Schwab M (1985) Oncogene amplification in tumor cells. A review. Adv. Cancer Res. 47:235

Alt FW, DePinho RA, Zimmerman K, Legouy E, Hatton K, Ferrier P, Tesfaye A, Yancopoulos GD, Nisen P (1986) The human *myc*-gene family. Cold Spring Harbor Symp. Quant. Biol. 51:931

Beckmann H, Sou L-K, Kadesch T (1990) TFE3: A helix-loop-helix protein that activates transcription through the immunoglobin enhancer μE3 motif. Genes Dev. 4:167

Bister K, Jansen HW (1986) Oncogenes in retroviruses and cells: Biochemistry and molecular genetics. Adv. Cancer Res. 47:99

Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN, Weintraub H (1990) Sequence-specific DNA-binding by the c-Myc protein. Science 250:1149

Blackwood EM, Eisenman RN (1991) Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251:1211

Blackwood EM, Lüscher B, Eisenman RN (1992) Myc and Max associate in vivo. Genes Dev. 6:71

Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, and Hunter T (1991) Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA-binding activity. Cell 64: 573

Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T, and Karin M (1988) The c-fos protein interacts with c-jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54: 541

Dang CV, Barrett J, Villa-Garcia M, Resar LMS, Kato GJ, Fearon ER (1991) Intracellular leucine zipper interactions suggest c-Myc hetero-oligomerization. Mol. Cell. Biol. 11:954

Fisher F, Jayaraman P-S, Goding CR(1991) C-myc and the yeast transcription factor PHO4 share a common CACGTG-binding motif. Oncogene 6:1099

Gorman CM, Moffat LF, Howard BE(1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044

Gregor PD, Sawadogo M, Roeder RG (1990) The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev. 4:1730

Halazonetis TD, Kandil AN(1991) Determination of the c-myc DNA-binding site. Proc. Natl. Acad. Sci. 88:6162

Harland R, Weintraub H (1985) Translation of mRNA injected into Xenopus oocytes is specifically inhibited by antisense RNA. J. Cell Biol. 101:1094

Kato GJ, Barrett J, Villa-Garcia M, Dang CV (1990) An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. Mol. Cell. Biol. 10:5914

Kerkhoff E, Bister K, Klempnauer K-H (1991) Sequence-specific DNA binding by Myc proteins. Proc. Natl. Acad. Sci. 88:4323

Landschulz WH, Johnson PF, McKnight SL (1988) The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 240:1759

Lüscher B, Eisenman RN (1990) New light on *myc* and *myb*. Genes Dev. 4:2025

Magrath I (1990) The pathogenesis of Burkitt's lymphoma. Adv. Cancer Res. 55:134

Murre C, McCaw PS, Baltimore D (1989) A new DNA binding and dimerization motif in immunoglobin enhancer binding, *daughterless, MyoD* and *myc* proteins. Cell 56:777

Nyborg JK, Matthews MAH, Yucel J, Wells L, Golde WT, Dynan WS, Wachsman W (1990) Interaction of host cell proteins with the human T-cell leukemia virus type I transcriptional control region. J. Biol. Chem. 265: 8237

Prendergast GC, Lawe D, Ziff EB (1991) Association of Myn, the murine homolog of Max, with c-Myc stimulates mythylation-sensitive DNA binding and Ras cotransformation. Cell 65:395

Prendergast GC, Ziff EB (1991) Methylation-sensitive sequence-specific DNA binding by the c-myc basic region. Science 251:186

Ptashne M (1988) How transcriptional activators work. Nature 335:683

Spencer CA, Groudine M (1990) Control of *c-myc* regulation in normal and neoplastic cells. Adv. Cancer Res. 56:1

van Lohuizen M, Breuer M, Berns A (1989a) N-myc is frequently activated by proviral insertion in MuLV-induced T cell lymphomas. EMBO J 8:133

Watt R, Stanton LW, Marcu KB, Gallo RC, Croce, CM, and Rovera G (1983) Nucleotide sequence of cloned cDNA of human c-*myc* oncogene. Nature 303:727

Weinberg RA (ed) (1989) Oncogenes and multistep carcinogenesis. In Oncogenes and the molecular origins of cancer p. 307 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Weintraub H, Davis R, Lockshon D, Lassar A (1990) MyoD binds cooperatively to two sites in a target enhancer sequence: Occupancy of two sites is required for activation. Proc. Natl. Acad. Sci. USA 87:5623

c-*myc* Transactivates the Ornithine Decarboxylase Gene

C. Bello-Fernandez[1] and J. L. Cleveland[1]

[1]Department of Biochemistry, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105

Introduction

Several observations suggest that the c-*myc* proto-oncogene is a key regulator of cell growth and differentiation. First, expression of c-*myc* RNA and protein is tightly regulated by mitogens and are suppressed by growth inhibitory agents (Luscher and Eisenman, 1991). Second, removal of c-*myc* protein appears required for withdrawal from the cell cycle since enforced c-*myc* expression promotes cell cycle progression (Askew et al., 1991; Eilers et al., 1991; Evan et al., 1992) and inhibits terminal differentiation (Prochownik and Kukowska, 1986; Coppolla and Cole, 1986). Finally, inhibiton of c-*myc* expression by antisense oligonucleotides or RNA prevents entry of cells into S phase and accelerates differentiation (Heikkela et al, 1987; Prowchownik et al., 1988). The precise function that c-*myc* provides to promote cell cycle progression is unknown. *Myc* family proteins have domains characteristic of known transcription factors, including a basic-helix-loop-helix-(B-HLH) motif and a leucine zipper (LZ) domain, and have therefore been proposed to function as transcription factors (Luscher and Eisenman, 1991). Furthermore, it has recently been demonstrated that c-*myc* functions as a sequence-specific DNA binding protein (Blackwell et al., 1990; Prendergast and Ziff, 1991; Halazonetis and Kandil, 1991) and through dimerization with a novel B-HLH-LZ partner, max, *myc* binds with high affinity to the "E-box" motif CACGTG (Blackwood and Eisenman, 1991). In addition, regions of the amino terminus of c-*myc* have been shown to function as transactivation domains when fused to the DNA binding domain of the transcription factor GAL4 (Kato et al., 1990).

The identification of gene targets which are directly regulated by c-*myc* have remained elusive. We have reasoned that potential gene targets for c-*myc* which are associated with it's ability to promote cell cycling are themselves likely required for progression into S phase. One of the enzymes necessary for G1 progression into S phase is ornithine decarboxylase (ODC), which is the rate-limiting enzyme of polyamine biosynthesis. Specific inhibitors of ODC enzyme activity or depletion of polyamines (Bowlin et al., 1986) arrests cells in G1. ODC expression is also tightly regulated by growth factors and is induced as an early class gene following growth factor stimulation (Askew et al., 1991). We have previously demonstrated that constitutive c-*myc* expression in interleukin-3 (IL-3)-dependent myeloid cell lines relieves the growth factor dependent expression of ODC RNA (Askew et al., 1991; Dean, et al., 1987). To evaluate whether c-*myc* directly regulates ODC expression, we have tested whether the activity of murine ODC promoter-reporter gene constructs can be modulated by c-*myc*. We demonstrate that c-*myc* is a potent transactivator of ODC transcription which requires a conserved repeat of the *myc* binding sequence, CACGTG, found in ODC intron 1. These results demonstrate that c-*myc* is a transcription factor and suggest that ODC is a physiologically relevant transcriptional target of c-*myc*.

Current Topics in Microbiology and Immunology, Vol. 182

Results

C-*myc* Transactivates the Murine ODC Promoter

Enforced c-*myc* expression in interleukin-3 (IL-3)-dependent myeloid cell lines promotes G1 progression and relieves the growth factor dependent expression of ODC RNA (Askew et al., 1991; Dean, et al., 1987). Multiple copies of the ODC gene occur in the mammalian genome, yet only one directs synthesis of active enzyme (Kahana, and Nathans, 1984). The murine ODC promoter contains several consensus transcription factor binding sites (Fig. 1, Katz and Kahana, 1988). In addition, two potential *myc* binding sites (CACGTG), separated by 34 nucleotides, are present in ODC intron 1 just downstream from the end of ODC exon 1 (Fig. 1). This position and sequence are highly conserved in mammals (data not shown).

To test whether enforced c-*myc* expression could influence ODC promoter activity we initially examined the ability of human c-*myc* to promote transcription from two murine ODC-promoter CAT constructs (Brabant et al., 1988), termed ODC CAT and ODCΔCAT (kindly provided by Dr. Philip Coffino, UCSF), which contain the ODC promoter region, exon 1, all of intron 1 and a portion of exon 2 (Fig. 1). Co-transfection experiments with increasing amounts of c-*myc* expression plasmid demonstrated that c-*myc* was a very potent transactivator of the ODCΔCAT promoter construct in fibroblast cells (> 300 fold, Fig.2). Similar results were obtained with the ODC CAT promoter construct (data not shown). By contrast, titration of the *myc* vector in cotransfection experiments in myeloid cells demonstrated a more moderate transactivation (approximately 15-fold induction, Bello-Fernandez and Cleveland, submitted). Activation of the ODC promoter was c-*myc*-specific since transfection with constructs expressing mutants of c-*myc* failed to promote CAT activity (see below).

Conserved *myc* Binding Sites in ODC Intron 1 Are Required for c-*myc* Transactivation

To evaluate whether the conserved CACGTG sequences present in ODC intron 1 were necessary for c-*myc* transactivation, an internal deletion promoter construct was tested which lacks a 186 bp NcoI fragment in which both *myc* boxes are deleted (termed ODC CATΔN). Transient co-transfection experiments demonstrated that this deletion abolished the ability of c-*myc* to transactivate the ODC promoter in fibroblast cells (Fig. 3) and also in myeloid cells (data not shown).

The position of the *myc* binding site in ODC intron 1 suggests that it may function as a c-*myc*-specific transcriptional enhancer. To evaluate this possibility we cloned a a double stranded oligonucleotide containing this sequence both 5' of the minimal TK promoter of the pBL2 CAT vector and also 3' of CAT. Transient co-transfection experiments demonstrated that c-*myc* was also a potent transactivator of all these constructs (Bello-Fernandez and Cleveland, submitted). Therefore, these conserved *myc* binding sites can function as an enhancer.

Domains of c-*myc* Required for Transactivation of the ODC Promoter

To identify domains of c-*myc* which are necessary for transactivation of the ODC promoter, we employed several well-defined deletion mutants (Stone et al., 1987) and B-HLH point mutants (Halazonetis and Kandil, 1991) of human c-*myc* (kindly provided by William Lee and Thanos Halazonetis) in transient co-transfection assays with ODCΔCAT. While removal or mutation of anticipated domains of c-*myc* abolished transactivation, such as the B-HLH region and the amino terminal regions necessary for transformation (amino acids 1-103), deletion of other regions did not inhibit transactivation (Fig. 4). Surprisingly this included a deletion which removes two of four leucines of the LZ domain (deletion of amino acids 414-433, Fig. 4), and takes the last leucine, Leu^{434}, out of context. To confirm this

result, we also tested point mutants of the murine c-*myc* LZ domain and found that only mutation of Leu^{413}, directly juxtaposed to the B-HLH domain, abolished transactivation of the ODC promoter (Bello-Fernandez and Cleveland, submitted). In addition, deletion mutants which lacked specific regions of the c-*myc* amino terminus (amino acids 56-93 and 106-143) and a region comprising the central third of human c-*myc* (amino acids 145 to 262) displayed increased ability to transactivate the ODC promoter (at least 3 fold more active than wild-type c-*myc*, Fig. 4).

Discussion

ODC is an attractive transcriptional target for c-*myc* since, similar to c-*myc*, ODC is required for G1 progression (Bowlin et al., 1986) and can replace c-*myc* in primary fibroblast co-transformation assays with activated *ras* oncogenes (Hibshoosh et al., 1991). In addition, induction of ODC RNA follows that of c-*myc* transcripts when ligand starved cells are treated with mitogens, and enforced c-*myc* expression drives constitutive, growth factor-independent expression of ODC in IL-3 dependent myeloid cells (Askew et al., 1991; Dean, et al., 1987). Here we have shown that c-*myc* is a potent activator of the ODC promoter which requires conserved consensus *myc* binding sites in ODC intron 1. Moreover, when fused to a heterologous promoter, this element can function as an *myc*-responsive enhancer (Bello-Fernandez and Cleveland, submitted). These criteria indicate that c-*myc* is a transcription factor and identify ODC as a c-*myc* target.

Identification of c-*myc* domains required for trans-activation of the ODC promoter revealed that specific regions were necessary and these generally agree with those domains required for c-*myc* transforming potential (Stone et al., 1987; Halazonetis and Kandil, 1991). The B-HLH and two noncontigous amino-terminal domains (amino acids 1-50 and 93-103) were required for transactivation. Both of these amino terminal regions have been shown to function as transactivation domains (Kato et al.,1990). Interestingly, deletion of other regions (for example amino acids 56-93 and 106-262) increased the ability of c-myc to transactivate the ODC promoter, suggesting that these domains may contain phosphorylation sites which modulate c-*myc* activity or that these regions may interact with negative regulatory factors. In this context, it is perhaps noteworthy that the retinoblasotoma tumor suppressor gene product binds to c-*myc* *in vitro* in overlappping regions (Rustgi et al., 1991). In addition, we have observed substantial differences in the trans-activating potential of c-*myc* in NIH 3T3 fibroblasts versus murine myeloid cells (Bello-Fernandez and Cleveland, submitted), perhaps suggesting that interactive factors may be differentially expressed.

The most surprising region which was dispensable for c-*myc* transactivation of the ODC promoter was most of the LZ domain. The c-*myc* LZ domain is required for dimerization with max *in vitro* (Blackwood and Eisenman, 1991) and *in vivo* (Kato et al. 1992) and for binding of an *in vitro* translated *myc*/max complex (Blackwood and Eisenman, 1991). Since c-myc LZ domain deletion or point mutants can transactivate the ODC promoter, we speculate that *myc*, at least in the context of transactivation of the ODC promoter, can function independent of it's identified heterodimeric partner. Recent findings have demonstrated that other B-LZ proteins (c-*jun* and c-*fos*) can interact with B-HLH proteins (MyoD) or B-HLH-LZ proteins (FIP) through their respective dimerization motifs (Bengal et al., 1992; Blanar and Rutter, 1992). Therefore it is possible that c-*myc* may also interact with novel dimerization partners through other HLH-HLH or HLH-LZ interactions to transactivate ODC.

Acknowledgements

We are grateful for the technical expertise of Brenda Simmons and Elsie White, and Joyce Galle and Kathy Fry for secretarial assistance. We are also indebted to investigators who kindly provided necessary reagents: ODC CAT and ODCΔCAT plasmid DNA (Phil Coffino); pSV7humycmj1 and pSV7humycmj2 (Thanos Halazonetis), pM21 and human c-*myc* deletion mutants (William Lee). This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant DK44158 (J.L.C.), the National Cancer Institute Center Support (CORE) grant PO CA21765 and by the American Lebanese Syrian Associated Charities (ALSAC).

References

Askew, D. S., Ashmun, R. A., Simmons, B. C. & Cleveland, J. L. (1991). Constitutive c-*myc* expression in an IL-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 6, 1915-1922.

Bengal, E., Ransone, L., Scharfmann, R., Dwarki, V.J., Tapscott, S.J., Weintraub, H. & Verma, I.M. (1992). Functional antagonism between c-*jun* and MyoD proteins: a direct physical association. Cell 68, 507-519.

Blackwell, T. K., Kretzner, L., Blackwood, E. M., Eisenman, R. N. & Weintraub, H. (1990). Sequence-specific DNA binding by the c-*myc* protein. Science 250, 1149-1151.

Blackwood, E. M., and Eisenman, R. N. (1991). Max: A helix-loop-helix zipper protein that forms a sequnce-specific DNA-binding complex with *myc*. Science 251, 1211-1217.

Blanar, M.A., & Rutter, W.J. (1992). Interaction cloning: Identification of a helix-loop-helix zipper protein that interacts with c-*fos*. Science 256, 1014-1018.

Bowlin T.L., McKnown, B. & Sunkara, P. (1986). Ornithine decarboxylase induction and polyamine biosynthesis are required for growth of interleukin-2 and interleukin-3 dependent cell lines. Cell Immunol. 98, 341-350, 1986.

Brabant, M., McConlogue, Van Daalen Wetters, T. & Coffino, P. (1988). Mouse ornithine decarboxylase gene: Cloning, structure and expression. Proc. Natl. Acad. Sci. USA 85, 2200-2204.

Coppolla, J.A. & Cole, M.D. (1986). Constitutive c-*myc* oncogene expression blocks mouse erythroleukemia cell differentiation but not commitment. Nature 320, 760-763.

Dean, M., Cleveland, J.L., Rapp, U. R., and Ihle, J. N. (1987). Role of myc in the abrogation of IL-3 dependence myeloid FDC-P1 cells. Oncogene Res. 1: 279-296.

Eilers, M., Schirm, S., and Bishop, J. M. (1991). The *Myc* protein activates transcription of the alpha-prothymosin gene. EMBO J 10, 133-141.

Evan, G.I., Wylie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-*myc* protein. Cell 69, 119-128.

Heikkela, R., Schwab, G., Wickstrom, E., Lohie, S. L., Pliznik, D.H., Watt, R. and Nacres, L. M. (1987). A c-*myc* antisense oligonucleotide inhibits entry into S phase but not progression from G0 to G1. Nature 328, 445-449.

Halazonetis, T. D. and Kandil, A. N. (1991). Determination of the c-*myc* DNA binding site. Proc. Natl. Acad. Sci. USA 88, 6162-6166.

Hibshoosh, H., Johnson, M. and Weinstein, I. B. (1991). Effects of overexpression of ornithine decarboxylase (ODC) on growth control and oncogene-induced cell transformation. Oncogene 6, 739- 743.

Luscher, B. & Eisenman, R. (1990). New light on *Myc* and *Myb*. Part I: *Myc*. Genes Dev. 4: 2025-2035.

Kahana, C. & Nathans, D. (1984). Nucleotide sequence of murine ornithine decarboxylase mRNA. Proc. Natl. Acad. Sci. USA 81, 3645-3649.

Karn, J., Watson, J. V., Lowe, A. D., Greene, S. M. & Vedeckis, W. (1989). Regulation of cell cycle duration by c-*myc* levels. Oncogene 4, 773-781.

Kato, G. J., Barrett, J., Villa-Garcia, M. & Dang, C. V. (1990). An aminoterminal c-*myc* domain required for neoplastic transformation activates transcription. Mol. Cell. Biol. 10, 5914-5920.

Kato, G. J., Lee, W. M. F., Chen, L. & Dang, C. V. (1992). Max: Functional domains and interaction with c-*myc*. Genes Dev. 6, 81-92.

Katz, A. & Kahana, C. (1988). Isolation and characterization of the mouse ornithine decarboxylase gene. J. Biol. Chem. 263, 7604-7609.

Prendergast, G.C. & Ziff, E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-*myc* basic region. Science 251, 186-189.

Prochownik, E.V. & Kukowska, J.F. (1986). Deregulated expression of c-*myc* by murine erythroleukemia cells prevents differentiation. Nature 322, 848-850.

Prowchownik, E. V., Kukowska, J. & Rodgers, C. (1988). c-*myc* antisense transcripts accelerate differentiation and inhibit G1 progression in murine erythroleukemia cells. Mol. Cell. Biol. 8, 3683-3695.

Rustgi, A. K., Dyson, N. & Bernards, R. (1991). Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the retinoblastoma gene product. Nature 352, 541- 544.

Stone, J., De Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H. & Lee, W. (1987). Definition of regions in human c-*myc* that are involved in transformation and nuclear translocation. Mol. Cell. Biol. 7, 1697-1709.

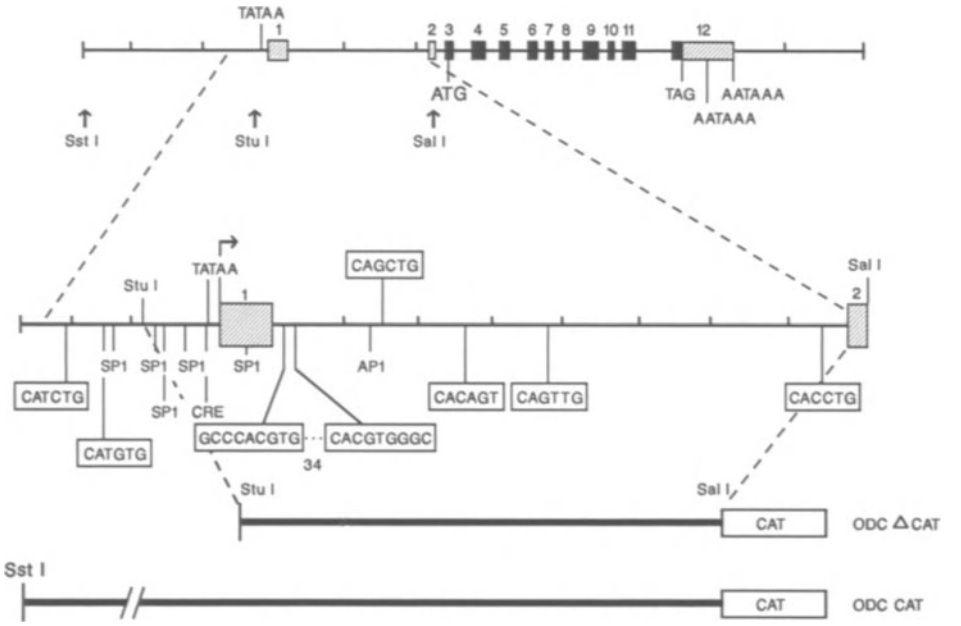


Fig. 1. Structure of the murine ODC gene is shown at the top and an expanded diagrammatic of the ODC promoter, exon 1, intron 1 and exon 2 is shown beneath. The location of putative transcription factor binding sites are boxed, including E-box sequences and two *myc* binding sites, CACGTG, present in ODC intron 1. The structure of murine ODC promoter CAT constructs is shown beneath.

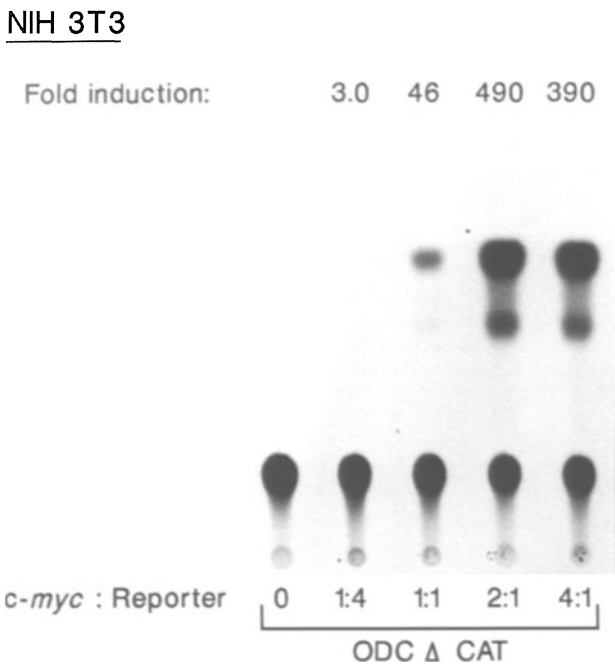


Fig. 2. c-*myc* transactivates the ODC promoter. A representative transient co-transfection titration experiment of ODCΔCAT and c-*myc* expression plasmid are shown for NIH 3T3 Cl7 cells. The fold induction of CAT activity by c-*myc* is indicated above individual assays. The molar ratio of c-*myc* expression plasmid to the reporter construct is given below.

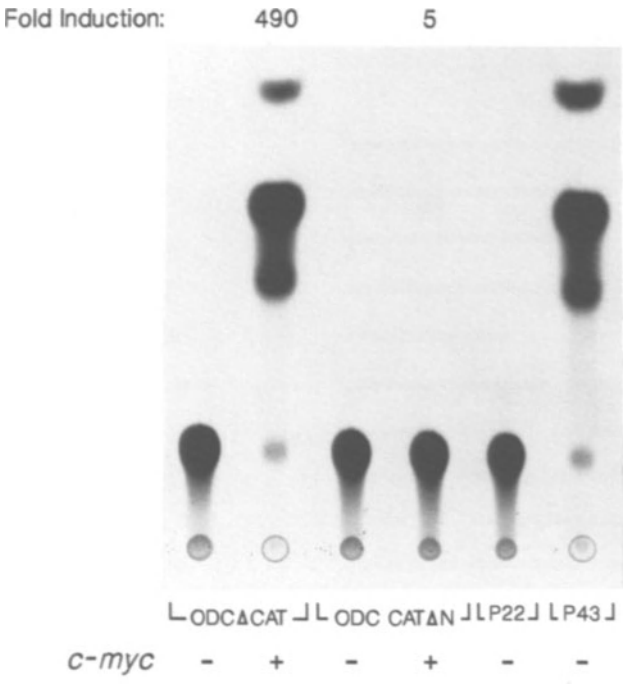


Fig. 3. Transactivation of the ODC promoter requires the *myc* binding sites in ODC intron 1. A representative transient transfection experiment with the ODCΔCAT and ODC CATΔN reporter constructs and c-*myc* expression plasmid (at a 2:1 molar ratio) was carried out in NIH 3T3 cells and the extracts normalized for transfection efficiency. The fold induction is indicated above individual assays. P22 is a promoter-less CAT construct and P43 is a MoMuLV-LTR CAT construct.

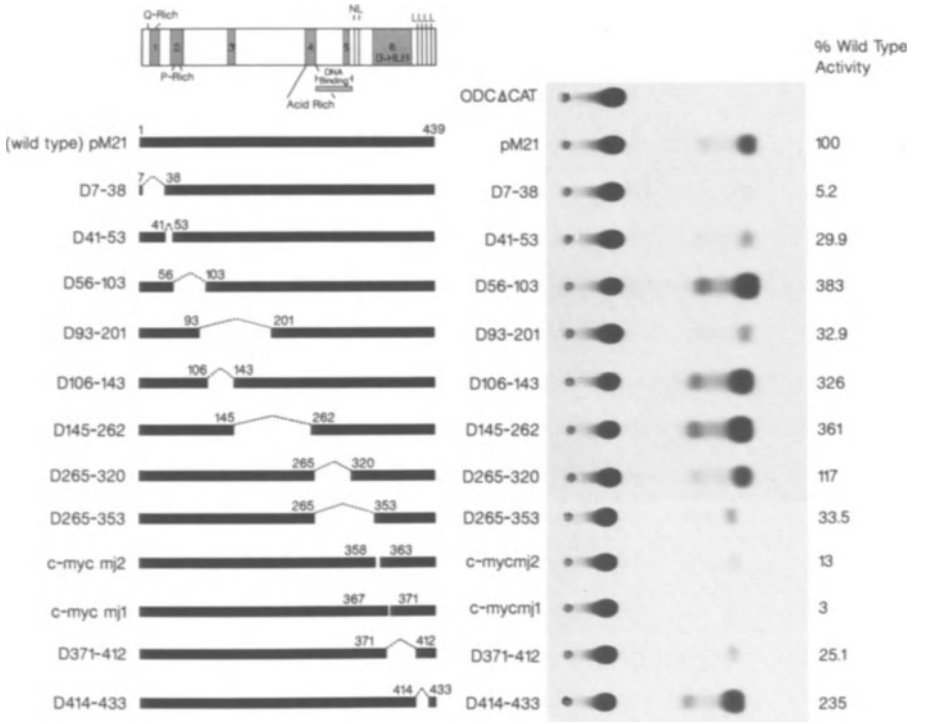


Fig. 4. Domains of c-*myc* required for transactivation of the ODC promoter. At left is shown the domain structure of human c-*myc* and beneath the structure of human c-*myc* deletion and point mutants. At right is shown data from a representative CAT assay using standardized extracts from NIH 3T3 cells transiently transfected with a 2:1 molar ratio of the indicated c-*myc* expression plasmids and the ODCΔCAT reporter. The fold induction of the ODCΔCAT reporter is depicted as % activity of wild-type (PM21) c-*myc*.

v-Raf/v-Myc Synergism in Abrogation of IL-3 Dependence: v-Raf Suppresses Apoptosis

J. TROPPMAIR[1], J. L. CLEVELAND[2], D. S. ASKEW[2] and U. R. RAPP[1]

[1]Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, MD 21702-1201,
[2]Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38015

Introduction

Raf-1 serine/threonine protein kinase is a mitogen-regulated cytosolic enzyme that is essential for growth of NIH/3T3 cells induced by serum, PDGF, PKC or ras (Kolch et al., 1991) as well as myeloid FDC-P1 cells induced by IL-3 or erythropoietin (Carroll et al., 1991). The requirement for growth induction correlates with its requirement for AP-1/Ets dependent transcriptional trans-activation (Bruder et al., 1992) and is consistent with the function of Raf kinase as a shuttle enzyme that connects activation of growth factor receptors at the cell membrane with cell cycle progression-related transcriptional events in the nucleus (Rapp, 1991). Several lines of evidence have demonstrated a role of Raf family protein kinases in growth and differentiation of T -/B-lymphoid and myeloid lineage cells (Table 1)

Table 1: Raf-1 kinase/B cell connection

1. Exogenous oncogenic *raf* synergizes with *myc* in plasmacytoma induction, B cell immortalization and induction of the B cell/myeloid switch (Morse and Rapp, 1988; Rapp et al. 1985; Principato et al., 1988, 1989; Klinken et al., 1988, Troppmair et al., 1988, 1989)

2. Raf-1 functions as a second messenger in the signal transduction from sIgM, receptors for IL-2 and stem cell factor, but apparently not in the signalling from IL-4 and IL-6 receptors (Turrner et al., 1991, Miyazawa et al., 1991, and unpublished findings)

3. Raf-1 activates Ap-1/ets as well as NF-kB dependent transcription (Bruder et al., 1992, Bruder et al., manuscript submitted)

Previously we have described synergistic abrogation of myeloid FDC-P1 cells by *raf* and *myc* oncogenes (Rapp et al., 1990) and examined the contribution of *myc* to the synergism (Dean et al., 1987, Cleveland et al., 1988b; Cleveland et al., 1989). Briefly, constitutive expression of exogenous v-*myc* or c-*myc* promotes competence for G1 progression in several cell systems (Rapp et al., 1985a,b; Langdon et al., 1986; Cleveland et al., 1988a; Dean et al., 1987; Cleveland et al., 1988b; Cleveland et al., 1989; Rapp et al., 1990; Askew et al., 1991). The degree to which exogenous v- or c-*myc* is sufficient for growth factor abrogation is cell context dependent and ranges from complete abrogation at extreme levels of v-*myc* overexpression in FDC-P1 cells (Rapp et al., 1985a,b; Rapp et al., 1988; Cleveland et al., 1989) to partial abrogation of the same cells at near normal levels of constitutive c-*myc* expression (Dean et al., 1987) and finally to acceleration of apoptotic cell death in 32D cells which have a more stringent requirement for IL-3 control (Askew et al., 1991). This pattern of *myc* effects presumably reflects cell type specific differences in the activity of pathways, such as a *raf* pathway, that synergize with *myc* in growth factor abrogation. Here we will present data that identify the contribution of activated Raf kinase to the observed *raf/myc* synergism.

Results

Effects of Raf and Myc Oncogenes on Growth of 32D cells in Methylcellulose

Earlier studies have established the relative efficiency of viruses carrying *raf* plus *myc* or exogenous tyrosine kinase oncogenes for induction of IL-3 independent growth of FDC-P1 and 32D cells (Rapp et al., 1990). Because of their roughly equal efficiency we speculated that intrinsic or associated receptor tyrosine kinases normally regulate growth by activating two complementary pathways, a *myc* inducing pathway and a Raf kinase stimulating pathway. The experiment in Fig. 1 illustrates the IL-3 abrogation by v-*raf* and v-*myc* in methylcellulose colony assays. 32D cells transfected with v-*raf* or v-*myc* alone yielded no IL-3 independent colonies whereas the combination of v-*raf* plus v-*myc* resulted in efficient IL-3 abrogation.

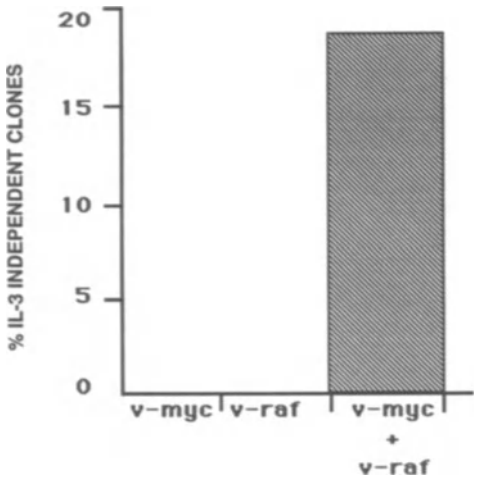


Figure 1: IL-3 abrogation in oncogene carrying 32D cells. Retroviral constructs used in this experiment have been described previously (Cleveland et al., 1989) DNAs were introduced into 32D cells along with pMNCneo by electroporation (v-*myc*, v-*raf*) or infection with helper virus (leuk) pseudotypes (v-*trk*, v-*src*). Cells were seeded in methylcellulose in the absence of IL-3 and the cultures scored for the number of G418 resistant IL-3 independent clones.

Activated Raf Promotes Proliferation of 32D Cells in IL-3

To test the contribution of Raf in the growth factor abrogation by v-*myc*/v-*raf* we transfected activated *raf* (v-*raf*) or normal full length *raf* (c-*raf*) into 32D cells and assayed the resulting clones for cell doubling time. As shown in Fig. 2 overexpression of normal *c-raf* had no effect on proliferation. By contrast v-*raf* clones grew faster and to higher cell densities than 32D cells or c-*raf* clones. The increased growth of v-*raf* clones was associated with an increase in the proportion of cells in the S phase of the cell cycle at the expense of the number of cells in G1 phase (data not shown).

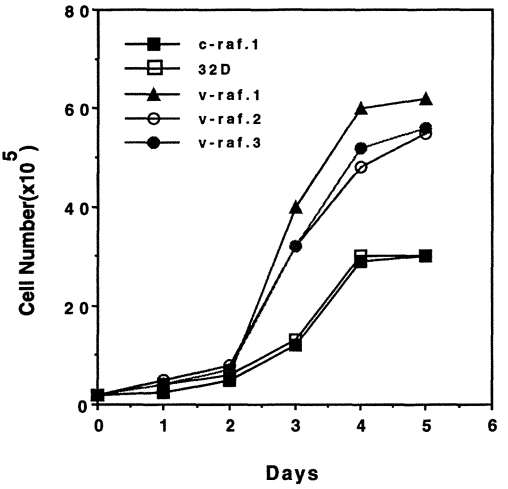


Figure 2: Activated Raf accelerates growth of IL-3 dependent 32D cells. Retroviral expression vectors carrying full length c-*raf*-1 or constitutively activated gag-v-*raf* together with a neo selectable marker were electroporated into 32 cells and neo clones were analyzed for growth in IL-3.

Activated Raf Promotes Survival of 32D Cells in the Absence of IL-3

We have previously demonstrated that constitutive expression of c-*myc* in 32D cells promotes cell cycle progression in the absence of IL-3 and accelerates cell death (Askew et al., 1991). We have now analyzed the effect of v-*raf* on 32D cell growth in the absence of IL-3. Control cells and v-*raf* expressing 32D clones were transferred to media lacking IL-3 and examined for viability. 24 hours after IL-3 withdrawal 90% of the 32D cells had died (Fig. 3A). By contrast, v-*raf* clones persisted for extended periods in the absence of IL-3. No secreted factor was present in conditioned medium from these v-*raf* clones which promoted survival of control cells (Cleveland, Askew, Troppmair and Rapp, manuscript in preparation). When genomic DNA was assayed 20 hrs after IL-3 deprivation 32D cells showed the typical DNA ladder characteristic of apoptotic death (Fig. 3B). In contrast in v-*raf* clones no signs of DNA degradation were present. From these data we conclude that activated Raf kinase promotes 32D cell survival by delaying apoptosis.

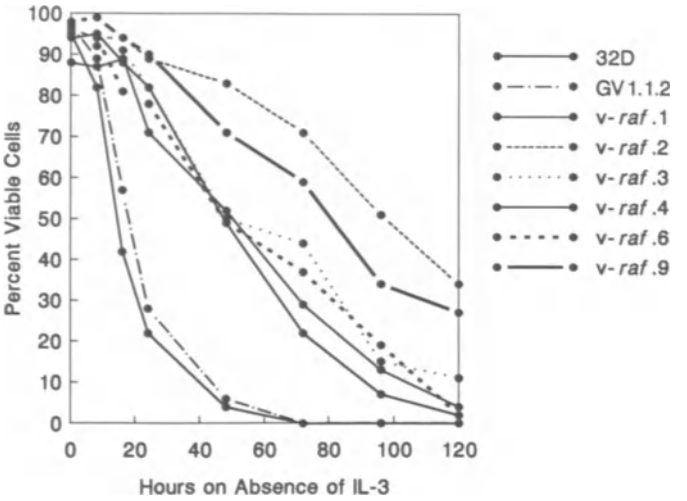


Figure 3 A: Activated Raf prolongs survival of 32D cells in the absence of IL-3. 32D cells and 32D clones harboring gag-v-*raf* (v-*raf* clones) were transferred to medium lacking IL-3 and cell viability was monitored using trypan blue dye exclusion.

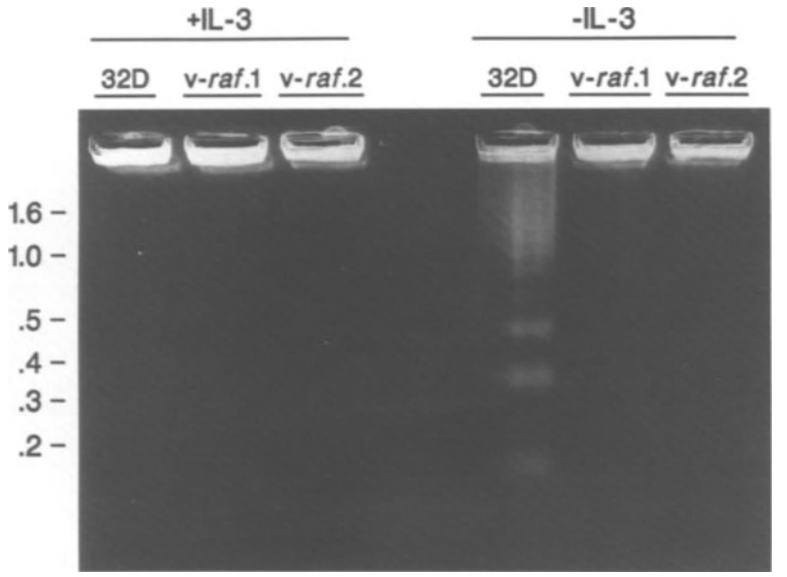


Figure 3 B: Activated Raf prevents apoptotic death in 32D cells after IL-3 withdrawal. Genomic DNA was isolated from 32D and v-*raf* clones grown in IL-3 and from 32D and v-*raf* clones 20 hours after IL-3 withdrawal and analyzed on 2% agarose gels for DNA degradation characteristic of apoptosis.

Discussion

We have previously demonstrated that IL-3 growth factor abrogation in 32D cells jointly requires two signaling pathways, a *myc* and a *raf* pathway (Rapp et al., 1988, Rapp et al., 1990 and Fig.4). Whereas enforced *myc* expression overcomes the G1 arrest normally associated with IL-3 withdrawal and allows the cells to progress into late G1 and S it also accelerates cell death (Askew et al., 1991). *Myc* overexpression *per se* does not appear to be deleterious to cell survival and cell cycle progression except at high density when cells are grown in the presence of IL-3. Growth factor stimulation therefore must activate an additional pathway which promotes survival. The data presented here are consistent with activated Raf

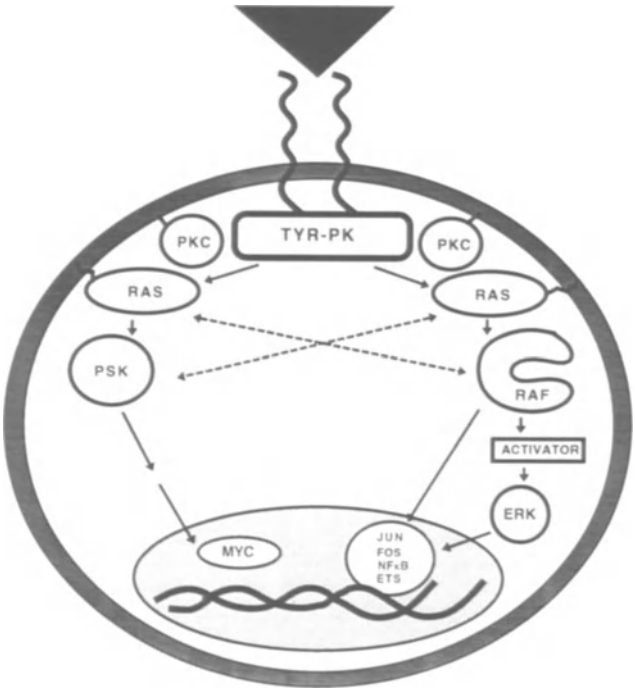


Figure 4: Signaling pathways originating from the IL-3 receptor. The ability of IL-3 to regulate a myc induction pathway and Raf-1 kinase has been established (Rapp et al., 1985a,b; 1988, 1990). The involvement of Ras and MAP/erk kinase(s) is speculative and based on work with other protein tyrosine kinase dependent receptors in fibroblasts (Troppmair et al., in press; Kyriakis et al., in press)

kinase providing such a survival function. It can be envisioned that Raf functions by cancelling a program induced by *myc* which although initially required for cell cycle progression is incompatible with cell survival.

The mechanism of transformation by v-*raf* involves transcriptional regulation of specific growth factor sensitive genes (Wasylyk et al., 1989; Kaibuchi et al., 1989; Jamal and Ziff, 1990; Qureshi et al., 1991). Whether the effect of v-*raf* on cell survival also depends on v-*raf* regulated genes or is a consequence of phosphorylation of already expressed proteins that do not feed back on transcription is not clear. Analysis of immediate early gene expression in 32D cells and v-*raf* clones demonstrated that the presence of activated Raf kinase failed to replace the IL-3 requirements for all ligand-regulated genes analyzed so far including c-*myc*, *jun*B, c-*fos*, *egr*-1, ODC and pim 1 (Cleveland, Askew, Troppmair, and Rapp, manuscript in preparation).

Based on the observed synergism between v-*raf* and v-*myc* in transformation and growth factor abrogation we have previously speculated that v-*raf* acts as a progression factor and thereby complements the competence factor *myc* (Rapp et al., 1988). Cell cycle analysis of v-*raf* clones after IL-3 removal demonstrates that activated *raf* by itself is insufficient to promote cell cycle progression during G1. In contrast, when v-*raf* clones were tested for growth in the presence of IL-3 (Fig. 2) analysis of cell cycle distribution showed that accelerated growth resulted from shortened G1 phase. This demonstrates that activated *raf* can affect cell cycle progression if an additional signal is provided.

V-*raf* shares the ability to prolong cell survival by counteracting apoptosis with another gene, *bcl-2* (Vaux et al., 1988). This activity of *bcl*-2 was initially identified in *in vitro* culture of hemopoietic cells (Vaux et al., 1988) and later confirmed by findings in transgenic animals where Eμ-driven expression of *bcl*-2 results in a prolonged life span of certain T and B cell subpopulations (McDonnell et al., 1989, Strasser et al., 1990). Expression of *bcl*-2 by itself is insufficient to induce the transformed phenotype which requires cooperation of *bcl*-2 with c-*myc* (Vaux et al., 1988). In 32D cells constitutive expression of *bcl* - 2 prolongs cell survival in the absence of IL-3 with efficiencies comparable to v-*raf* (J.Reed, unpublished results). These findings raise the possibility that both genes converge on the same signal transduction pathway for survival although v-*raf* presumably has additional growth stimulatory effects (e.g. cell cycle progression) as it is sufficient for both rapid tumor induction in mice (Rapp et al., 1983) and induction of DNA synthesis in NIH3T3 cells (Smith et al., 1990) whereas *bcl*-2 is not. We have tested a possible Bcl-2/Raf connection by examining NIH3T3 cells overexpressing Bcl-2 and found that it had no effect on Raf-1 kinase activity, demonstrating that Raf-1 is not a Bcl-2 effector. It remains to be established, however, whether Bcl-2 functions in a signalling pathway downstream of Raf or whether its action is Raf independent.

References

Askew D.S., Ashmun R.A., Simmons B.C., and Cleveland J.L. (1991) Constitutive c-*myc* expression in IL-3 dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. Oncogene 6, 1915-1922.

Bruder J.T., Heidecker G., and Rapp U.R. (1992) Serum-, TPA-, and Ras-induced expression from AP-1/*ets*-driven promoters require Raf-1 kinase. Genes and Development 6, 545-556.

Bruder J.T., Tan T.H., Heidecker G., Derse D., and Rapp U.R. Raf transactivation of HIV-LTR driven expression via NF-κB binding sites (manusrcipt submitted)

Carroll M.P., Clark-Lewis I., Rapp U.R., and May W.S. (1990) Interleukin-3 and granulocyte-macrophage colony stimulating factor mediate rapid phosphorylation and activation of cytosolic c-Raf. J.Biol.Chem. 265, 119812-19817.

Carroll M.P., McMahon M., Weich N., Spivak J.L., and Rapp U.R. (1991) Erythropoietin induces Raf-1 activation and Raf-1 is required for erythopoietin-mediated proliferation. J.Biol.Chem. 266, 14964-14969.

Cleveland J.L., Morse ,III, H.C., and Rapp U.R. (1988a) *Myc* oncogenes and tumor induction. In: ISI Atlas of Science: Biochemistry. 1, pp 93-100.

Cleveland J.L., Dean M., Wang J.Y., Hedge A.M., Ihle J.N., and Rapp U.R. (1988b) Abrogation of Il-3 dependence of myeloid FDC-P1 cells by tyrosine kinase oncogenes is associated with induction of c-*myc*. In: Potter M., Melchers F. (eds.): Curr.Top.Microbiol.Immunol.141. Berlin, Springer Verlag, pp.300-309.

Cleveland J.L., Dean M., Rosenberg N., Wang J.Y.J. and Rapp U.R. (1989) Tyrosine kinase oncogenes abrogate interleukin-3 dependence of murine myeloid cells through signalling pathways involving c-*myc*: conditional regulation of c-*myc* transcription by temperature-sensitive v-*abl*. Mol.Cell.Biol. 9, 5685-5695.

Dean M., Cleveland J.L., Rapp U.R., and Ihle J.N. (1987) Role of *myc* in the abrogation of IL-3 dependence of myeloid FDC-P1 cells. Oncogene Res. 1: 279-296.

Heidecker G., Cleveland J.L., Beck T., Huleihel M., Kolch W., Storm S.M., and Rapp U.R. (1989) Role of *raf* and *myc* oncogenes in signal transduction. In: Colburn N. (ed.): Genes and Signal Transduction in Multistage Carcinogenesis. pp.339-374, New York, M.Dekker.

Jamal S., and Ziff E. (1990) Transactivation of c-*fos* and beta-actin genes by *raf* as a step in early response to transmembrane signals. Nature 344:463-466.

Kaibuchi K., Fukumoto Y., Oku N., Hori Y., Yamamoto T., Toyoshima K., and Takai Y.(1989) Activation of the serum response element and 12-O-tetradecanoylphorbol-13-acetate response element by the activated c-*raf*-1 protein in a manner independent of protein kinase C. J.Biol. Chem 264: 20855-20858.

Kyriakis J.M., App H., Zhang X., Banerjee P., Brautigan D.L., Rapp U.R., and Avruch J. (1992) Activation of MAP kinase-kinase by Raf-1. Nature, in press.

Klinken S.P., Alexander W.S., Adams J.M. (1988) Hemopoietic lineage switch: v-*raf* oncogene converts Eμ-*myc* transgenic B cells. Cell 53: 857-867.

Kolch W., Heidecker G., Lloyd P., and Rapp U.R. (1991) Raf-1 protein kinase is required for growth of NIH/3T3 cells induced by serum, TPA and *ras* oncogenes. Nature 349, 426-428.

Langdon W.Y., Harris A.W., Cory S., and Adams J.M. (1986) The c-*myc* oncogene perturbs B lymphocyte development in E-μ-*myc* transgenic mice. Cell 47, 11-18.

McDonnell T.J., Deane N., Platt F.M., Nunez G., Jaeger U., McKearn J.P., and Korsmeyer S.J. (1989) *Bcl*-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular proliferation. Cell 57, 79-88.

Miyazawa K., Hendrie P.C., Mantel C., Wood K., Ashman L.E., and Broxmeyer H.E. (1991) Comparative analysis of signaling pathways between mast cell growth factor (c-*kit* ligand) and granulocyte-macrophage colony-stimulating factor in a human factor-dependent myeloid cell line involves phosphorylation of Raf-1, GTPase-activating protein and mitogen-activated protein kinase. Exp.Hematol 19, 1110-1123.

Morse, III, H.C., Hartley J.W., Fredrickson T.N., Yetter R.A., Majumdar C., Cleveland J.L., and Rapp U.R. (1986) Recombinant murine retroviruses containing avian v-*myc* induce a wide spectrum of neoplams in newborn mice. PNAS 83, 6868-6872.

Morse, III, H.C., and Rapp U.R. (1988) Tumorigenic activity of artificially activated c-*onc* genes. In: Klein G. (ed): Cellular Oncogene Activation. Marcel Dekker Inc., New York, pp. 335-364.

Principato M., Klinken S.P., Cleveland J.L., Rapp U.R., Holmes K.L., Pierce J.H., and Morse H.C. (1988) *In vitro* transformation of murine bone marrow cells with a v-*raf*/v-*myc* retrovirus yields clonally-related mature B cells and macrophages. In: Potter M., Melchers F. (eds.): Curr.Top.Microbiol.Immunol.141. Berlin, Springer Verlag, pp. 31-41.

Principato M., Cleveland J.L., Rapp U.R., Holmes K.L., Pierce J.H., Morse, III, H.C. and Klinken S.P., (1990) Transformation of murine bone marrow cells with combined v-*raf*/v-*myc* oncogenes yields clonally-related mature B cells and macrophages. Mol.Cell.Biol. 10, 3562-3568.

Qureshi S.A., Rim M., Bruder J., Kolch W., Rapp U., Sukhatme V.P., and Foster D.A. (1991) An inhibitory mutant of c-Raf-1 blocks v-Src induced activation of the Egr-1 promoter. J.Biol.Chem. 266, 20594-20597.

Rapp U.R., Goldsborough M.D., Mark G.E., Bonner T.I.,, Groffen J., Reynolds Jr., F.H., and Stephenson J. (1983) Structure and biological activity of v-*raf*, a unique oncogene transduced by a retrovirus. PNAS 80, 4218-4222.promoter.

Rapp U.R., Cleveland J.L., Brightman K., Scott A., and Ihle J.N. (1985a) Abrogation of IL-3 and IL-2 dependence by murine retroviruses expressing v-*myc* oncogenes. Nature 317, 434-438.

Rapp U.R., Cleveland J.L., Fredrickson T.N., Holmes K.L., Morse, III, H.C., Jansen H.W., Patschinsky T. and Bister K. (1985b) Rapid induction of hemopoietic neoplasms in newborn mice by a *raf(mil)/myc* recombinant murine retrovirus. J.Virol. 55, 23-30.

Rapp U.R., Heidecker G., Huleihel M., Cleveland J.L., Choi W.C., Pawson T., Ihle J.N. and Anderson W.B. (1988) In: Cold Spring Harbor Symp. on Quant.Biol.: Signal Transduction, Vol.53, Cold Spring Harbor Laboratory Press: New York, pp. 173-184.

Rapp U.R., Troppmair J., Carroll M., and May S. (1990) Role of Raf-1 protein kinase in IL-3 and GM-CSF-mediated signal transduction. In: Potter M., Melchers F. (eds.): Curr.Top.Microbiol.Immunol.166. Berlin, Springer Verlag, pp.129-139.

Rapp U.R. (1991) Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene 6, 495-500.

Smith M.R., Heidecker G., Rapp U.R., and Kung H. (1990) Induction of transformation and DNA synthesis after injection of raf proteins. Mol.Cell. Biol. 10, 3828-3833.

Strasser A., Harris A.W., Bath M.L., and Cory S. (1990) Novel primitive lymphoid tumors induced in transgenic mice by cooperation between *myc* and *bcl* -2. Nature 348, 331-333.

Thompson P.A., Ledbetter J.A., Rapp U.R., and Bolen J.B. (1991) The Raf-1 serine threonine kinase is a substrate for the p56^{lck} protein tyrosine kinase in human T cells. Cell Growth and Diff. 2, 609-617.

Troppmair J., Huleihel M., Cleveland J., Mushinski J.F., Kurie J., Morse, III, H.C., Wax J.S., Potter M., and Rapp U.R. (1988) Plasmacytoma induction by J series of recombinant v-*myc* retroviruses: Evidence for the requirement of two (*raf* and *myc*) oncogenes for transformation. In: Potter M., Melchers F. (eds.): Curr.Top.Microbiol.Immunol.141. Berlin, Springer Verlag, pp. 110-114.

Troppmair J., Potter M., Wax J.S., and Rapp U.R. (1989) An altered v-*raf* is required in addition to v-*myc* in J3 virus for the acceleration of murine plasmacytomagenesis. PNAS 86, 9941-9945

Troppmair J., Bruder J.T., App H., Cai H., Szeberenyi J., Cooper G.M., and Rapp U.R. (1992) Ras controls coupling of growth factor receptors and protein kinase C in the membrane to Raf-1 and B-Raf protein serine kinases in the cytosol. Oncogene, in press

Turner B.C., Rapp U.R., App H., Green M., Dobashi K., and Reed J.C. (1991) Interleukin 2 (IL-2) induces tyrosine phophorylation and activation of p72-74 Raf-1 kinase in T cell line lymphocyte. PNAS 88, 1227-1231.

Vaux D.L., Cory S., Adams J.M. (1988) *Bcl*-2 gene promotes hemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. Nature 335:440-442.

Wasylyk C., Wasylyk B., Heidecker G., Huleihel M., and Rapp U.R. (1989)Expression of *raf* oncogenes activates the PEA1 transcription factor motif. Mol.Cell.Biol. 9: 2247-2250.

Cyclins in the Cell Cycle: An Overview

J. HANLEY-HYDE

Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 USA

Great strides have been made in the search to understand the origins of neoplasia in cells of the immune system. Within the last decade a large number of oncogenes have been identified and characterized. Their nucleotide and amino acid sequences are known, as well as mutations that make them transforming. Even the physiological properties and certain aspects of function are known for some of them. Nonetheless, it is not known exactly how they transform cells. Since the common feature of transformation is unregulated proliferation, it may be possible to explain the actions of many oncogenes by defining their interactions with the gene products that drive the cell division cycle. Progress in this regard has been impeded by a lack of understanding of the biochemistry of cell division. Recently, some of the molecular components required for cell cycle in several systems have been described, and in each case, control has been shown to center on the activity of heterodimeric complexes containing a protein serine/threonine kinase and a regulatory subunit called a cyclin. Both the kinases and their cyclins are members of multigene families. The kinases each contain the amino acid motif "PSTAIRE" as well as a moderate overall sequence similarity to the CDC2 gene product from *Schizosaccharomyces pombe* [1]. As many as eight distinct kinases have been identified [2]. The two kinases that have been studied most thoroughly are active during different phases of the cell cycle.

As depicted in Fig. 1, $p33^{CDK2}$ binds to and is activated by the cyclins expressed earlier in the cell cycle, the so-called G_1/S or interphase cyclins, while $p34^{CDC2}$ binds to the mitotic cyclins and is active during G_2 and M phase [reviewed in 3-8]. In addition, the proteins in these complexes are transiently phosphorylated in a cell cycle-dependent manner.

On the basis of amino acid sequence comparisons, cyclins can be divided into two classes. They all show a moderate level of similarity within a centrally located region, designated the "cyclin box"[6]. However, the mitotic cyclins have an amino terminal sequence that permits ubiquitin-dependent degradation during anaphase [9-11]. The interphase cyclins lack this amino terminal sequence, but instead have a carboxyl terminal extension rich in serine, proline, threonine and glutamate that serves the same purpose [5,12]. Cyclins of vertebrates have been studied most extensively in HeLa cells, *Xenopus laevis* oocytes, and the mouse with yeast systems serving as a paradigm. Six or seven cyclins have been cloned from each system

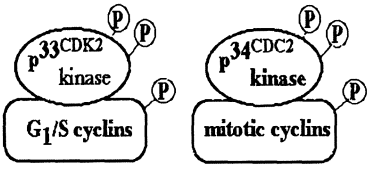


Fig. 1 Kinases are activated both by binding to cyclin and by transient phosphorylation

"G1/S" Cyclins

Yeast	Human	Mouse
CLN1	CYC-D [PRAD 1]	CYL-1
CLN2		CYL-2
CLN3	CYC-C	CYL-3
	CYC-E	

Cyclin A is required for S phase and for mitosis.

"Mitotic" Cyclins

Yeast		Human	*X. laevis*	Mouse
ScB 1	CLB1	A	A	A
ScB 2	CLB2	B1	B1	B1
ScB 3		B2	B2	B2
ScB 4				

Fig. 2 A list of known cyclins

Current Topics in Microbiology and Immunology, Vol. 182

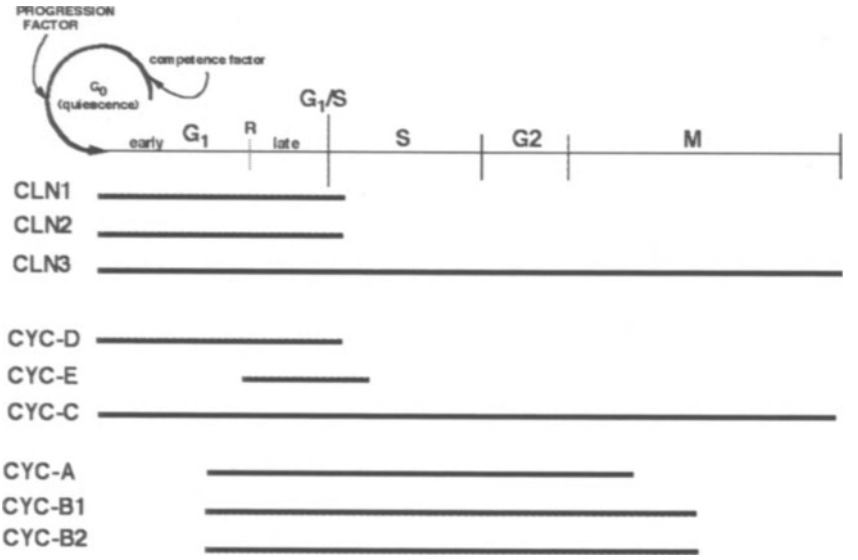


Fig. 3 Schematic representation of the timing of expression of the various cyclins during the cell cycle.

[3-8, and Hanley-Hyde, unpublished]; they are listed in Fig. 2 in an order that indicates the apparent similarities in structure, function, and the timing of expression. A graphic representation of expression of the cyclins is presented in Fig.3; the dark horizontal lines show when, during the cell cycle, detectable levels of either mRNA or protein appear.

The presence of detectable levels of mRNA or protein does not always correlate with the activities of individual cyclin-kinase complexes. Fig.4 shows a schematic representation of the activities of each set of complexes during the cell cycle. While most of the G_1/S complexes are active throughout interphase and

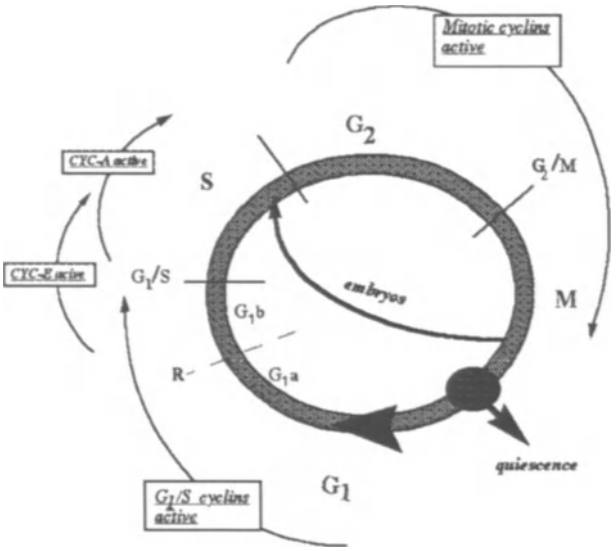


Fig. 4 A diagram of the cell cycle showing when individual cyclin-kinase complexes are most active.

are degraded shortly after the G_1/ S transition, in humans the cyclin E variant is active across that transition and into S phase[13]. Cyclin A was originally described as a mitotic cyclin, however the complexes containing cyclin A are translocated into the nucleus at the G_1/S transition and are active throughout S phase as well as during G_2 [14]. Mitotic cyclins become active during G_2, most profoundly at the G_2/M transition, and are rapidly degraded during anaphase[12]. As depicted by the arrow across the center of the cycle, embryos do not exhibit an interphase between mitosis and replication early in development, and therefore have a fore-shortened cell cycle. This characteristic proved very useful in analyzing the biochemistry of cyclin-kinase interactions during the latter half of the cell cycle. Much more is known about these interactions than about those that occur during G_1. As shown in Fig. 5, CDC2 kinase binds to a cyclin early in the cycle. It is phosphorylated doubly at its active site, on Thr-14 and on Tyr-15 [15,16]. As the cycle progresses, the cyclin itself is phosphorylated and subsequently the kinase is dephosphorylated at the active site by a phosphatase that is the homologue of CDC25 of *S. pombe*. This phosphatase is activated *in vitro* by either of the B-type cyclins. Thus the cyclins activate bound $p34^{CDC2}$ kinase both by complexation and by stimulating the removal of inhibitory phosphates at the active site. After this dephosphorylation, the kinase becomes even more highly activated by phosphorylation at a location well-removed from the active site. This is the most active form of the complex and it occurs near the G_2/M transition. After metaphase, however, the complex must be removed so that the cells can exit anaphase. To accomplish this, the cyclin is degraded via a ubiquitin-dependent pathway. The processes

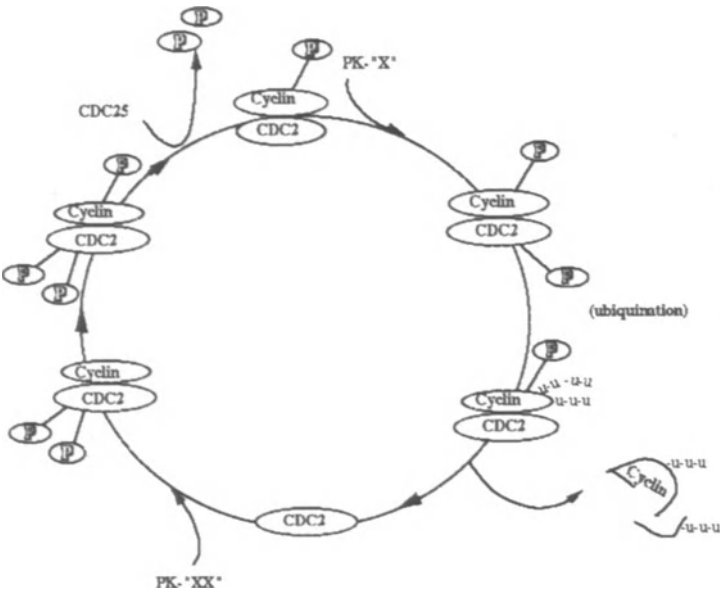


Fig. 5 Regulation of $p34^{cdc2}$ kinase activity requires a tightly controlled series of events including transient phosphorylation and nuclear localization at the end of S phase while in complex with cyclin; the cyclin is degraded at the end of anaphase and the kinase becomes inactive.

SUBSTRATES

G_1 and S	G_2 and M
Rb	**Lamins**
p53	**nucleolin**
RNA polymerase II CTD	**CBP**
histones	**myosin light chain**
RF-A	**pp60 *src***
c-abl	**histones**

Fig. 6 A list of known substrates for active cyclin-kinase complexes.

that govern the activity of the G_1/S complexes have not been as thoroughly characterized but seem to have similarities in that the kinase protein is phosphorylated and may persist while the cyclins are degraded.

The role of cyclin-kinase complexes in the regulation of cell cycle can be inferred from a list of their substrate specificities (shown in Fig. 6[6,7]). The complexes active during G_1 and S phases act to phosphorylate the tumor-suppressor genes Rb and p53, as well as RF-A of the replication initiation complex and RNA polymerase II C-terminal domain (CTD). These events are part of the phosphorylation cascade known to potentiate entry into cell cycle and replication. Recently, it has been shown that cyclin A binds to p107, a so-called "pocket binding protein" like Rb. The transcription factor, E2F, also binds this protein. Both cyclin A and E2F can be dislodged from p107 by the adenoviral protein, E1a, as depicted in Fig.7. This transforming protein binds tightly to cyclin A, and can be co-immunoprecipitated when using antibody to cyclin A [19]. At present, it is not clear precisely how these interactions might affect gene transcription but it is most likely that they do. Complexes active during G_2 and M phases phosphorylate the nuclear lamins and nucleolin, myosin light chain, centromere binding protein (CBP) and histones. These events permit nuclear envelope breakdown, organization of mitotic spindles, and packaging of chromatin for mitosis.

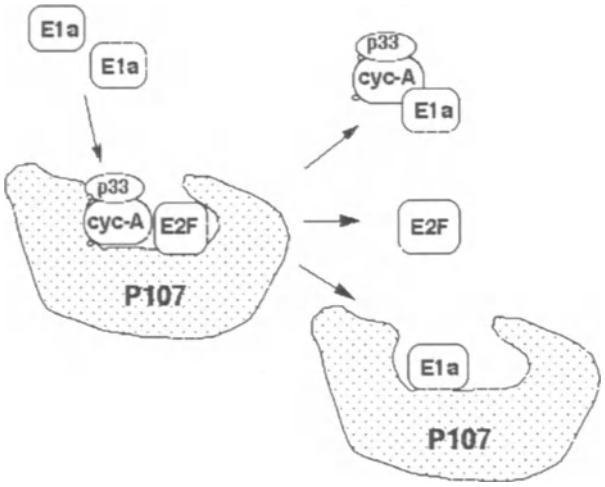


Fig. 7 Cyclin A forms a complex with the transcription factor E2F and both bind P107. These complexes are disrupted by adenoviral protein E1a [17,18].

So far, these processes have been defined predominantly in yeast, HeLa cells, and *Xenopus* oocytes. To study their behavior in murine cells of hematopoietic origin, we made a preliminary survey of a panel of tumor cells and normal tissues.

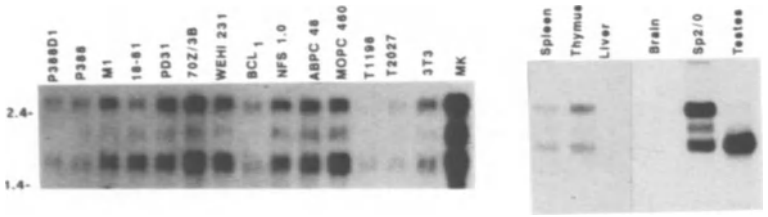


Fig. 8 Northern hybridization analysis of poly(A)+ mRNAs from a panel of transformed cell lines and normal tissues using cyclin B1 cDNA as a probe.(Adapted from [20].)

Figures 8 and 9 show northern analyses of various cell lines and mRNAs from several normal tissues with murine cDNA probes to cyclin B1, CYL-1, and CYL-3. There are three cyclin B1 transripts in samples from each of the cell lines and tissues in which cells are dividing. The CYL-3 probe hybridizes to one or two transcripts from each source, and we found these transcripts to be present in every tissue. In contrast, the CYL-1 cDNA probe displays tissue-specific expression, hybrizing strongly to two transripts in mRNA from cells of myeloid, fibroblastic, or epithelial origin, but weakly to a smaller transcript in samples from each source when moderately stringent conditions are used for hybridization and washing. CYL-1 is a murine homologue of human cyclin D(PRAD1) [21]. We are in the process of isolating the cDNA for the transcript expressed in B lymphocytes.

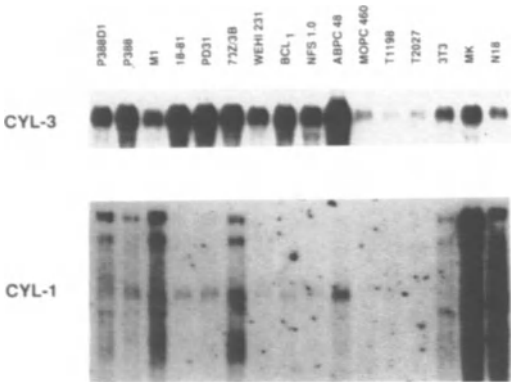


Fig. 9 Northern analyses of the same panel of mRNAs as in Fig. 8 with cDNA probes encoding CYL-3 and CYL-1 demonstrate that CYL-1 expression is cell type-specific but CYL-3 is present in each sample.

During these studies, we also observed that the steady-state level of mRNAs for cyclin B1 is decreased in 3T3 cells that are not dividing as a result of serum deprivation. As shown in Fig.10, addition of serum and FGF to these cells induces a rapid and dramatic increase in detectable levels of mRNA for cyclin B1. Also, there is an apparent "superinduction" when the cells are stimulated in the presence of cycloheximide.

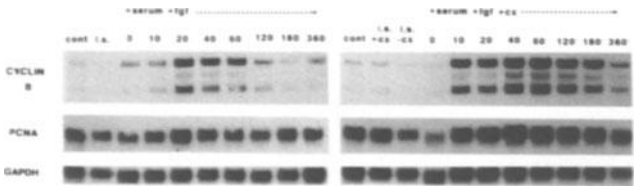


Fig. 10 Cyclin B1 expression is modulated in response to serum in serum-deprived 3T3 cells and is "super-induced" by refeeding in the presence of cycloheximide. (Adapted from [20].)

Interestingly, the levels of CYL-3 were found to remain relatively constant, as was seen for PCNA mRNA. To analyze the regulation of cyclin mRNA expression in factor-dependant cells, the IL-6 dependant plasmacytoma,TEPC-1165, was starved for IL-6 for 16 hours and refed. The steady-state levels of transcripts encoding cyclin B1, murine $p34^{cdc2}$ kinase, and CYL-3 from four timepoints thereafter were analyzed by northern hybridization. As shown in Fig.11, while the levels of mRNAs for cyclin B1 were modulated, there was no apparent change in levels of transcripts for either the kinase or CYL-3. From this it would appear that transformation might prevent a timely exit from the cell cycle by inducing constitutive expression of some of the gene products that regulate events occuring early in this process, thus providing for continuous re-entry into G_1.

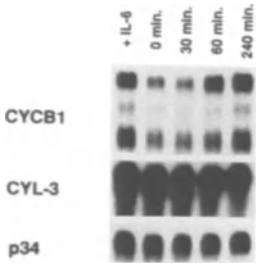


Fig 11. When TEPC 1165 plasmacytoma cells are starved for IL-6 and refed after 16 hours, cyclin B1 levels vary over time but not mRNAs for $p34^{cdc2}$ kinase or CYL-3.

Clearly, all of these studies are "work-in-progress". Even so, it is somewhat satisfying to see connections emerge between known oncogenes or tumor suppressors and the macromolecules that control cell division, as the pathway(s) of the cell division cycle unfold.

The Localization of the Products of the c-*cbl* and v-*cbl* Oncogenes During Mitosis and Transformation

W. Y. LANGDON, K. G. HEATH and T. J. BLAKE

Hanson Centre for Cancer Research, Adelaide, South Australia, 5000, Australia.

Introduction

The study of proto-oncogenes has been responsible for the elucidation of a wide range of genes that encode proteins with functions that are critical in regulating cell growth and development. Although the number of proto-oncogenes is large there presently appears to be only three biochemical mechanisms by which these genes act (Bishop, 1991). These are the phosphorylation of proteins with serine, threonine and tyrosine as substrates, the transmission of signals by GTPases and the regulation of gene transcription. However, the functions of some oncogenes remain unknown and as such it is possible that this list of mechanisms may be expanded in the future.

The c-*cbl* proto-oncogene is in the category where a biochemical mechanism has yet to be determined. This oncogene was discovered in the acutely transforming Cas NS-1 retrovirus which induces pre-B cell lymphomas in mice and transforms fibroblast cell lines *in vitro* (Langdon et al, 1989). The transforming protein of the Cas NS-1 retrovirus is a 100kD *gag*-v-*cbl* fusion protein of which 40kD is encoded by v-*cbl*. The human and mouse homologs of v-*cbl* have been cloned and sequenced and the sequence has revealed that v-*cbl* is a markedly truncated form of c-*cbl* that encompasses 355 N-terminal amino acids of 913 amino acids in the complete protein (Blake et al, 1991). The sequence also demonstrated a high level of conservation between the mouse and human genes with 98% identity within the v-*cbl* region and 93% identity over the entire coding region. Searches of DNA and protein data bases have not revealed any known gene with significant similarities to c-*cbl*, and as such the sequence has not provided definitive clues for a possible function. To aid our studies investigating the function of c-*cbl* we have raised antibodies that identify a 120kD protein. In this report we describe the characteristics of this protein, and $p100^{gag\text{-}v\text{-}cbl}$, during the cell cycle and following transformation.

Materials and Methods

Anti-cbl Antibodies

Fragments from the human c-*cbl* cDNA which encode amino acids 143-450 and 540-906 were inserted into pGEX expression vectors (Smith and Johnson, 1988) and expressed in *E. coli* as glutathione-S-transferase (GST)-cbl fusion proteins. Rabbits were immunized with each fusion protein and antibodies affinity purified on Affi-Gel (BioRad) - GST-cbl columns.

Immunofluorescence

HeLa cells were grown on glass slides and fixed in methanol for 5 min and acetone for 2 min at -20°C. HEp.2 cells grown on glass slides and fixed using an acetone-based fixative were purchased from Protrac Industries, San Antonia, Texas. A sheep anti-rabbit Ig-FITC was purchased from Silenus, Australia. Immunofluorescent cells were viewed using an Olympus fluorescent microscope attached to a confocal microscope (BioRad).

Cell Fractionation and Western Blotting

Cells were fractionated into nuclear and cytoplasmic fractions in PBS containing 1% Triton X-100, 1mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin. Total cellular membranes were prepared in 0.25M sucrose, 10mM Hepes pH7.5 plus protease inhibitors. Cells at $5x10^6$/ml were dounce homogenized, nuclei removed at 4,000g for 15 min and membranes and cytoskeleton pelleted at 100,000g for 40 min. The supernatant contained the cytosolic fraction. Proteins in each fraction were analysed by Western blotting as previously described (Blake and Langdon, 1992).

Centrifugal Elutriation

HeLa cells at 10^7/ml in 15ml of PBS, 0.3mM EDTA, 1% FCS and 0.1% glucose were loaded at 6.5ml/min with a rotor speed of 1550rpm using a Beckman JE-5.0 elutriator centrifuge with a Sanderson chamber. Following a 500ml of buffer wash, fractions were elutriated by increases of pump speed from 7.5-18.5ml/min. Fractions were analysed for stages of the cell cycle by propidium iodide staining of DNA and flow cytometry and for cbl protein by Western blotting.

Results

Localization of p120 in the Cytosolic and Membrane Fractions

Antibodies to c-*cbl* readily detect a 120kD protein in a wide range of cell lines. This protein is the same size as *in vitro* translated c-*cbl* in rabbit reticulocyte lysates and is commonly detected as a doublet suggestive of hyperphosphorylation of the upper band. Cells fractionated into cytoplasmic and nuclear components and examined by Western blotting clearly show the 120kD protein to be localized in the cytoplasm (Fig. 1). Additional

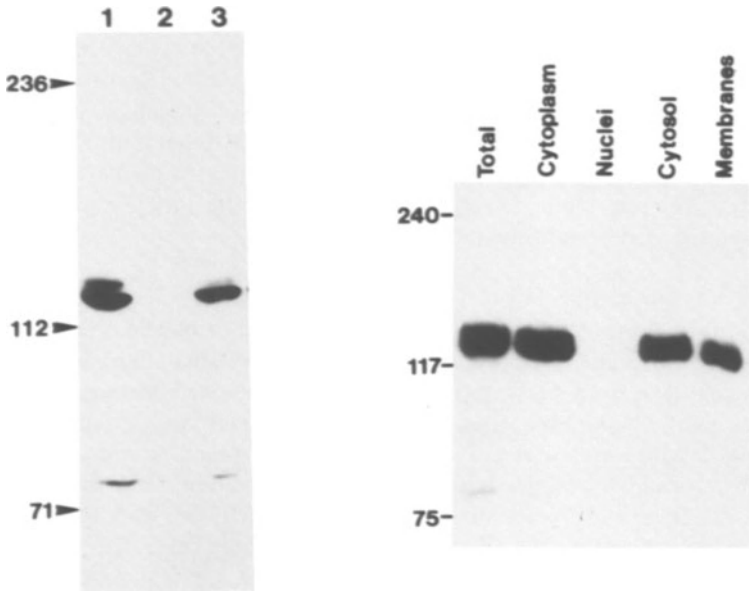


Fig. 1. Western blot of Jurkat cells probed with rabbit antibodies to the C-terminal region of c-*cbl*. Lane 1. Cytoplasm; 2. Nuclei; 3. Total cell extract.

Fig. 2. Western blot of NIH3T3 cells infected with a pJZenNeo retrovirus containing the complete human c-*cbl* coding region. The fractionated cell extracts were probed with rabbit antibodies to the C-terminal of c-*cbl*.

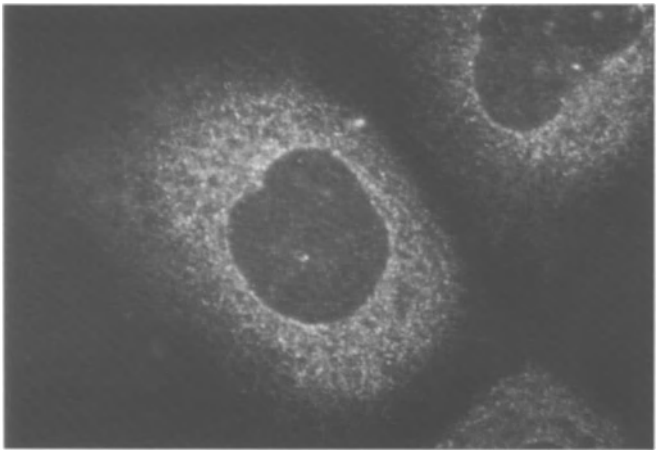


Fig. 3. Cytoplasmic immunofluorescence of HeLa cells incubated with rabbit antibodies to the C-terminal region of c-*cbl*.

fractionation of the cytoplasm into cytosolic and membrane components indicate p120 is evenly distributed between these components (Fig. 2). Immunofluorescence studies of HeLa and Hep-2 cells are consistent with these findings since a reticular pattern is evident in the cytoplasm which suggests p120 may be associated with the endoplasmic reticulum (ER) (Fig. 3). However, we have not discounted the possibility that p120 may associate with the cytoskeleton and experiments that separate membranes from the cytoskeleton will be informative.

Bright Vesicular Staining of Cells During Mitosis

A striking feature of the immunofluorescence studies of HeLa and Hep-2 cells is the bright vesicular staining observed in mitotic cells (Fig. 4). This pattern is first evident from early prophase and is maintained through all stages of mitosis until cytokinesis when the vesicles disperse and a region of intense staining is concentrated at the midbody. The fragmentation of the ER and the formation of small vesicles has been observed in a number of cell lines during mitosis (Zeligs and Wollman, 1979). These small vesicles reassemble after cell division to reform the ER of each new daughter cell. The vesicles shown in Figure 4 appear larger than those normally seen for fragments of ER during mitosis, and this observation adds some doubt whether they are ER-derived vesicles.

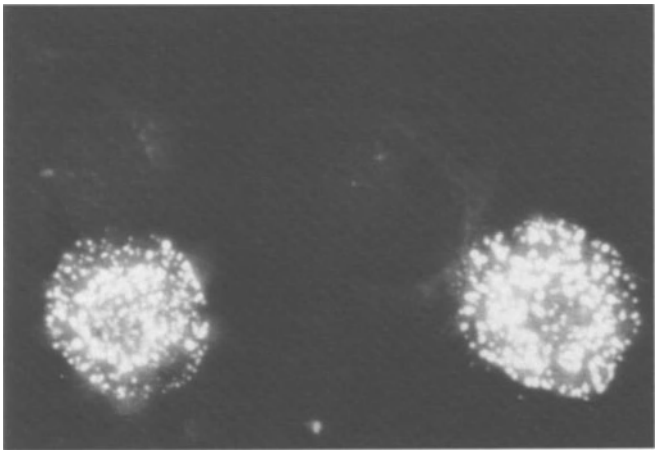


Fig. 4. Bright vesicular immunofluorescence of mitotic HeLa cells incubated with rabbit antibodies to the C-terminal region of c-cbl. An interphase cell is located between the two mitotic cells.

The intense immunofluorescence of the vesicles in mitotic cells is suggestive of a quantitative change in the level of cbl protein as the cells enter mitosis. To examine this possibility HeLa cells were fractionated by centrifugal elutriation to obtain cells enriched for each stage of the cell cycle. Cells harvested throughout the cell cycle and analysed by Western blotting showed no quantitative or qualitative changes to p120 (Fig. 5). This finding indicates another mechanism must be responsible for the intense vesicular staining. It is possible that a conformational change, or the release of an associated protein, may reveal additional antigenic sites for antibody binding. Alternatively, vesicle formation may concentrate p120 to a level where an intense fluorescence signal is observed. However, references to a similar phenomena of ER or cytoskeletal proteins have not been found in the literature. Another explanation could be a redistribution of the cytosolic p120 to the ER (or cytoskeleton) and this could account for the increased fluorescence. Fractionation of the soluble and insoluble components of the cytoplasm during the cell cycle would answer this possibility.

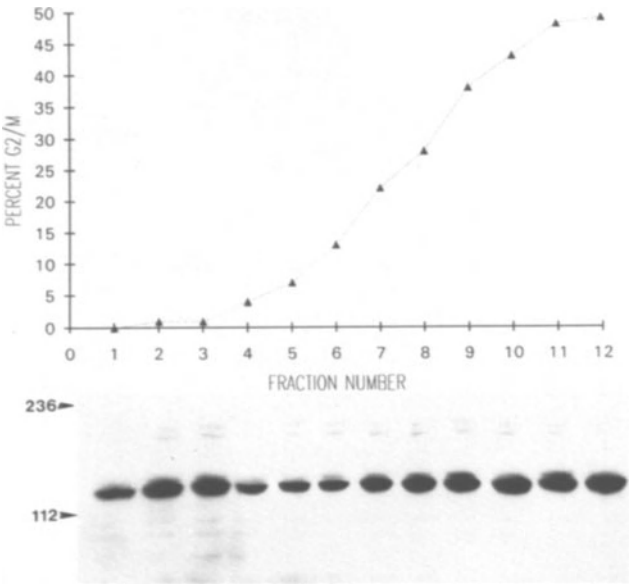


Fig. 5. Constant cell cycle regulation of p120. HeLa cells were separated by elutriation centrifugation and analysed at an enriched (100%) G_1 population i.e. fraction 1., through to an enriched (50%) G2/M population, i.e. fraction 12. The levels of p120 at progressive stages of the cell cycle were analysed by Western blotting.

p100$^{gag\text{-}v\text{-}cbl}$ is Localized to the Nucleus and Membranes

The results described above clearly indicate that p120 is localized in the cytoplasm. Furthermore, overexpression of c-*cbl* in the retroviral vector pJZenNeo did not alter this distribution (see Fig. 2). In contrast, however, p100$^{gag\text{-}v\text{-}cbl}$ is distributed both in the nucleus and the cytoplasm (Fig. 6). A protein this large requires an active nuclear localization sequence to the transported to the nuclear pore and into the nucleus (Siver, 1991) and the amino terminal region of c-*cbl*, which encompasses v-*cbl*, does contain a four residue consensus nuclear localization sequence of KKTK. Further fractionation of the cytoplasm into membrane and cytosolic components showed p100$^{gag\text{-}v\text{-}cbl}$ is entirely membrane associated in the cytoplasm. A likely explanation for this is the myristylation of gag which allows attachment to the plasma membrane.

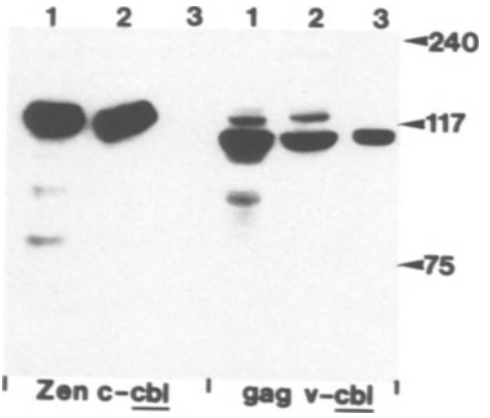


Fig. 6. Differential subcellular localizations of p120$^{c\text{-}cbl}$ and p100$^{gag\text{-}v\text{-}cbl}$. NIH3T3 cells infected with a pJZen c-*cbl* retrovirus and the Cas NS-1 retrovirus were examined by Western blotting using rabbit antibodies to the v-*cbl* region of c-*cbl*. Lane 1. Total cell extract; Lane 2. Cytoplasm; Lane 3. Nuclei.

In contrast to p100$^{gag\text{-}v\text{-}cbl}$ (which has 360 N-terminal amino acids of c-*cbl*) another truncated form of *cbl* which was found in HUT78 T cell lymphoma cells and encodes 647 N-terminal amino acids (Blake and Langdon, 1992) is totally cytoplasmic in its localization. Furthermore, the ability to bind DNA correlates with nuclear localization, i.e. the v-*cbl* encoded protein binds DNA whereas the HUT78 *cbl* and the full length c-*cbl* encoded proteins show no evidence of DNA binding (Fig. 7).

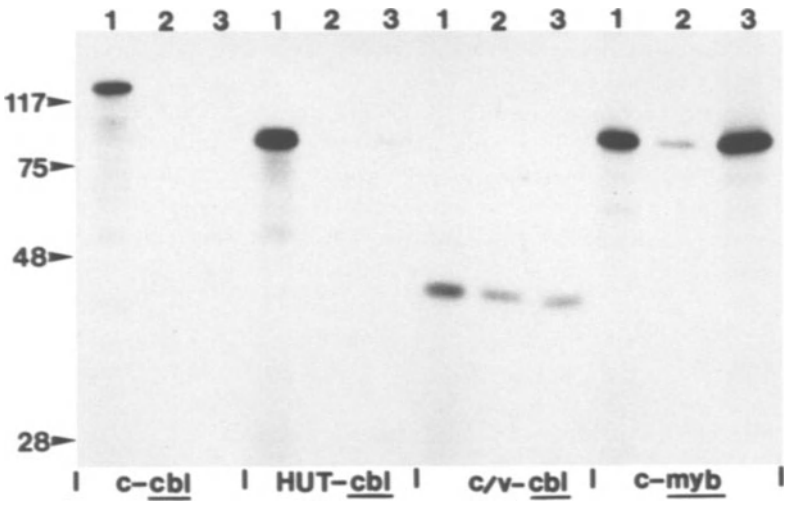


Fig. 7. Differential DNA-binding of c-*cbl* and v-*cbl* encoded proteins. *In vitro* translated c-*cbl* (906aa), HUT c-*cbl* (655aa) cellular v-*cbl* (360aa) and c-*myb* encoded proteins were examined for abilities to bind dsDNA cellulose. Lane 1. Unbound; Lane 2. Protein eluted by 500mM NaCl wash; Lane 3. Retained protein.

Discussion

The sequence of the c-*cbl* oncogene suggests it does not encode a kinase or a GTPase, and the cytoplasmic localization of p120 precludes the possibility that this protein functions as a transcription factor. Furthermore, its association with the ER (or cytoskeleton), and its bright vesicular staining during mitosis, distinguish it from other proteins encoded by known oncogenes. This would suggest that p120 has a biochemical function not yet defined for an oncogene. However, the nuclear localization and DNA binding of the truncated v-*cbl* encoded protein may be providing clues that a naturally occurring truncated form of cbl may exist and could function as a transcription factor. Our studies with the v-*cbl* encoded protein clearly indicate that removal of 556 C-terminal amino acids dramatically alters its localization and DNA binding. This suggests a masking of the nuclear localization sequence and DNA binding domains by the C-terminal region. A mechanism of intramolecular masking of nuclear localization has recently been described for the p110 precursor for the p50 NF-κB subunit (Henkel et al, 1992). To date we have not identified a p50 equivalent for c-*cbl*, however an examination of a larger number of cell lines and activation regimes may enable the detection of such a protein.

A possible explanation for our inability to detect a truncated form of cbl protein in the nucleus could be its instability. We have noted very low levels of the 40kD cellular version of v-*cbl* (i.e. c/v-*cbl*) expressed in a retroviral vector compared to complete c-*cbl* in the same vector. However low levels of the 40kD protein are still acutely transforming suggesting this protein has a potent effect on the regulation of cell growth. Indeed in some transformed clones infected with the c/v-*cbl* retrovirus we have been unable to detect the 40kD protein. This observation suggests it may be a difficult task to detect a "naturally" truncated form of *cbl* in the nucleus.

References

Bishop MJ (1991) Molecular themes in oncogenesis. Cell 64:235-248.

Blake TJ, Shapiro M, Morse HC, Langdon WY (1991) The sequences of the human and mouse c-*cbl* proto-oncogenes show v-*cbl* was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. Oncogene 6:653-657.

Blake TJ, Langdon WY (1992) A rearrangement of the c-*cbl* proto-oncogene in HUT78 T lymphoma cells results in a truncated protein. Oncogene 7 (in press).

Henkel T, Zabel U, vanZee K, Muller JM, Fanning E, Baeverle PA (1992) Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-κB subunit. Cell 68:1121-1133.

Langdon WY, Hartley JW, Klinken SP, Ruscetti SK, Morse HC (1989) v-*cbl*, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. Proc Natl Acad Sci USA 86:1168-1172.

Smith DB, Johnson KS (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-S-transferase. Gene 67:31-40.

Silver PA (1991) How proteins enter the nucleus. Cell 64:489-497.

Zelligs JD, Wollman SH (1979) Mitosis in rat thyroid epithelial cells in vivo. Ultrastructural changes in cytoplasmic organelles during the mitotic cycle. J Ultrastruct Res 66:53-77.

V-*rel* and C-*rel* Modulate the Expression of Both Bursal and Non-Bursal Antigens on Avian B-Cell Lymphomas

E. H. HUMPHRIES and G. ZHANG

Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV. 26506

Introduction

Infection of chickens with the avian retrovirus, Reticuloendotheliosis virus-T [REV-T(CSV)], results in the rapid development of a variety of hematopoietic tumors [2,4]. Typically, the birds die between 1 and 2 weeks bearing pauciclonal tumors with extensive metastatic disease. Examination of cell surface lineage specific markers demonstrates that these tumors can be derived from B-cells, T-cells and cells within the myeloid lineage [1,3]. Genetic experiments have demonstrated that tumor development results from the expression of the viral oncogene, v-*rel* [5,6]. V-*rel* is related to the proteins p50 and p65 that comprise the transcriptional complex NF-*k*B [7,17,18,21]. Extensive analysis has demonstrated that this complex regulates transcription through the formation of heterodimers that bind DNA [15,22]. Transcriptional regulation of a number of proteins may be regulated in part through the interaction of NF-*k*B protein with NF-*k*B consensus sequence in the promoter region of these genes [7,15]. Transcription would appear to be activated by external signals that interact with cell surface receptors and result in the conversion of NF-*k*B from a cytoplasmic protein complex to a nuclear complex that can bind DNA [16].

A variety of *rel* -related proteins have been identified and it seems likely that transcriptional regulation may be mediated in the form of several different heterodimers and, perhaps homodimers [19]. The mechanism through which v-*rel* induces transformation is unknown at present but two observations suggest that the induction of aberrant transcription plays a significant role in this process. First, v-*rel* is a mutant protein that has, in addition to several point mutations and small deletions, a major deletion of the carboxy terminus that functions in assays that measure the transactivation component of *in vitro* transcription [19,20]. Therefore, while v-*rel* may be able to bind DNA and form dimeric protein complexes, its ability to regulate transcription is probably compromised through the formation of aberrant protein complexes or the inability to form normal complexes. Normal regulation is almost certainly altered further as a result of the high levels of v-*rel* that are initiated from the retroviral LTR. Second, tumor development is normally a multistep process so that a stochastic accumulation of several mutations collaborate over an extended period of time to promote tumor development [8]. In contrast, v-*rel*-induced tumors develop within a very short period of time indicating that the expression of this oncogene induces multiple genetic and\or epigenetic alterations. One model that fulfils this functional requirement proposes that v-*rel* induces several aberrant transcriptional changes characterized by both the induction and repression of gene expression.

In this report, we describe seven cell surface markers that are modulated by the expression of v-*rel*. Five of these antigens are found on the surface of normal B-cells. Two others are apparently not present on bursal B-cells and appear to be expressed ectopically on the v-*rel*

Current Topics in Microbiology and Immunology, Vol. 182

induced B-lymphoma. The functional significance of this aberrant expression for tumor development has not yet been addressed.

Results

Characterization of Two Non-Bursal Antigens

Last year we described the isolation of three mouse monoclonal antibodies that reacted with cell surface proteins that are absent from the DT40 cell line but that appear following infection of that cell line with wild type REV-T(CSV) [14]. These monoclonal antibodies are designated HY78, HY82 and HY84. They immunoprecipitate proteins of appoximately 120kD, 80kD and 35kD (Fig. 1). This analysis reveals that all three monoclonal antibodies precipitate two major species of protein. Labelling studies with ^{35}S-methionine and 2-deoxyglucose indicate that the larger of the forms is glycosylated (data not shown). Immunoprecipitation of the non-glycosylated proteins demonstrates that they are approximately 90kD, 60kD and 30kD in mass. The proteins precipitated by HY84 from the two cell lines examined differ and may represent differences related to the fact that one line is B-cell in origin while the other is a non-B\non-T cell.

As an initial attempt to characterize these three proteins, their presence on hematopoietic cell was quantified by flow cytometric analysis. A typical profile is shown in Fig. 2 where bursal, splenic and bone marrow cells from a 4 week old chick were stained for the expression of p120. This analysis reveals that less than 5% of bursal cells express p120. Direct microscopic analysis of these bursal cells reveals no significant staining of this population. Similar results were obtained following the staining of splenic cells (Table 1). While approximately 20% of these cells stain faintly by flow cytometric analysis, no significant staining is observed microscopically. These and other data obtained from a similar analysis of both thymus and bone marrow, indicate that in some tissues a significant population may express very low levels of p120. Whether or not this weak staining represents a low abundance of p120 or a technical artifact remains to be resolved. In contrast, approximately 15-20% of the bone marrow population expressed high levels of

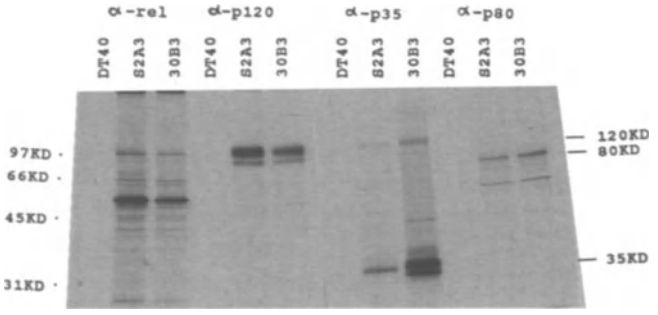


Fig 1. Immunoprecipitation of proteins by monoclonal antibodies HY78, HY82 and HY84. ^{35}S labeled lysates from three cell lines, DT40 (an avian B-cell line that does not express v-*rel*), S2A3 (a non-B\non-T cell line transformed by v-*rel*) and 30B3 (a B-cell transformed by v-*rel*) were reacted with monoclonal antibodies HY78, HY82 and HY84 and the proteins analyzed by immunoprecipitation and 10% polyacrylamide gel electrophoresis.

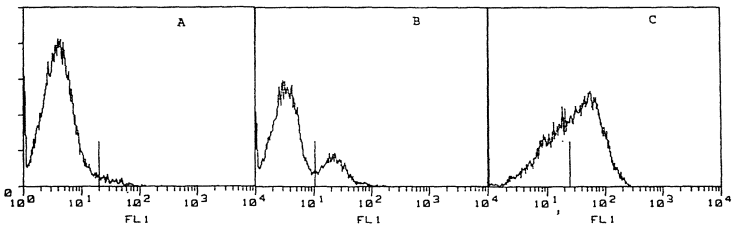


Fig. 2. Flow cytometric analysis of avian hematopoietic tissues for the expression of p120. Single cell suspensions of bursa, spleen and bone marrow were stained with HY78. The vertical line indicates the position of less than 5% staining with FITC conjugated goat anti-mouse Ig antibody alone.

p120 and could be detected microscopically. This population consisted of cells that were large and possessed a large and granular cytoplasm similar to that observed in granulocytes. A larger population, approximately 50% stained only weakly as detected by flow cytometric analysis. These data are summarized in Table 1 and differ slightly from data published earlier in that the low level staining was not previously included [14]. A similar analysis of other avian embryonic and hematopoietic cell suspensions is also summarized in Table 1. There are three notable features regarding the distribution of these antigens. First, p80 is

Table 1. Distribution of p35, p80 and p120 in avian embryonic/hematopoietic cell suspensions

	p35	p80	p120
Embryonic cell suspension			
2 day	-	-	-
4 day	-	-	20%
7 day	-	-	10%
12 day	-	-	5%
Embryonic bursa			
11 day	-	-	-
15 day	25%	-	-
Adult chicken tissue suspension (3-4 wks old)			
Bursa	45%(80%)	-	<1%(<5%)
Spleen	35%(50%)	-	<1%(20%)
Thymus	5%(40%)	-	<1%(50%)
Bone Marrow	20%(50%)	-	15%(60%)
White Blood Cells	15%(?)	-	<1%(?)
Red Blood Cells	-	-	- -

Percentage positive cells are presented for both microscopic analysis and flow cytometric analysis ( ).

not present in any of the cells analyzed. Second, p120 is not present on bursal B-cells. Whether or not it is present on any Ig positive cells in the spleen, thymus or bone marrow is currently under investigation. Third, the distribution and mass of p35 suggest a similarity to avian MHC class II molecules (Hrdlickova,R., Nehyba, J. and Humphries, E. Unpublished data).

The distribution of these proteins was examined further using *in situ* immunohistochemistry. Frozen sections from different tissues were incubated with monoclonal antibodies HY78, HY82 and HY84 and developed with horse radish peroxidase. P120 was detected in a large number of tissues, including gut, liver, spleen and kidney. It was most commonly present at tissue boundries including venules and sinusoids of the liver, blood vessels in the spleen and liver and the mucosal boundry of the gut. One of its most striking distributions was in the bursa where it appeared present on cells that divide the cortex and medullary compartments of the follicles (Fig. 3). Radiating from these cells, p120 was also on the denditric or stromal cells that are found within the medulla. Consistent with the analysis

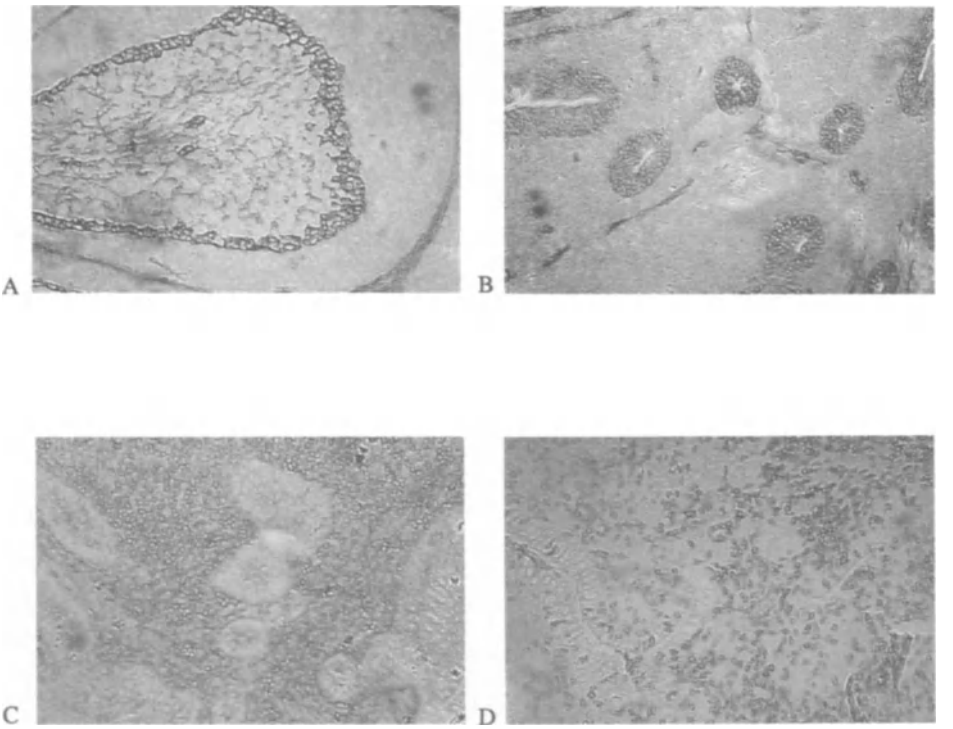


Fig. 3. Frozen tissue from bursa (A) and caecal tonsil (B,C and D) were analyzed by immunohistochemistry using horse radish immunoperoxidase following staining with HY78 (A), HY82 (B), anti-IgM (C) or anti-CT3 (D).

already presented, it was not present on the bursal B-cell population. It was also absent from the major populations of hematopoietic cells within the spleen although staining of a small percentage of B or T cells could not be excluded. In contrast, p80 was observed in only one tissue examined, the caecal tonsil (Fig. 3). Staining by HY82 was confined to epithelial cells within the glands of Lieberkuhn. Areas that contain B-cells (stained by IgM) or T-cells (stained by CT3) were negative for the expression of p80 (Fig. 3).

These data support one major conclusion. The development of B-cell lymphomas as a consequence of the expression of v-*rel* results in the expression of two cell surface proteins on the tumor that have not been detected on normal B-cells. One of these proteins, p80, has been observed only in epithelial cells in the glands of Lieberkuhn within the caecal tonsils. This protein has not been detected in any other tissue examined thus far. The other protein, p120, while detected in a variety of tissues, does not appear to be present on lymphoid cells including bursal B-cells. It is present in bursal tissue but only in association with stromal or denditric cells. While it is possible that either or both of these proteins may be expressed on normal B-cells during some restricted phase of differentiation or at the time of a specialized physiological response, at present it appears that p80 and p120 may be expressed ectopically on v-*rel* induced B-cell lymphomas.

Induction of bursal and non-bursal antigens

In order to establish a system with which v-*rel*-induced gene expression could be examined, a B-lymphoid cell line established by ALV insertional mutagenesis of the c-*myc* locus, DT95 [10] was cocultivated with fibroblasts infected with a retroviral vector expressing either v-*rel* or c-*rel* (Hrdlickova, R., Nehyba, J. and Humphries, E. Unpublished data). Following 8 hours of cocultivation, the cell line was recovered and cultured for an additional 72 hours. The expression of v-*rel* and c-*rel* as well as p35, p80 and p120 were monitored by dual color immunofluorescence. This analysis (Fig. 4) shows that, within 24 hours post cocultivation, nearly all DT95 cells express either v-*rel* or c-*rel*. The three cell surface proteins were induced within 48 hours. While it appeared that p120 was induced most rapidly and p80 most slowly, potential differences in sensitivity based upon monoclonal antibody affinities makes such conclusions impossible at present. A similar experiment was

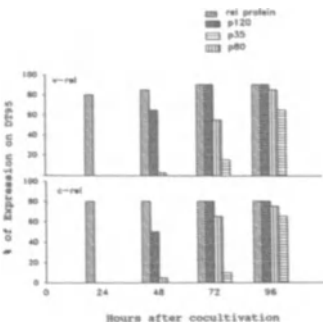


Fig. 4. Induction of p35, p80 and p120 expression in DT95 following expression of v-*rel* or c-*rel*. DT95 in the absence of infection with either retroviral vector does not express detectable *rel* protein.

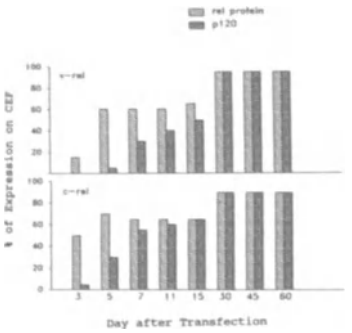


Fig. 5. Induction of p120 expression in fibroblasts following transfection with retroviral vectors that express either v-*rel* or c-*rel*.

conducted to examine the induction of these proteins in fibroblasts (Fig. 5). Following transfection of uninfected fibroblasts with plasmid constructs carrying the helper virus CSV and an REV-T based retroviral vector expressing either v-*rel* or c-*rel*, cultures were examined for the expression of p35, p80 and p120 (Fig. 5). Unlike the expression observed in DT95, only p120 was induced during the first 60 days of observation. In neither experiment was the expression induced by v-*rel* different from that induced by c-*rel*. These results are summarized in Table 2. The induction of these proteins was analyzed further in cell lines established following *in vitro* transformation of splenic mononuclear cells. Clones transformed by v-*rel* or infected by c-*rel* were examined for the expression of the three cell surface proteins by immunofluoresence (Table 2). All clones transformed by v-*rel* expressed p35, p80 and p120. In contrast, while all the clones infected by c-*rel* expressed p35 and p120, only half expressed this protein indicating that the expression of p80 may be regulated differentially by v-*rel* and c-*rel*.

A slightly different system was utilized in order to examine the effect of v-*rel* on the expression of bursal cell antigens. The DT40 cell line, a B-cell lymphoma like DT95 that was established following ALV insertion within the c-*myc* locus, as well as different clones

Table 2. The expression of p80 is differentially regulated by v-*rel* and c-*rel* and has a tissue-specific induction pattern

	p120	p80	p35
DT95/v-*rel*	+	+	+
DT95/c-*rel*	+	+	+
CEF/v-*rel*	+	-	-
CEF/c-*rel*	+	-	-
v-*rel* splenic clones	24/24	24/24	24/24
c-*rel* splenic clones	27/28	16/28	28/28

Table 3. Rel-inducted modulation of bursal and non-bursal antigens

	CTI	CLAI	IgM	Bu1	Bu2	p35	Hy28	Hy29	p80	p120
DT40	-	+	+	+	+	-	-	-	-	-
DT40(CSV)	-	+	+	+	+	-	-	-	-	-
DT40(T)	-	+	-	-	-	+	-	-	-	+
DT40(C)	-	+	+/-	-	-	+	-	-	-	+
30B3	-	+	+	-	+	+	-	-	+	+
Bursa	-	+	+	+	+	85%	65%	+	-	-
Spleen	15%	+	30%	45%	40%	45%	<1%	<5%	-	<1%

of DT40 infected with a retroviral vector that expresses either v-*rel* or c-*rel*, were examined for the presence of IgM heavy chain, IgM light chain as well as the two bursal antigens Bu-1 and Bu-2 (Table 3). A clone of DT40 that expresses only the helper virus, CSV, as well as a v-*rel*-induced B-cell lymphoma derived cell line, 30B3, were examined in parallel as controls. DT40 is most likely to have developed from a preneoplastic bursal B-cell [9]. It is characterized by ongoing gene conversion of the variable region in the light chain locus, a property shared with most bursal B-cells [11,12,13]. It is also similar to bursal B-cells in the expression of surface IgM, Bu-1 and Bu-2. The results indicate that, while infection with CSV does not alter the surface phenotype of DT40, the expression of either v-*rel* or c-*rel* inhibited the expression of not only the heavy and light chain proteins of IgM, but also of Bu-1 and Bu-2. In contrast to the expression of v-*rel* in DT40, 30B3 continues to express Bu-2. This apparent contradiction may result from the fact that 30B3 exhibits a more mature phenotype. Equally, the DT40 cell expresses high levels of *myc* and may represent a rather artificial system in which to observe *rel* regulation. In support of the former possibility, neither v-*rel* nor c-*rel* induce p80 in DT40 (Table 3) while both induce p80 in DT95 (Fig. 4 and Table 2). While both DT40 and DT95 express high levels of *myc*, based upon at least three different parameters, DT95 is a more mature B-cell [10]. The expression of CT-I, CLA-I, HY28 and HY29 serve as controls for the specificity of both induction and repression.

These observations demonstrate the feasibility of expressing either the v-*rel* or c-*rel* gene in B-cell lines immortalized by *myc* in order to examine the mechanism through which gene expression can be induced or repressed. The ability to examine these processes in a B-cell provides an additional tool relevant to the study of B-lymphoma development. These data also support the notion that elevated levels of either v-*rel* or c-*rel* establish, at least in part, similar aberrant expression profiles. Finally, it not surprising to find that both the induction and repression of gene expression involve tissue specific and, probably differentiation specific, factors.

Conclusions

The data presented in this report suggest that at least two non-bursal cell surface proteins are expressed ectopically on v-*rel*-induced B-cell lymphomas. Whether or not these proteins are ever expressed on any lymphoid cells remains to be determined. However, the absence of p80 and p120 on the surface of normal B-cells, cells in which c-*rel* is expressed at physiological levels, as well as the presence of these proteins on epithelial or stromal cells, makes this issue one of significant interest. Given the documented role of NF-*k*B in lymphoid cell differentiation in man and the mouse, it is not surprising that v-*rel* should be

observed to regulate normal bursal cell antigens. Using the two cell lines, DT40 and DT95, we have established an experimental system in which the data demonstrate that both v-*rel* and c-*rel* modulate five proteins normally associated with the bursal B-cell phenotype. Four of these proteins are repressed while one, p35, is induced. This experimental system provides several important opportunies. First, it makes available isogenic paired cell lines that differ only in the type of *rel* protein that they express. These lines, therefore, provide an excellent opportunity to identify additional genes that are regulated by *rel* proteins. These cell lines also make feasible the characterization of mutant *rel* genes that differ with respect to their ability to regulate transcription, aberrantly or normally. Finally, we have estimated from two dimensional protein gel electrophoresis that there are approximately 100 differences in expressed proteins that distinguish the DT40(CSV) and DT40(REV-T) cell lines. The identification of genes that play a significant functional role in the development of v-*rel*-induced B-cell lymphomas will probably be a difficult task. The approach we have described here, employed in conjunction with *rel* protein mutants ought to make that task easier.

Acknowledgements

We thank Radmila Hrdlickova, Kenneth Landreth and Jiri Nehyba for their assistance and discussions throughout this project. This work has been supported by Public Health Service Grant CA-41450 from the National Institutes of Health.

References

1. Barth, C.F., D.L. Ewert, W.C. Olson and E.H. Humphries (1990) Reticuloendotheliosis virus REV-T(REV-A)-induced neoplasia: Development of tumors within the T-lymphoid and myeloid lineages. Journal of Virology 64:6054-6062
2. Barth, C.F. and E.H. Humphries (1988) A nonimmunosuppressive helper virus allows high efficiency induction of B-cell lymphomas by reticuloendotheliosis virus strain T. Journal of Experimental Medicine 167:89-108
3. Barth, C.F. and E.H. Humphries (1988) Expression of v-*rel* induces mature B-cell lines that reflect the diversity of avian immunoglobulin heavy- and light-chain rearrangements. Molecular and Cellular Biology 8:5358-5368
4. Olson, L.D. (1967) Histopathologic and hematologic changes in moribund stages of chicks infected with T-virus. American Journal of Veterinary Research 28:1501-1507
5. Hoelzer, J.D., R.B. Lewis, C.R. Wasmuth and H.R. Bose, Jr. (1980) Hematopoietic cell transformation by reticuloendotheliosis virus: Characterization of the genetic defect. Virology 100:462-474
6. Hoelzer, J.D., R.B. Franklin and H.R. Bose, Jr. (1979) Transformation by reticuloendotheliosis virus: Development of a focus assay and isolation of a nontransforming virus. Virology 93:20-39
7. Lenardo, M.J. and D. Baltimore (1989) NF-*k*B: A pleiotropic mediator of inducible and tissue-specific gene control. Cell 58:227-229
8. Foulds, L. (1954) The experimental study of tumor progression: A review. Cancer Research 14:327-339
9. Baba, T.W. and E.H. Humphries (1985) Formation of transformed follicle is necessary but not sufficient for development of an avian leukosis virus-induced lymphoma. Proceedings of the National Academy of Science USA 82:213-216
10. Baba, T.W., B.P. Giroir and E.H. Humphries (1985) Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology 144:139-151
11. Ratcliffe, M.J.H. (1985) The ontogeny and cloning of B-cells in the bursa of Fabricius. Immuno Today 6:223-227

12. Reynaud, C-A., V.Anquez, H. Grimal and J-C. Weill (1987) A hyperconversion mechanism generates the chicken light chain preimmune hypertoire. Cell 48:379-388
13. McCormack, W.T., L.W. Tjoelker, C.F. Barth, L.M. Carlson, B. Petryniak, E.H. Humphries and C.B. Thompson (1989) Selection of B cells with productive IgL gene rearrangements occurs in the bursa of Fabricius during chicken embryonic development. Genes & Development 3:838-847
14. Humphries, E.H. (1991) Mechanisms of B cell neoplasia. Melchers, F. and Potter,M. (ed) Editiones Roche, Basel, Switzerland pp. 223-228.
15. Baeuerle, P.A. (1991) The inducible transcription activator NF-*k*B: Regulation by distinct protein subunits. Biochim. Biophys. Acta 1072:63-80
16. Bours, V., J. Villalobos, P.R. Burd, K. Kelly and U. Siebenlist (1990) Cloning of a mitogen-inducible gene encoding a *k*B DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. Nature (London) 348:76-80
17. Gosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan and D. Baltimore (1990) Cloning of the p50 DNA binding subunit of NF-*k*B: Homology to *rel* and dorsal. Cell 62:1019-1029
18. Gilmore, T.D. (1990) NF-*k*B, KBF1, dorsal and related matters. Cell 62:841-843
19. Gilmore, T.D. (1991) Malignant transformation by mutant *rel* proteins. Trends Genet. 7:318-322
20. Kamens, J., P. Richardson, G. Mosialos, R. Brent and T.D. Gilmore (1990) Oncogenic transformation by v-*rel* requires an amino-terminal activation domain. Mol. Cell. Biology 10:2840-2847
21. Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M.B. Urban, P. Kourilsky, P.A. Baeuerle and A. Israel (1990) The DNA binding subunit of NF-*k*B is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell 62:1007-1018
22. Blank, V., P. Kourilsky and A. Israel (1992) NF-*k*B and related proteins: *Rel*/dorsal homologies meet ankyrin-like repeats. TIBS 17:135-140

Why Don't Germline Mutations in *RB1* Predispose to Leukemia?

R. A. PHILLIPS[1,4,5], R. M. GILL[1,5], E. ZACKSENHAUS[5], R. BREMNER[5], Z. JIANG[5], M. SOPTA[5], B. L. GALLIE[1,3,5] and P. A. HAMEL[2]

Departments of [1]Medical Genetics, [2]Pathology, [3]Ophthalmology and [4]Immunology, University of Toronto, and [5]Division of Immunology and Cancer Research, The Hospital for Sick Children, Toronto, Canada M5G 1X8

Retinoblastoma and the *RB1* Gene

Retinoblastoma, a rare tumor of childhood, is interesting because it exists in both heritable and non-heritable forms (for review see [1]). In the non-heritable form, affected individuals develop only a single tumor in one eye. In contrast, in the heritable form, the affected individuals develop multiple tumors usually affecting both eyes. Heritable retinoblastoma has high penetrance with more than 90% of individuals carrying a germline mutation in the retinoblastoma gene (*RB1*) on chromosome 13 ultimately developing tumors. In addition, patients with a germline *RB1* mutation are susceptible to multiple other tumors, primarily osteosarcoma, fibrosarcoma, melanoma, small cell carcinoma of the lung and bladder carcinoma [2, 3]. However, such individuals appear not to have an increased risk for leukemias or other malignancies of the hematopoietic system [4].

In 1971 Knudson proposed that only two mutations are necessary to convert a normal retinal cell into a malignant retinoblastoma tumor cell [5]. In normal individuals, both mutations must occur in the same retinal progenitor cell. Since the time available for these mutations to occur is relatively short (retinoblastoma seldom develops in children older than 3 years), few individuals develop these mutations, and the incidence of retinoblastoma in the population is low, approximately 1 in 20,000 births. However, in individuals with a germline mutation, all cells in the retina have the one mutated *RB1* allele so that a single mutation of the remaining normal *RB1* allele in any retina cell will lead to retinoblastoma; since only a single mutation can cause cancer, such individuals are likely to accumulate mutations in more than one retinal cell and, thus, usually develop multiple tumors.

The cloning of the retinoblastoma gene [6] led to studies which have confirmed the basic elements of the two-hit hypothesis of Knudson [7]. Namely, in retinoblastoma tumors, both alleles of the *RB1* gene carry mutations. Individuals with heritable retinoblastoma have a mutation in one of their *RB1* alleles. In most cases, the mutations predisposing to retinoblastoma result in non-functional protein [7-9]. Thus, tumors arise when all functions of the retinoblastoma gene are destroyed by mutation, hence the name recessive oncogene or tumor suppressor gene.

The Product of the *RB1* Gene

Subsequent characterization of the protein product of the retinoblastoma gene produced many surprises. As shown by Lee et al., the retinoblastoma protein is a 110 kd nuclear phosphoprotein with phosphorylation detectable only on serine and threonine residues [10]. The major surprises were the findings that the *RB1* protein, $p110^{RB1}$, is expressed in almost all dividing cells [6, 8, 11, 12] and that it appears to play an important role in regulating proliferation in almost all cells. Given the limited number of tumors to which individuals with germline mutations in the *RB1* gene are predisposed, most investigators had predicted that the product of the *RB1* gene would be expressed only in a limited number of tissues. In particular, it was unexpected to find such high levels of $p110^{RB1}$ in hematopoietic cells since the incidence of leukemia is not increased in patients having heritable retinoblastoma. Another surprise was the finding that proteins produced by several DNA tumor viruses physically bound $p110^{RB1}$ [13-15]. In fact, disruption of the $p110^{RB1}$-binding sites on these viral proteins prevented the transforming ability of the tumor viruses. Thus, ability to bind $p110^{RB1}$ appears essential for the transformation initiated by these viruses.

Phosphorylation of p110^{RB1} During the Cell Cycle

Early studies on p110^{RB1} demonstrated that it was phosphorylated in a cell cycle-dependent manner (for review see [16]), being hypophosphorylated in quiescence cells and during G1, and hyperphosphorylated during S and G2 [17-19]. During mitosis, unknown phosphatases return p110^{RB1} to a hypophosphorylated state. The long half-life of the *RB1* protein [7, 8], greater than the cycle length of many rapidly proliferating tumor cells, indicated that phosphorylation was likely the most important mechanism for regulating its function. In addition, the observation that SV40 large T antigen preferentially bound the hypophosphorylated form of p110^{RB1} supported the conclusion that the hypophosphorylated form was functional and that the virus inactivated p110^{RB1} function by binding it to large T antigen [20]. Presumably, cells normally inactivate the p110^{RB1} by phosphorylating critical serines and threonines.

At least eleven potential p34^{cdc2} phosphorylation sites exist in p110^{RB1} (Figure 1), only a few of which have been unequivocally identified [21]. As well, the sites critical for controlling *RB1* function have not yet been defined. Hamel et al. showed that mutation of ser-804 in murine p110^{RB1} (equivalent to ser-811 in human, see Fig. 1) prevented the mobility shift associated with phosphorylation of the wild type protein but had no detectable effect on the overall level of phosphorylation [22]. Mutation of the other sites had no effect on the mobility shift. Simultaneous mutation of eight sites, those in exon 23 and the ones 5' of the T antigen-binding domains, dramatically decreased phosphorylation, but mutation of other sites either singly or in groups of 3 or 4 had no detectable effects on phosphorylation.

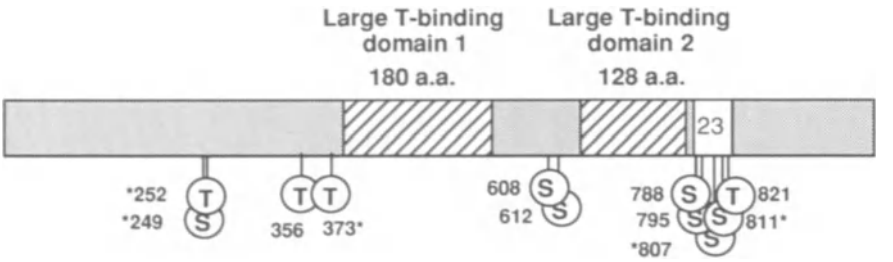


Fig. 1. Schematic representation of *RB1* protein, showing 11 potential p34^{cdc2} phosphorylation sites and the two domains required for binding to large T antigen. The amino acids coded by exon 23 are indicated because of the cluster of potential sites in this region. The five sites indicated with asterisks have been positively identified [21].

The kinases responsible for phosphorylating p110^{RB1} during the cell cycle have not yet been characterized. The cell cycle dependence of phosphorylation suggests that p34^{cdc2} or a related kinase phosphorylates p110^{RB1} [23, 24]. p107, a protein related to p110^{RB1} [25], is probably phosphorylated by a complex consisting of p33^{cdk2}, a p34^{cdc2}-related kinase, and cyclin A, since it is possible to isolate from dividing cells a complex containing p107 together with these proteins[24, 26]. By analogy, one may expect that p110^{RB1} will be phosphorylated by a similar cyclin complex, perhaps p33^{cdk2} with one of the newly isolated G1 cyclins (for review see[16]).

p110^{RB1} Regulates Transcription of c-*myc* and *RB1*

Several experiments suggest that in normal cells p110^{RB1} serves as an important regulator of transcription [16]. Although many cellular proteins can bind to p110^{RB1} fixed to a matrix [27], the best characterized interaction is the one between p110^{RB1} and a transcription factor called E2F [28-30]. E2F was initially identified as a cellular factor required for transcription of the adenovirus E2 protein [31]; it is now known that E2F also plays a role in the regulation of several cellular genes, particularly some of those involved in progression through the cell cycle. Of particular interest are DHFR [32] and c-*myc*. [33]. Promoters of both of these genes contain E2F-binding elements. In both promoters, mutation of the E2F-binding sites dramatically down

regulates expression. We have demonstrated that p110^{RB1} can down regulate the P2 promoter in c-*myc* and that this down regulation by *RB1* requires an intact E2F-binding site [33]. On the basis of our observations and work from other laboratories showing interaction between p110^{RB1} and E2F, we suggest that this interaction regulates transcription of c-*myc* and other cell cycle-dependent genes (cd-genes) [33]. Thus, in G_0/G_1, hypophosphorylated p110^{RB1} binds E2F preventing it from stimulating c-*myc* transcription. Subsequent phosphorylation of p110^{RB1} in late G_1 and S changes its conformation, resulting in release of E2F and stimulation of c-*myc* transcription (Fig. 2). Confirmation that the hypophosphorylated form of p110^{RB1} is functional comes from our observation that mutant forms of p110^{RB1} which are unable to be phosphorylated are super repressors of the c-*myc* promoter [33].

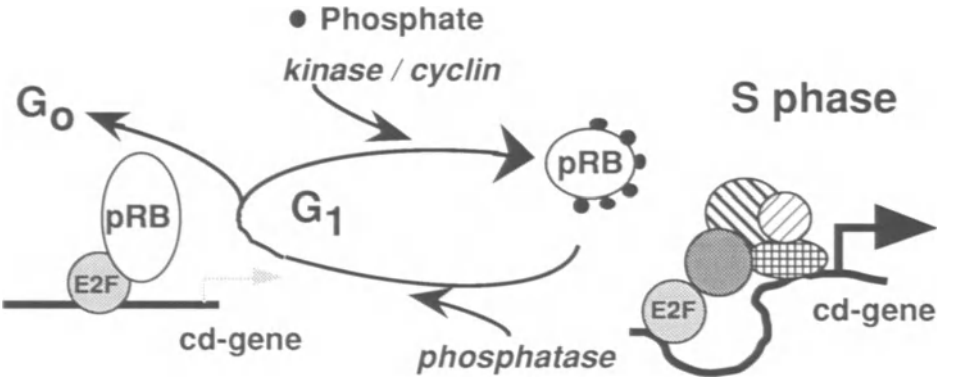


Fig. 2. Model for role of E2F and c-*myc* during cell cycle. In G_0 and G_1, p110^{RB1} binds to E2F and prevents transcription of cd-genes such as c-*myc* required for cell cycle progression. Following activation of undefined G_1 cyclins and kinases, p110^{RB1} becomes phosphorylated, allowing E2F to interact with various transcription factors required for transcription of cd-genes.

Interestingly, p110^{RB1} also appears to act as an autoregulator of its own transcription [33]. Thus, high levels of p110^{RB1} expression down regulate *RB1* transcription. Although both the human (Gill et al., in preparation) and mouse (Zacksenhaus et. al., submitteed) *RB1* promoter are highly conserved and contain an E2F-binding domain within the minimal promoter region, the precise mechanism by which p110^{RB1} represses *RB1* transcription remains to be elucidated.

It is unlikely that all the effects of p110^{RB1} on the cell are mediated entirely through E2F. Kaelin et al. identified approximately eight proteins which can bind to p110^{RB1} [27]. One of these proteins is clearly E2F. Two other proteins have also been cloned but have no known function [34]. Interestingly, Rustigi et al. have presented evidence that p110^{RB1} can also bind to c-*myc* [35], an observation confirmed by R. Bremner (Unpublished data). Given that p110^{RB1} can regulate the transcription of c-*myc*, this observation is intriguing and may indicate that *RB1* may modulate both the transcription of c-*myc* and the regulatory activities of c-*myc*.

Significance of Mutations in p110^{RB1} in Leukemia

The above conclusions on the roles of p110^{RB1} in the cell seem inconsistent with what is known about genetic changes associated with leukemia. The above data indicate that p110^{RB1} plays an important role in the regulation of c-*myc* expression. Since unregulated c-*myc* expression is probably the most common genetic abnormality in leukemia, particularly lymphoid leukemia, one might expect mutations in *RB1* to occur frequently in leukemia. Normal lymphoid cells and their progenitors express high levels of p110^{RB1} indicating that *RB1* gene expression plays an important role in regulating proliferation and differentiation of lymphoid cells. Hatzfeld et al. found that incubation of human bone marrow cells in medium containing antisense oligonucleotides specific for *RB1* significantly increased the number of hematopoietic progenitors subsequently detected in a colony assay [36]. They suggeste that p110^{RB1} helps maintain progenitors in a quiescent state where they are relatively non-responsive to growth

factors; inhibition of *RB1* function puts cells into a proliferative state where they are sensitive to stimulation by growth factors. These observations indicate that $p110^{RB1}$ is an important regulator of myeloid as well as lymphoid cells.

If one function of $p110^{RB1}$ is to repress expression of c-*myc*, then loss of $p110^{RB1}$ expression should lead to unregulated c-*myc* expression, much in the same way that other mutations, insertions and translocations produce unregulated c-*myc* expression in lymphoid leukemias. However, there is no evidence that patients with germline mutations in *RB1* are predisposed to leukemias. Given the high levels of proliferation in the hematopoietic system and assuming normal mutation rates, everyone must produce hundreds of cells every day with an inactivating mutation in one *RB1* allele. In patients with a germline mutation in one *RB1* allele, a mutation in the remaining *RB1* gene in any lymphoid cell should eliminate *RB1* function producing unregulated cells. For this reason, individuals carrying a germline mutation in one *RB1* allele should produce many mutant cells each day totally lacking functional $p110^{RB1}$. Yet there is no indication that these mutations predispose to leukemic transformation in patients with heritable retinoblastoma.

Confounding this issue are the frequent reports of mutations in *RB1* in many types of human leukemia. Ahuja et al. examined leukemic cells from 215 patients for $p110^{RB1}$ expression and found no protein in 26 samples; 14 of those samples had detectable structural abnormalities in the *RB1* gene [37]. All types of leukemia had *RB1* abnormalities. Furukawa et al. detected loss of $p110^{RB1}$ expression in 15 of 56 samples; they did not characterize the structure of the *RB1* gene in these samples [38]. Others have obtained similar data: Ginsberg et al. found deletion of the *RB1* gene in 4 of 85 fresh leukemic samples and in 2 of 9 leukemic cell lines [39]; Hillion et al. observed deletion in 2 of 50 leukemic samples [40]. In another large study, Chen et al. detected a lower frequency of abnormal *RB1* genes, only 2 out of 130 leukemic samples [41]. Two groups have reported studies on a single type of leukemia. Raghoebier et al. reported no *RB1* deletions in 44 cases of chronic B cell leukemia [42]. In contrast, Towatari et al. examined $p110^{RB1}$ expression in 17 CML patients with different types of blast crisis; all 5 cases of megakaryoblastic crisis failed to express detectable $p110^{RB1}$ despite making *RB1* mRNA, but no *RB1* abnormalities were observed in other forms of blast crisis [43].

From the above summary, one can conclude that *RB1* function is deleted by mutation in approximately 5% of leukemic samples. If all of studies on lack of $p110^{RB1}$ expression actually result from *RB1* mutations, the frequency may be as high as 15-30%. These high frequencies indicate that *RB1* mutations contribute to the malignant phenotype in a significant proportion of human leukemias. On the basis of these results, one would expect then that germline *RB1* mutations should result in a marked predisposition to many types of leukemia, a prediction not supported by the clinical data. Thus, we are left with two seemingly contradictory conclusions: 1. Somatic mutations in the *RB1* gene contribute to leukemogenesis. 2. Germline mutations in the *RB1* gene do not predispose to leukemia.

One can only speculate about possible explanations for these apparently contradictory conclusions. The currently available data on the function of the *RB1* gene indicate that it has multiple functions since its product can interact with several cellular proteins. Thus, it is likely that $p110^{RB1}$ has different functions in different cells. It is also clear from several systems, that important cellular processes frequently have redundant regulatory mechanisms, probably to protect the cells from the inadvertent loss of one mechanism. Perhaps retina is unique in its susceptibility to *RB1* mutations because it does not utilize other mechanisms to control its proliferation. In contrast, cells with extensive proliferative ability, like most of the cells in the hematopoietic system, may use several mechanisms to control proliferation and are less susceptible to loss of a single one, such as $p110^{RB1}$. Alternately, $p110^{RB1}$ may carry out an essential function in normal hematopoietic cells, so that loss of this function is lethal. While one might imagine that normal hematopoietic cells are unaffected by loss of *RB1* function, this conclusion is difficult to accept since hematopoietic cells produce high levels of $p110^{RB1}$ and since mutations in leukemic cells indicate growth regulatory functions for the *RB1* gene. If *RB1* function is essential for survival of normal hematopoietic cells, *RB1* mutations may be tolerated only after a cell has accumulated enough mutations to become malignant. Once the cell is leukemic, it can tolerate loss of *RB1* function and such loss then increases the growth or survival advantage allowing selection of leukemic cells with *RB1* mutations.

Regardless of the explanation for the apparent difference between somatic and germline mutations in regard to leukemia, these observations may provide information about the nature and type of mutations contributing to the final leukemic phenotype. If we accept that both

conclusions are correct, then we must conclude that *RB1* mutations are not important in leukemogenesis. That is, mutations in the *RB1* gene play no role in converting a normal hematopoietic cell to a malignant cell. If *RB1* mutations could contribute to the initiation of leukemia, retinoblastoma patients with germline mutations in *RB1* should have an increased incidence of leukemia, and they do not. However, after a hematopoietic cell has become malignant and escaped most growth control mechanisms, subsequent *RB1* mutations may increase growth rates and contribute to progression of the malignant phenotype, perhaps by altering c-*myc* expression.

Acknowledgements

The research described in this paper was supported by The National Cancer Institute of Canada with funds from the Canadian Cancer Society and from the Terry Fox Marathon of Hope, by the Medical Research Council and by the Retinoblastoma Family Association. B.L.G. is a Research Associate of the Ontario Cancer Foundation. E.Z., M.S. and P.A.H. were supported by Fellowships from the National Cancer Institute of Canada.

References

1. Gallie BL, Squire JA, Goddard A, Dunn JM, Canton M, Hinton D, Zhu XP, Phillips RA (1990) Mechanism of oncogenesis in retinoblastoma. Lab Invest 62:394-408
2. Roarty JD, McLean IW, Zimmerman LE (1988) Incidence of second neoplasms in patients with bilateral retinoblastoma. Opthalmology 95:1583-1587
3. Sanders BM, Jay M, Draper GJ, Roberts EM (1989) Non-ocular cancer in relatives of retinoblastoma patients. Br J Cancer 60:358-365
4. White L, Ortega JA, Ying KL (1985) Acute non-lymphocytic leukemia following multimodality therapy for retinoblastoma. Cancer 55:496-498
5. Knudson AGJ (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820-823
6. Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643-646
7. Dunn JM, Phillips RA, Zhu X, Becker AJ, Gallie BL (1989) Mutations in the RB1 gene and their effects on transcription. Mol Cell Biol 9:4596-4604
8. Horowitz JM, Yandell DW, Park S-H, Canning S, Whyte P, Buchkovich K, Harlow E, Weinberg RA, Dryja TP (1989) Point mutational inactivation of the retinoblastoma antioncogene. Science 243:936-940
9. Fung YK, Murphree AL, Tang A, Qian J, Hinrichs SH, Benedict WF (1987) Structural evidence for the authenticity of the human retinoblastoma gene. Science 236:1657-1661
10. Lee W-H, Shew JY, Hong FD, Sery TW, Donoso LA, Young LJ, Bookstein R, Lee EY (1987) The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature 329:642-645
11. Goddard AD, Balakier H, Canton M, Dunn J, Squire J, Reyes E, Becker A, Phillips RA, Gallie BL (1988) Infrequent genomic rearrangement and normal expression of the putative RB1 gene in retinoblastoma tumors. Mol Cell Biol 8:2082-2088
12. Horowitz JM, Park S-H, Bogenmann E, Cheng J-C, Yandell dW, Kaye FJ, Minna JD, Dryja TP, Weinberg RA (1990) Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. Proc Natl Acad Sci USA 87:2775-2779
13. Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E (1988) Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334:124-129
14. DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54:275-283
15. Egan C, Jelsma TN, Howe JA, Bayley ST, Ferguson B, Branton PE (1988) Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol Cell Biol 8:3955-3959
16. Hamel PA, Gallie BL, Phillips RA (1992) The retinoblastoma protein and cell cycle regulation. Trends Genet 8:180-185

17. DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnica-Worms H, Huang CM, Livingston DM (1989) The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58:1085-1095
18. Buchkovich K, Duffy LA, Harlow E (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58:1097-1105
19. Chen PL, Scully P, Shew JY, Wang JY, Lee W-H (1989) Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58:1193-1198
20. Ludlow JW, DeCaprio JA, Huang C-M, Lee W-H, Paucha E, Livingston DM (1989) SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell 56:57-65
21. Lees JA, Buchkovich KJ, Marshak DR, Anderson CW, Harlow E (1991) The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J 10:4279-4290
22. Hamel PA, Cohen BL, Sorce LM, Gallie BL, Phillips RA (1990) Hyperphosphorylation of the retinoblastoma gene product is determined by domains outside the Simian virus 40 large-T-binding -regions. Mol Cell Biol 10:6586-6595
23. Lin BT, Gruenwald S, Morla AO, Lee W-H, Wang JY (1991) Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator *cdc2* kinase. EMBO J 10:857-864
24. Hu QJ, Lees JA, Buchkovich KJ, Harlow E (1992) The retinoblastoma protein physically associates with the human cdc2 kinase. Mol Cell Biol 12:971-980
25. Ewen ME, Xing Y, Lawrence JB, Livingston DM (1991) Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell 66:1155-1164
26. Devoto SH, Mudryj M, Pines J, Hunter T, Nevins JR (1992) A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: $p33^{cdk2}$ is a component of the E2F-cyclin A complex. Cell 68:167-176
27. Kaelin Jr. WG, Pallas DC, DeCaprio JA, Kaye FJ, Livingston DM (1991) Identification of cellular proteins that can interact specifically withthe T/E1A-binding region of the retionblastoma gene product. Cell 64:521-532
28. Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR (1991) The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053-1061
29. Bagchi S, Weinmann R, Raychaudhuri P (1991) The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. Cell 65:1063-1072
30. Shirodkar S, Ewen M, DeCaprio JA, Morgan J, Livingston DM, Chittenden T (1992) The transcripton factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell 68:157-166
31. Kovesdi I, Reichel R, Nevins JR (1987) Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proc Natl Acad Sci USA 84:2180-2184
32. Blake MC, Azizkhan JC (1989) Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol Cell Biol 9:4994-5002
33. Hamel PA, Gill RM, Phillips RA, Gallie BL (1992) Transcriptional repression of the E2-containing promoters EIIaE, c-*myc* and *RB1* by the product of the *RB1* gene. Mol Cell Biol (in press):
34. Defeo-Jones D, Hunang PS, Jones RE, Haskell KM, Vuocolo GA, Hanobik MG, Huber HE, Oliff A (1991) Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. Nature 352:251-254
35. Rustgi AK, Dyson N, Bernards R (1991) Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the reinoblastoma gene product. Nature 352:541-544
36. Hatzfeld J, Li ML, Brown EL, Sookdeo H, Levesque JP, O'Toole T, Gurney C, Clark SC, Hatzfeld A (1991) Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor b1 or Rb oligonucleotides. J Exp Med 174:925-929
37. Ahuja HG, Jat PS, Foti A, Bar-Eli EM, Cline MJ (1991) Abnormalities of the retinoblastoma gene in the pathogenesis of acute leukemia. Blood 78:3259-68
38. Furukawa Y, DeCaprio JA, Belvin M, Griffin JD (1991) Heterogeneous expression of the product of the retinoblastoma susceptibility gene in primary human leukemia cells. Oncogene 6:1343-1346

39. Ginsberg AM, Raffeld M, Cossman J (1991) Inactivation of the retinoblastoma gene in human lymphoid neoplasms. Blood 77:833-840
40. Hillion J, Larsen CJ, Berger R (1991) Hemizygous deletion of the 3' end of the RB1 gene in a case of Philadelphia positive-acute lymphoblastic leukemia. Cancer Genet Cytogenet 54:197-203
41. Chen YC, Chen PJ, Yeh SH, Tien HF, Wang CH, Tang JL, Hong RL (1990) Deletion of the human retinoblastoma gene in primary leukemias. Blood 76:2060-2064
42. Raghoebier S, van Krieken JH, Kluin-Nelemans JC, Gillis A, van Ommen GJ, Ginsberg AM, Raffeld M, Kluin PM (1991) Oncogene rearrangements in chronic B-cell leukemia. Blood 77:1560-1564
43. Towatari M, Adachi K, Kato H, Saito H (1991) Absence of the human retinoblastoma gene product in the megakaryoblastic crisis of chronic myelogenous leukemia. Blood 78:2178-2181

Loss of p53 Expression in Myc-induced B Lineage Tumors

R. H. SCHEUERMANN and S. R. BAUER

[1]Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9072, and
[2]Laboratory of Molecular Immunology, FDA, Bethesda, MD 20892.

Introduction

Transgenic mice carrying an activated oncogene have been used extensively to investigate the mutational requirements for tumorigenesis. Transgenic mice carrying the *c-myc* gene under the control of the immunoglobulin heavy chain enhancer express the transgene exclusively in lymphoid tissues (Adams et al 1985; Harris et al 1988; Schmidt et al 1988; Scheuermann and Bauer 1990). At an early age these mice develop normally with only small effects on the normal lymphoid compartments. Invariably the mice develop lymphoid tumors after a latent period of about 10 weeks. The tumors are clonal, that is they derive from a single precursor cell, as judged by the rearrangement status of the antigen receptor genes. The fact that the tumors which arise in these mice are clonal and develop after a certain latent period suggests that inappropriate expression of *c-myc* is not sufficient for tumorigenesis, and that additional events, probably mutational, are necessary for transformation. Thus, these transgenic mice provide an excellent model system for the identification of genes which collaborate with activated myc in lymphoid tumorigenesis.

Three types of approaches have been used to try and identify genes which will collaborate with activated *c-myc* in transformation in these transgenic mice. Myc transgenic mice have been mated with transgenic mice carrying other activated oncogenes to see if tumorigenesis is accelerated in the double transgenic mice (e.g. Verbeek et al 1991). Myc transgenic mice have been infected with transforming retroviruses carrying activated oncogenes (Langdon et al 1989), where it was found that *v-raf* and *v-ras* could accelerate the time of tumor onset, suggesting a collaboration, whereas *v-abl* did not. Finally, transgenic mice have been infected with the non-acutely transforming Moloney murine leukemia virus (Mo-MuLV) which does not carry an activated oncogene (van Lohuizen et al 1991; Haupt et al 1991). Integration of the retroviral DNA close to another gene can lead to transcriptional activation by the viral LTRs. Transgenic mice infected with Mo-MuLV succumb to B lineage tumors with a shorter latency than uninfected mice. The virus is found to integrate preferentially in certain sites within the genome of tumor cells. At least two genes have been identified which are often transcriptionally activated by these retroviral insertions, *pim-1* and *bmi-1*, suggesting that these genes are good candidates to be myc collaborators.

One drawback to these approaches is that in each case the mice have been deliberately perturbed in some way. In addition, the first two approaches are limited not only by the need to guess which known oncogenes might collaborate with myc but also by the need to generate recombinant retroviral constructs and viruses, or the generation of new transgenic lines. The third approach, while not limited by these constraints, is designed only to detect genes which can be activated by retroviral insertion. It would miss genes like ras which require missense point mutations for activation, tumor suppressor genes in which two copies of the gene need to be altered, or genes in regions of the genome which are refractory to retroviral insertion and subsequent transcriptional activation. These approaches would therefore provide a somewhat biased view of oncogene collaboration We have begun an analysis designed to complement the approaches described above by examining expression of a large set of known oncogenes and tumor suppressor genes in a panel of tumors which have arisen spontaneously in *c-myc* transgenic mice. Unusual expression of an oncogene would implicate this gene in the

transformation of a particular tumor and encourage further investigation into the mechanism of the altered expression. Since they arise spontaneously, these tumors might more accurately reflect the kinds of collaborations which occur in naturally arising tumors in man.

Results

p53 Alterations

In an initial investigation into genes which can collaborate with myc, the tumor suppressor gene p53 was analyzed for alterations in structure and expression. Changes in the p53 gene are the most common genetic defects associated with tumorigenesis in man (reviewed in Levine et al 1991). p53 gene mutations have been found frequently in colon and lung carcinomas, osteosarcomas, brain and breast cancers, and certain leukemias and lymphomas. Alterations in tumor suppressor genes are examples of genetic changes which might be involved in tumorigenesis which would not be detected by the three approaches described above.

We have selected 17 B lineage tumors for detailed analysis. Total RNA was isolated from the tumor material by the guanidinium-thiocyanate method (Chomczynshi and Sacchi 1987)) and cDNA prepared with Moloney reverse transcriptase, for gene expression analysis by quantitative PCR (Saiki et al 1988; Scheuermann and Bauer 1992). The main drawback to the use of PCR to quantify mRNA levels is that differences in PCR reaction efficiencies between samples can have profound effects on the amount of product obtained following many cycles of amplification. Therefore in simple PCR reactions it is not possible to be certain that the difference in the amount of PCR product between samples reflects a difference in the amount of specific mRNA or a difference in reaction efficiency. One approach to alleviate this problem is to measure the PCR reaction efficiencies for each sample; if the efficiencies are the same in the samples analyzed then the amount of product generated will be proportional to the amount of starting mRNA. This can be achieved simply by performing the PCR reactions with varying cycle numbers. The amount of product, N_C, generated in a PCR reaction following c cycles of amplification is

$$N_C = N_0(1 + f)^c \qquad \text{Equation 1}$$

where N_0 is the amount of specific template at the beginning of the reaction and f is the efficiency of the reaction. Then

$$\log N_C = \log N_0 + c \log(1 + f) \qquad \text{Equation 2}$$

In a graph of $\log(N_C)$ versus c, a straight line indicates that the reaction efficiency is the same throughout the amplification process; the PCR reaction efficiency is directly related to the slope of this line ($\log(1 + f)$). When multiple samples are analyzed, if the slopes of the lines are the same then the PCR reaction efficiencies are the same and the amount of product generated from each sample will be proportional to the relative amount of starting material in each sample.

An example of this analysis is given in Figure 1. PCR reactions using ^{32}P-labelled PCR primers were terminated after 26, 27, 28 and 29 cycles and the reaction products analyzed by agarose gel electrophoresis. The amount of product generated with primers specific for *ß-actin* is similar between the four samples, especially between the pairs 15/18 and 16/17 (Fig. 1A). Using p53-specific primers, PCR product bands are seen even at 26 cycles (first lane in each set) with cDNAs from tumors 15, 16 and 17, and the amount of product increases with increasing cycle numbers. On the other hand, little if any p53-specific product is detected with cDNA from tumor 18, even after 29 cycles. The amount of radioactivity in the actin and p53 bands was quantified by phosphorimaging, and the log(product) graphed versus cycle number (Fig.1B and C, respectively). The slopes of the curves in each case are nearly identical indicating that the PCR reaction efficiencies are very similar between the samples, and thus the amount of product generated is proportional to the amount of specific template present in the cDNA mixes. Since the cDNA mixes from all four tumors gave comparable amount of actin-specific product, it indicates that there is little if any p53 mRNA in this last tumor. The small

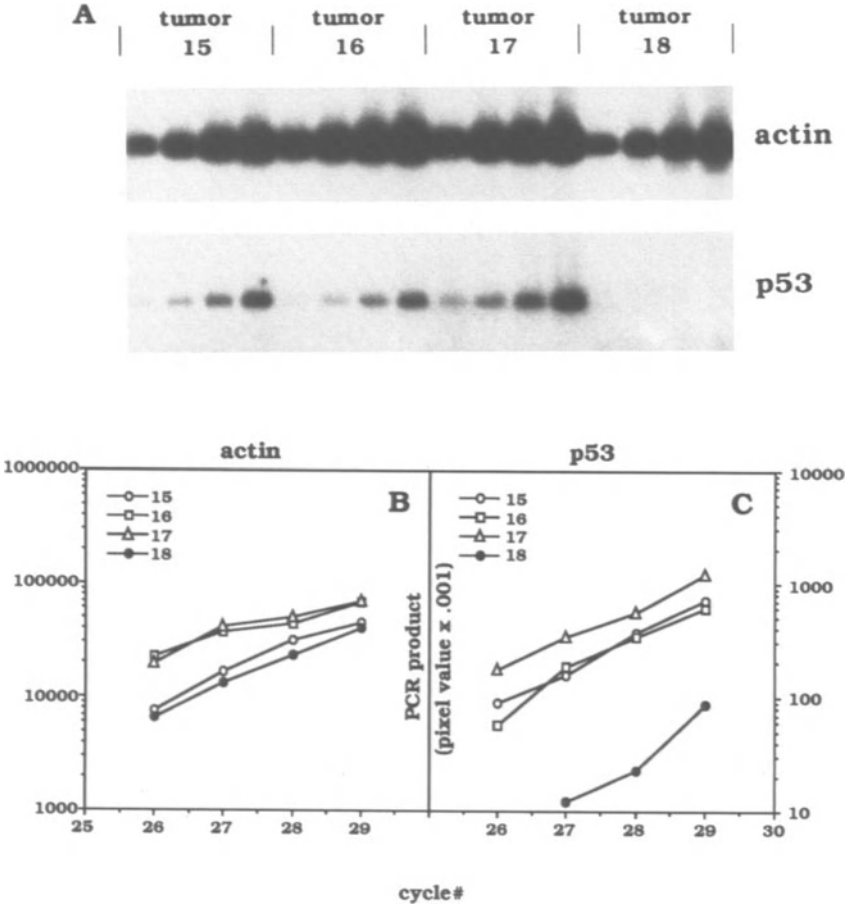


Fig.1. PCR-based quantification of p53 mRNA in myc-induced mature B cell tumors. Tumors arising spontaneously in four different myc transgenic mice were classified as mature B based on the cell surface expression of B220, IA and Ig (Scheuermann and Bauer 1990). The methods for RNA isolation, cDNA synthesis and PCR amplification, and PCR primer sequences are described elsewhere (Scheuermann and Bauer 1992). cDNA was synthesized from 5 μg of total RNA and 1/200 of this mixture used as a template in PCR reactions containing 150,000 cpm ^{32}P-labelled primers. Following electrophoresis in 2% agarose gels the PCR products were transfered to nylon membranes and quantified using a Molecular Dynamics Phosphorimager.

amount of p53 PCR signal which is invisible in the autoradiogram but detected during phosphorimaging may arise from the small number of normal cells present in the tumor mass from which the RNA was isolated.

In an analysis of 17 randomly selected B lineage tumors for p53 expression, 5 showed little if any p53 mRNA by this PCR method. Thus, in 30% of myc-induced, B-lineage tumors, p53 expression was lost, suggesting that alterations in p53 can effectively collaborate with inappropriate myc expression for the transformation of B lineage cells *in vivo*. Loss of p53 expression was found in 2 of 9 preB and 3 of 8 mature B type tumors. This result contrasts with previous work in several ways. In an analysis of human lymphatic leukemias loss of p53

expression was seen in some cases of myeloid malignancy; however, in T and B cell malignancies no loss of p53 expression was seen (Prokocimer et al 1986). In a recent study of human lymphomas, mutations in the p53 coding sequence were frequently observed in B-CLL, B- and preB-ALL, and Burkitt's lymphoma, but not in T cell derived tumors (Giadano et al 1991), however the expression of p53 was not examined. The results described here provide evidence that loss of p53 expression can also occur frequently in malignancies of B lymphocytes.

Since loss of p53 mRNA occurs at such a high frequency it suggests that loss of p53 function may be required for tumorigenesis in these mice. This would predict that in tumors which express p53 message, loss of p53 function might be achieved through effects on p53 translation or the presence of mutations which alter p53 protein activity. Indeed, point mutations are the most common form of p53 alterations in various types of tumors. These point mutations cluster within the middle of the protein, from codon 130 in exon 5 to codon 290 in exon 8 (Levine et al 1991). A method has been developed which allows the rapid screening of many samples for the presence of point mutations- Single-Stranded Conformational Polymorphism (SSCP, Orita et al 1989). The technique relies on the fact that the migration of nucleic acids in non-denaturing gels is dependent not only on their length but also on their structure. Specific sequences are amplified with radioactively-labelled primers by PCR. The amplified DNA is denatured with alkali and heat and then loaded onto a non-denaturing acrylamide gel. As the DNA enters the gel, the denaturant is gradually removed allowing the single-stranded DNA to assume a particular three dimensional structure. Both the rate of renaturation and the structure formed will depend on the specific sequence. This procedure is so sensitive to these parameters that a single point mutation within a stretch of several hundred nucleotides can be detected. In a previous study 100% concordance between mutations detected by SSCP and by direct sequencing was seen (Giadano et al 1991), indicating the power of the technique.

PCR amplification of cDNA derived from normal samples and several of the B lineage tumors which express p53 mRNA, along with subsequent SSCP analysis is indicated in Figure 2. In lane 10 the labelled PCR product from one mature B cell tumor migrated more slowly under the SSCP conditions than product derived from normal tissue. This indicates that the sequence of the p53 gene in this tumor contains a point mutation. In addition, since no band is seen comigrating with the wild-type band the mutant allele is either homozygous or hemizygous. In this analysis 1/12 of the tumors which still express p53 mRNA showed an SSCP mobility change. Since the PCR primers used in this experiment are located in exons 3 and 6, these PCR products would only include half of the region within p53 frequently mutated. Therefore, this frequency is probably an underestimate.

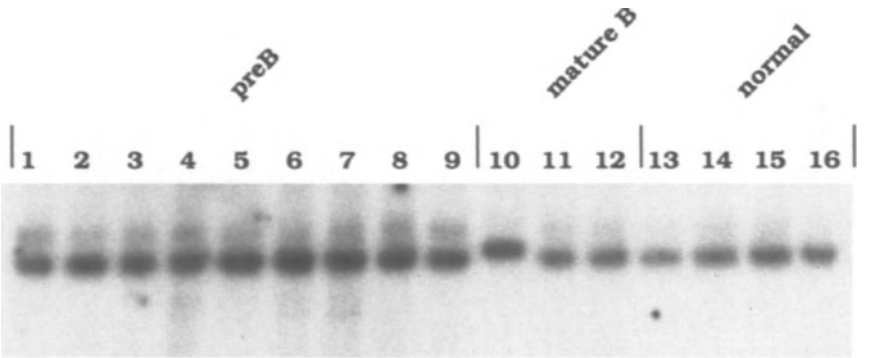


Fig.2. SSCP analysis of B lineage tumor cDNA using p53 specific primers. RNA isolated from preB cell tumors (lanes 1-9), mature B cell tumors (lanes 10-12), or tissue from a normal BDF_1 x C57/B6 mouse (lanes 13-16; bone marrow, lymph node, spleen and thymus, respectively) was converted to cDNA and amplified by PCR as in Fig.1. The PCR primers are derived from sequences present in exons 3 and 6 of the wild-type p53 gene. Samples were denatured at 95°C in 98% formamide, 10 mM EDTA, 20 mM NaOH and run on a 6% polyacrylamide gel in 1X TBE containing 10% glycerol at 10 watts constant power.

In total to date we have detected alterations in the p53 tumor suppressor gene in 35% of randomly selected B lineage tumors arising spontaneously in our myc transgenic mice. However, it remains to be determined how frequently p53 function is altered since we have not measured the level of protein expressed, or examined the whole coding sequence for point mutations.

A Search for Other Myc Collaborators

To extend the analysis described above the expression pattern of other oncogenes in the myc-induced tumors will be examined in order to identify other genes which can collaborate with *c-myc* in the spontaneous transformation of lymphocytes. In order to achieve sensitivity, accuracy and reproducibility we have revised our PCR-based system for mRNA quantification (Scheuermann and Bauer 1992). The technique relies on the use of a synthetic RNA molecule which is included in every reaction mix that acts not only as a control for tube-to-tube variations in reaction efficiencies, but also provides quantification in absolute terms allowing the direct comparison of data derived from different experiments. A schematic of the procedure is presented in Figure 3B. Total cellular RNA is isolated from any cell source by the guanidium-thiocyanate method (Chomczynski and Sacchi 1987). In parallel a standard RNA is synthesized from a vector containing PCR priming sites for the genes of interest cloned between an SP6 promoter and a polyA stretch. OQ-1 is an example of this type of vector which contains the priming sites for 20 different oncogenes and two control genes (Fig.3A). The synthetic RNA generated using SP6 RNA polymerase is purified by oligo(dT) chromatography and accurately quantified by U.V. absorbance. A mixture of cellular RNA and synthetic RNA is converted into cDNA using random hexamer primers (pdN_6) and reverse transcriptase (RT), and this cDNA mix is used as the template in PCR reactions. Primer pairs are chosen which are specific for the gene whose expression is to be analyzed, in this case specific oncogenes. If radioactively labelled primers are used, the amount of product following PCR amplification can be easily measured. The synthetic RNA is designed to contain sequences identical to the priming sites in the endogenous gene of interest. The amplified products from the synthetic RNA (s) and the endogenous cellular RNA (e) are designed to differ in size and can be distinguished following gel electrophoresis.

A comparison of the amount of PCR product derived from the known amount of standard RNA and the amount derived from the endogenous mRNA determines the number of molecules of specific mRNA present in the cDNA mixture. In the example given in Fig.3B RNA derived from normal tissue and two different tumors is analyzed for the expression of an oncogene. Samples are removed from PCR reactions following consecutive numbers of amplification cycles. The amount of product generated from the synthetic, standard RNA (s) is the same for all three samples indicating similar reaction efficiencies. In the normal and tumor 1 samples the amount of PCR product generated from the endogenous mRNA (e) is the same, and equal to 1/4 the amount generated from the standard. If these cDNA's was generated from 10^4 cells and 10^7 standard molecules it would indicate that there are 250 molecules of specific mRNA/cell for these two samples. On the other hand, the amount of product generated from the endogenous mRNA from tumor 2 is twice the amount of the standard indicating a level of 2000 molecules of specific mRNA/cell. This large increase in the level of mRNA would suggest that the normal expression of this gene had been altered in this particular tumor, and would warrant further investigation. Standard RNA generated from OQ-1 can be included in a cDNA mix and the level of 22 oncogenes determined simply by changing PCR primers, allowing the rapid analysis of oncogene expression in any sample. Details of this procedure and the structure of OQ-1 can be found in Scheuermann and Bauer (1992).

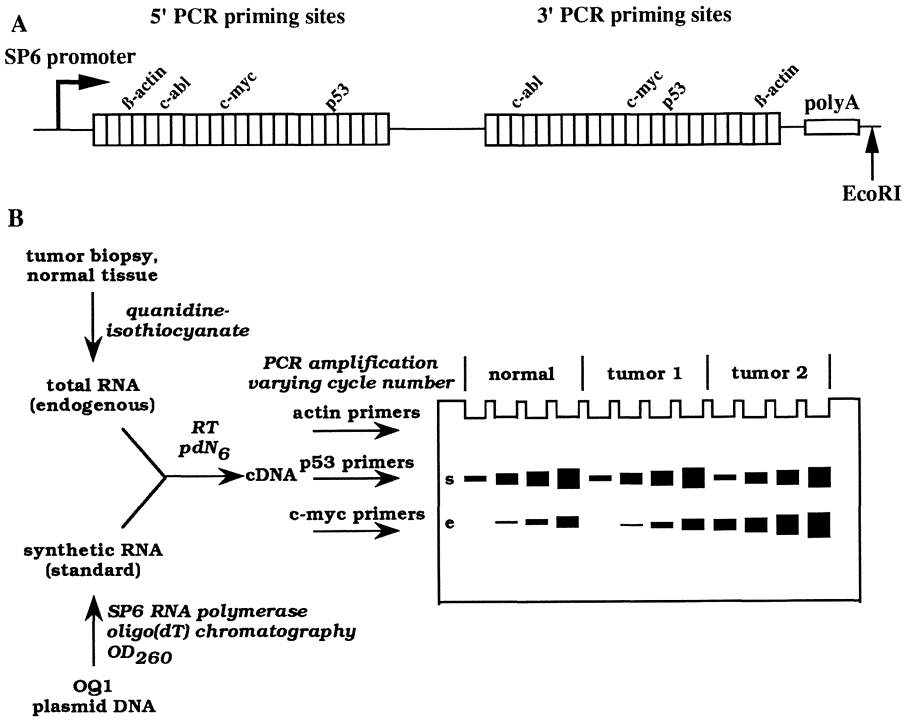


Fig. 3. PCR-based mRNA quantification using an internal standard **A.** OQ-1 insert. OQ-1 contains a 760 bp. synthetic stretch of DNA cloned into pSP64polyA. Included in this insert are primer pair sequences specific for two control genes (ß-actin and GAPDH) and 20 different oncogenes/tumor suppressor genes including *c-myc, c-abl, p53, c-raf, c-myb, lck, bcl-2*, and others. The structure and construction of OQ-1 are described in Scheuermann and Bauer (1992). **B.** A schematic representation of the procedure. See text for details.

Summary

Tumors are formed following the accumulation of several genetic changes in genes which normally function to regulate cell growth. As yet it is unclear why multiple mutations are required, which type of alterations can collaborate with each other, and if collaboration is cell-type specific. In our myc transgenic mouse model system both point mutations and loss of mRNA expression for the p53 tumor suppressor gene have been found in the myc-induced B-lineage tumors arising spontaneously in these mice. This demonstrates the collaboration between these two growth control genes in cellular transformation. The observation that alterations in the expression of p53 is a common phenomenon in tumors formed in myc transgenic mice as well as a variety of different types of human tumors suggests that inactivation of the p53 growth control pathway may be required for transformation, and that alterations in p53 itself might be the most efficient way to achieve this inactivation. An analysis of the molecular mechanism for p53 alterations has implications for what kind of factors, both environmental and physiological, can influence tumor formation. The identification of collaboration groups has implications for the process of tumor formation, growth regulation, and will some day be important for the diagnosis of cancer, the prognosis of the individual and the design of specific therapeutic agents for treatment.

References

Adams JM, Harris AW, Pinkert CA, Corcoran LM, Alexander WS, Cory S, Palmiter RD, Brinster RL (1985) The *c-myc* oncogene driven by immunoglobulin enhancers induce lymphoid malignancy in transgenic mice. Nature 318, 533-538.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chlororform extraction. Anal. Biochem. 162, 156-159.

Giadano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM, Dalla-Favera R (1991) p53 mutations in human lymphoid malignancies: Association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. U.S.A. 88, 5413-5417.

Harris AW, Pinkert CA, Crawford M, Langdon WY, Brinster RL, Adams JM (1988) The Eμ-myc transgenic mouse: A model for high-incidence spontaneous lymphoma and leukemia of early B cells. J. Exp. Med. 167, 353-371.

Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM (1991) Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in Eμ-myc transgenic mice. Cell 65, 753-763.

Langdon WY, Harris AW, Cory S (1989) Acceleration of B-lymphoid tumorigenesis in Eμ-myc transgenic mice by *v-Ha-ras* and *v-raf* but not *v-abl*. Oncogene Res. 4, 253-258.

Levine AJ, Momand J, Finlay CA (1991) The p53 tumour suppressor gene. Nature 351, 453-456.

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5, 874-879.

Prokocimer M, Shaklai M, BenBassat H, Wolf D, Goldfinger N, Rotter V (1986) Expression of p53 in human leukemias and lymphomas. Blood 68, 113-118.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487-494.

Scheuermann RH, Bauer SR (1990) Tumorigenesis in transgenic mice expressing the *c-myc* oncogene with various lymphoid enhancer elements. Curr. Topics Microbiol. Immunol. 166, 221-231.

Scheuermann RH, Bauer SR (1992) PCR-based mRNA quantification using an internal standard: A method for the analysis of oncogene expression. Methods Enzymol. in press.

Schmidt EV, Pattengale PK, Weir L, Leder P (1988) Transgenic mice bearing the human *c-myc* gene activated by an immunoglobulin enhancer: A pre-B-cell lymphoma model. Proc. Natl. Acad. Sci. U.S.A. 85, 6047-6051.

van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns, A. (1991) Identification of cooperating oncogenes in Eμ-myc transgenic mice by provirus tagging. Cell 65, 737-752

Verbeek, S., van Lohuizen, M., van der Valk, M., Domen, J., Kraal, G. and Berns, A. (1991) Mice bearing the Eμ-myc and Eμ-pim-1 transgenes develop pre-B-cell leukemia neonatally. Mol. Cell. Biol. 11, 1176-1179.