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Oncogenes in B-Cell Neoplasia

Workshop
at the National Cancer Institute,
National Institutes of Health,
Bethesda, MD, USA, March 5–7, 1984

Organized and Edited by
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With 65 Figures

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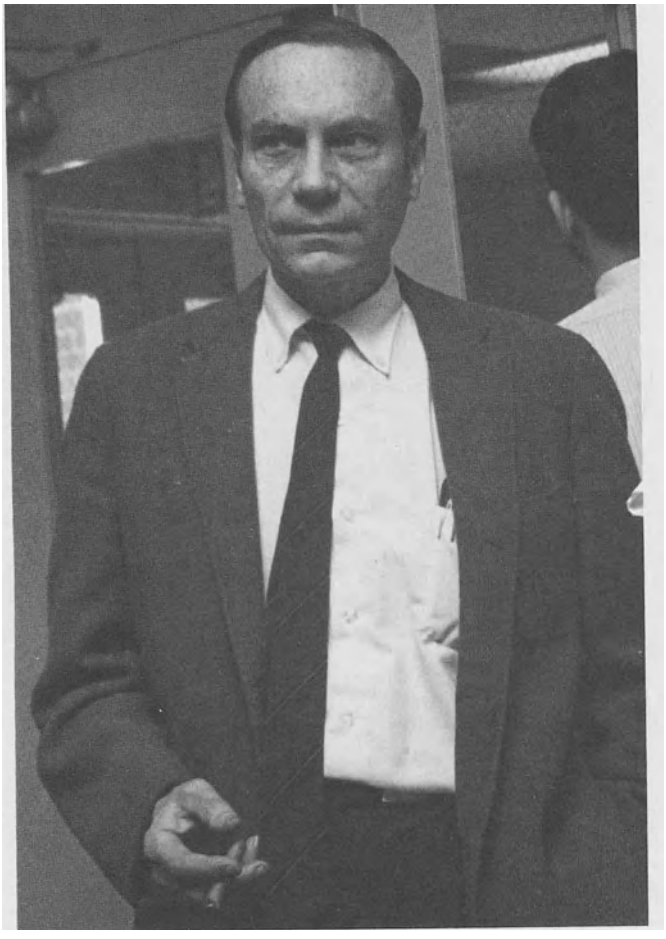
In Memoriam
Henry G. Kunkel (1916–1983)



Henry Kunkel planned to participate in this workshop. Much of his experimental work concerned B-cell neoplasm of man. In 1951 he, along with R.J. Slater and R.A. Good demonstrated the antigenic similarities of myeloma proteins and γ-globulins (immunoglobulins), and began using tumors of B-lymphocytes as a means for understanding the normal counterparts. The antigenic relationship between myeloma and normal immunoglobulin was the foundation for the subsequent elucidation of the heavy chain classes of immunoglobulins. The individual antigenic specificities of homogeneous myeloma proteins were the next important milestone (Slater et al., 1955), and the interpretation of these specificities at first presented a perplexing problem, but ultimately provided a direct conceptual pathway to the study of antibody diversity A very logical extension of Henry Kunkel's concept of homogeneous immunoglobulins produced by clones of B-cells, was that homogeneous

immunoglobulins could also possess functional (biological) activities, and he was among the first to find examples (Kritzman et al., 1961). The cross-specificities (shared idiotypic determinants) on groups of homogeneous immunoglobulins with similar functional properties had a great influence on subsequent structural studies of v-region genes and their corresponding structures (Kunkel, 1970).

Henry Kunkel inspired and trained a great number of today's most productive immunologists. A measure of his influence is illustrated by the dedication of an entire issue of the Scandinavian Journal of Immunology in September, 1976 to papers written by his students. This meeting surely owes many of its origins to the ideas of Henry Kunkel who facilitated the use of lymphoid tumors to examine normal processes by showing that many of the products of tumors were indeed similar to those made by normal counterparts. It is this familiarity that has provided a perspective for immunologists to question now, the origin of neoplastic change.

Kunkel HG, Slater RJ and Good RA (1951) Relation between certain myeloma proteins and normal gammaglobulin. Proc Soc Exp Biol Med 76: 190-193

Slater RJ, Ward SM and Kunkel HG (1955) Immunological relationships among the myeloma proteins. J Exp Med 101:85-108

Kritzman J, Kunkel HG, McCarthy J and Mellors RC (1961) Studies of a Waldenstrom-type macroglobulin with rheumatoid factor properties. J Lab Clin Med 57:905-917

Kunkel HG (1970) Individual antigenic specificity; cross-specificity and diversity of human antibodies. Fed Proc 29:55-58

Scandinavian Journal of Immunology (Sept 1976) 5:599-873

Preface

Michael Potter, Fritz Melchers, Martin Weigert

The second workshop on Mechanisms of B Cell Neoplasia was held in Bethesda, Maryland in Wilson Hall at the National Institutes of Health on March 5, 6, and 7, 1984. It followed a workshop on the same topic that was held at the Basel Institute for Immunology, March 15-17, 1983. That first meeting attempted to bring together cell biologists, experimental pathologists and molecular geneticists interested in B cells, to discuss pathogenetic processes in the development and maintenance of the neoplastic state. The impetus for this discussion emanated from two important developments: first, the discovery of the viral promoter insertion mechanism for activating the myc oncogene in bursal lymphomatosis by Hayward, Neil, and Astrin; second, the findings that the non-random chromosomal translocations involving the immunoglobulin gene chromosomes occurred in very high frequencies in murine plasmacytomas and human Burkitt's lymphomas. During the planning stages of that meeting Shen-Ong et al. discovered that non-random translocations activated the myc oncogene. Promoter insertions and non-random translocations were two mechanisms that caused transcription of the myc oncogene messages in three different kinds of well defined experimental and clinical B cell tumors. Unregulated myc gene transcription provided the first evidence of a specific biochemical lesion in B cell neoplasia. Immunologists have studied tumors of the B cell lineage for cellular and molecular aspects of normal B cell development, since they appear to be frozen states of various stages of this cellular development. From the comparison of normal and neoplastic B cells a wealth of data is available that provides a working basis for examining in detail the process of neoplastic transformation in B lymphocytes. In the intervening year between the workshops, rapid progress has been made in the elucidation of structures of oncogenes Different kinds of mutant c-oncogenes were discovered: 1) point mutations in coding sequences, e.g., the base substituted ras genes; 2) deletion mutants, e.g., the truncated oncogenes in transforming viruses, e.g., v-abl, erbB, etc.; 3) regulatory mutants of c-myc and 4) amplified c-oncogenes. Important insights were also gained in the understanding of the function of these oncogenes. In particular the normal counterparts of some oncogenes have been identified, e.g., the v-sis homology with platelet derived growth factor, the erbB homology with epidermal growth factor receptor, the Tlym-I homology with HMC class I genes, the B-lym homology with transferrin and the ras homology with the p21 subunit of the transferrin receptor.

The second workshop was as exciting as the first one. We thank the participants of the meeting for promptly submitting their manuscripts. This reflects, we think, the enthusiasm that pervaded this meeting and we are grateful that the proceedings can be published in such a short time. Rapid publication requires an efficient publisher. We therefore are indebted to Springer Verlag and their representative, Mr John Morgan, New York, for their interest, care and speed in editing and publishing these proceedings.

We are grateful to Ms Marion Millhouse of CSR, Inc., and Ms Victoria Rogers, manager of the Laboratory of Genetics, for organizing the meeting, making the local arrangements, assembling the manuscripts

and for working out all the demanding details of the meeting in a seemingly effortless way. Last, not least, the organizers of the meeting thank the National Cancer Institute for sponsoring the meeting, and Dr Alan S. Rabson, Director, Division of Cancer Biology and Diagnosis, for his enthusiastic support.

It is anticipated that the future will bring an even better understanding of the role of oncogenes in the process of B cell neoplasia This, in turn, should advance our understanding of the physiology and biochemistry of growth regulation of normal B lymphocytes. We are looking forward to the third workshop of this series in 1985, hoping that it will then still be possible to convene a representative selection of the major groups working in this fast moving field of biology and medicine as, we hope, we have done in 1984.

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Introductory Remarks

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25 years ago (Foulds 1958) Leslie Foulds formulated a number of "rules" for tumor progression, the process whereby "tumors go from bad to worse", in Peyton Rous' original description (Rous and Beard 1939). These rules are equally pertinent today and accessible to analysis by the powerful tools of modern biology.

Foulds has dissected the malignant phenotype into "unit characteristics", such as growth rate, invasiveness, metastasizability, hormone dependence, etc. In his own words (Foulds 1954):

"The behavior of tumors is determined by numerous characters that, within wide limits, are independently variable, capable of different combinations and assortments and liable to independent progression". This explains the biological individuality of tumors. No two tumors are exactly alike, even if induced by the same agent and in the same type of target cells of the same inbred host genotype. Foulds pointed out the need for "particularization of factorial analysis" (Foulds 1969)

Foulds' definition of the "unit characteristics" reflects his working area, experimental pathology. While it was mainly based on the studies of murine tumors, his examples also included human tumors. He emphasized the independent change of metastasizability and hormone responsiveness in human prostatic and thyroid carcinomas and the stepwise changes in the invasiveness of cervical, gastrointestinal and bladder carcinomas. In the mouse system, the possibility to maintain the tumor beyond the lifespan of the host revealed that progression does not reach an endpoint in the primary host. With the passage of tumor cell populations, new opportunities are created for further microevolutionary changes. They lead to increased independence from local, systemic or drug induced growth restrictions.

Foulds' rules were also applicable to changes in the expression of defined cellular markers like histocompatibility antigens (Klein and Klein 1957; Klein 1981) or differentiation related enzymes (Potter 1962).

In tumors of epithelial tissues the evolution of progressional changes could often be directly visualized. Increased invasiveness was detected in sharply outlined focal areas (Foulds 1954), suggesting a clonal event.

Experimental analysis of progressional changes in the characteristics of transplanted tumors has first shown that they are due to a Darwinian process of variation and selection within the cell population (Klein and Klein 1957; Klein 1981). Before the arrival of modern molecular biology, analytical approaches were hampered by the Janus-faced double nature of tumor cells. While capable of evolving by mutation and selection, like asexually reproducing microorganisms, their phenotype can also change by differentiation steps, like in normal somatic cells of higher organisms. Molecular distinctions between structural changes at the DNA level and epigenetic changes in regulation have only very recently become possible.

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Modern analysis will deal with more precisely defined phenotypic properties and their genetic control. The basic biological picture is still the same, however. We shall have to identify the phenotypic properties reflected by the "unit characteristics" of Foulds. Are some or all of them controlled by the presently known oncogenes or by oncogenes that remain to be identified? Is it possible to explain tumor progression as the sequential activation of multiple oncogenes?

The "oncogene world" has been perceived through four quite different windows: retroviral transfection, retroviral insertion near a cellular oncogene, DNA transfection and the "experiments of nature", reflected by the chromosomal translocations in some B-cell tumors. While each window allows a slightly different view, they all look at the same world and, remarkably, identify the same oncogenes. This increases the likelihood that a relatively limited number of genes control phenotypic properties that can play a key role in the development of neoplastic behavior.

Much discussion has centered on the question whether the tumor-associated oncogene activation reflects quantitative or qualitative changes. The potential significance of both has been demonstrated in model systems. The clearest examples of the quantitative model have been provided by the groups of Vande Woude (Oskarsson et al. 1980) and of Scolnick (DeFeo et al. 1981). They have linked the c-mos and the c-ras genes, respectively, to the appropriate viral promoter/enhancer carrying LTR sequences, and obtained DNA-mediated transformation of NIH-3T3 cells in vitro. The qualitative model has been proven by the single point mutations of the ras gene, discovered by Weinberg's group (Tabin et al. 1982) and confirmed by Aaronson and by Barbacid and their associates (Reddy et al. 1982; Yuasa et al. 1983). Thus, the ras gene can transform according to both the quantitative and the qualitative model.

In the case of c-myc activation by chromosomal translocations that juxtapose the oncogene and one of the immunoglobulin gene regions, the salient change may be more subtle than what can be measured as a gross increase in gene expression, or a mutational change in the gene product, occasional examples of both possibilities notwithstanding. Deregulation is gaining momentum as the key word. Dysregulation may be a better term, since it may be surmised that the transposed oncogene comes under the influence of immunoglobulin gene regulators. Although neither the normal function of the myc-product, nor its regulability during the cell cycle have been clarified, it is more than likely that its differentiation and cell cycle related expression differs from the immunoglobulin genes. The Ig-loci are permanently switched on in a B-cell and are not programmed to be switched off again. Precise cycle regulated studies on the transposed and the normal c-myc gene in comparison with the Ig-genes may provide crucial information on the mechanisms whereby the translocations contribute to the development of BL and MPC.

The regularity and precision of the translocations seen in MPC and BL and their absence from other types of murine and human B-cell tumors suggests, in itself, that this event is necessary for the tumorigenic process. The identical features of the translocations in the two tumors are probably highly meaningful. The breakpoints affect the c-myc oncogene in almost the same fashion (with only minor variations) in the two types of tumors, in spite of their disparate natural history and maturation stage of the cell of origin. Moreover, no other oncogenes than c-myc are known to be shared by the human chromosome

8 and murine 15. Human 8 but not mouse 15 carries c-mos, murine 15 but not human 8 carries c-sis.

A similar translocation has also been found in another B-cell derived tumor, the spontaneous immunocytoma of the Louvain strain of rats (Wiener et al. 1982). It arose from a reciprocal exchange between chromosomes 6 and 7 and showed striking banding homologies with the typical 12;15 translocation in the murine PC. Recently, our laboratory has localized c-myc to rat chromosome 7 (Sümegi et al. 1983). The rat immunoglobulin genes have not yet been localized on the chromosome map.

Currently, it is most reasonable to assume that the translocations play an essential role in the genesis of MPC and BL. This does not mean that they represent the only important change.

We shall hear about several cases where double or multiple oncogene activation occurs during neoplastic development. This area was opened by the study of the ALV-induced chicken B-cell lymphoma, where c-myc became activated through the insertion of a retroviral promoter (Hayward et al. 1981). Surprisingly, the transforming sequence detected by DNA transfection of NIH 3T3 cells was not c-myc but another (Cooper and Neiman 1981) later designated as Blym (Goubin et al. 1983). The same oncogene was also detected by transfecting NIH 3T3 with the DNA of six Burkitt lymphomas (Diamond et al. 1983), while in another BL line (Murray et al. 1983) the transfecting sequences were N-ras.

ALV-induced chicken B-lymphoma and human BL originates from B-cells at a similar intermediate stage of differentiation. The involvement of the same two oncogenes, c-myc and B-lym in the genesis of both is therefore particularly intriguing. Transfection experiments with DNA of mouse plasmacytomas have also identified an active oncogene but this was different from both c-myc and B-lym (Cooper 1982; Lane et al. 1982).

Similar double oncogene activations have been detected in other systems, like Abelson leukemia (Lane et al. 1982) and mammary tumor virus associated mouse mammary carcinoma (Lane et al. 1981; Nusse and Varmus 1982). Some or all of these cases may be relevant for the understanding of tumor progression.

Another road towards a progression-related functional analysis has been opened by the transfection of normal rat embryo fibroblasts with two different oncogenes (Land et al. 1983). The cooperative action of myc and ras was extended to some other oncogenes and has already led to a first rough definition of two complementation groups. H-ras could be substituted by N-ras or by polyoma middle T, whereas v-myc could be replaced by polyoma large T or by the E1a gene of adenovirus (Ruley 1983). Polyoma middle T is known to induce morphological changes and anchorage independence (Rassoulzadegan et al. 1982). This contrasting behavior stimulated speculations concerning the possible modes of action of the products of the two complementation groups. According to this model the genes belonging to the "myc complementation group" code for nuclear proteins, whereas the genes of the "ras group" products are membrane associated and are responsible for morphological and functional changes related to the social behavior of cells. Some of these, like anchorage independence may form an important part of tumorigenic behavior in vivo. It is particularly interesting that the oncogenes that can be assorted into the two complementation groups are not limited to the cell-derived oncogenes in the strict sense, but also include some of the virally coded oncogenes of the DNA tumor viruses.

Another example of double oncogene action is provided by the cases where the same transforming retrovirus has picked up two different, originally unlinked oncogenes. The cooperative effects of erb A- erb B, myc-mil, and myb-ets are some of the cases in point (Newmark 1983)

This session will deal with several of these problems. It is particularly gratifying that the interplay of different oncogene families, viral and cellular, have been brought into focus by the papers that have been selected by the organizers.

REFERENCES

Cooper GM (1982) Cellular transforming genes. Science 217:801-806

Cooper GM and Neiman PE (1981) Two distinct candidate transforming genes of lymphoid leukosis virus-induced neoplasms. Nature 292:857-859

DeFeo D, Gonda MA, Young HA, Chang EH, Lowy DR, Scolnick EM, Ellis RW (1981) Analysis of 2 divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc Natl Acad Sci 78:3328-3332.

Diamond A, Cooper GM, Ritz J, Lane MA (1983) Identification and molecular cloning of the human BLym transforming gene activated in Burkitt's lymphomas. Nature 305:112-116

Foulds L (1954) Tumor progression. Cancer Res 14:327-339.

Foulds L (1958) The natural history of cancer. J Chronic Diseases 8: 2-37.

Foulds L (1969) In: Neoplastic Development. Academic Press, London, New York, p 439

Goubin G, Goldman DS, Luce J, Neiman PE, Cooper GM (1983) Molecular cloning and nucleotide sequence of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. Nature 302:114-119

Hayward WS, Neel BG, Astrin SM (1981) Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 290:475-480

Klein G (1981) Mouse histocompatibility genetics and tumor immunology. Mammalian Genetics and Cancer. The Jackson Laboratory Fiftieth Anniversary Symposium. Alan R Liss, Inc. New York, pp 197-211.

Klein G, Klein E (1957) THe evolution of independence from specific growth stimulation and inhibition in mammalian tumor cell populations. Symp Soc Exp Biol 11:305-328

Land H, Parada LF, Weinberg RA (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596-602

Lane MA, Neary D, Cooper GM (1982) Activation of a cellular transforming gene in tumors induced by Abelson murine leukemia virus. Nature 300:659-661

Lane MA, Sainten A, Cooper GM (1981) Activation of related transforming genes in mouse and human mammary carcinomas. Proc Natl Acad Sci 78:5185-5189

Murray MJ, Cunningham JM, Parada LF, Dautry F, Lebowitz P, Weinberg RA (1983) THe HL-60 transforming sequence: A ras oncogene coexisting with altered myc genes in hematopoetic tumors. Cell 33:749-757

Newmark P (1983) Viral oncogene permutations. Nature 306:426

Nusse R, Varmus HE (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31:99-100

Oskarsson M, McClements WL, Blair DG, Maizel JV, Vande Woude GF (1980) Properties of a normal mouse cell DNA sequence (sarc) homologous to the src sequence of Moloney sarcoma virus. Science 207:1222-1224

Potter VR (1962) Enzyme studies on the deletion hypothesis of carcinogenesis. In: The Molecular Basis of Neoplasia. University of Texas Press, Austin. pp 367-399

Rassoulzadegan M, Cowie A, Carr A, Glaichenhaus N, Kamen R, Cuzin F (1982) The roles of individual polyoma virus early proteins in oncogenic transformation. Nature 300:713-718

Reddy EP, Reynolds RK, Santos E, Barbacid M (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 300:149-172

Rous P, Beard JW (1939) The progression to carcinoma of virus induced papillomas. J Exp Med 69:399-424

Ruley H E (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304:602-606

Sümegi J, Spira J, Bazin H, Szpirer J, Levan G, Klein G (1983) Rat c-myc oncogene is located on chromosome 7 and rearranges in immunocytomas with t(6;7) chromosomal translocation. Nature 306:497-498

Tabin CJ, Bradley SM, Bargmann CI, Weinberg RA, Papageorge AG, Scolnick EM, Dhar R, Lowy DR, Chang EH (1982) Mechanism of activation of a human oncogene. Nature 300:143-149

Wiener F, Babonits M, Spira J, Klein G, Bazin H (1982) Non-random chromosomal changes involving chromosomes 6 and 7 in spontaneous rat immunocytomas. Int J Cancer 29:431-437

Yuasa Y, Srivastava SK, Dunn CY, Rhim JS, Reddy EP, Aaronson SA (1983) Acquisition of transforming properties by alternative point mutations within c-bas/has human proto-oncogene. Nature 303:775-779

Activated Oncogenes in Burkitt's Lymphoma

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INTRODUCTION

Burkitt's lymphoma (BL) was recognized more than 25 years ago as a distinct pathoclinical entitity with peculiar epidemiological characteristics. The tumor, which occurs with a high incidence in certain areas of central Africa, has been suspected of having a viral etiology. This hypothesis was strengthened by the first isolation of the Epstein Barr virus (EBV) from a BL cell culture. More recently, we have learned that the association between the tumor and the EB virus is not as consistent as was initially hoped. The lack of viral assocation is especially true when tumors which arise in low-incidence areas such as Europe or the United States are considered. However, during the past few years it has become apparent that specific chromosomal rearrangements represent a crucial step in the development of the BL malignant process (Klein 1981, Leder 1983). The chromosomal rearrangement involves translocation of cellular proto-oncogenes, and may lead to their activation. In light of the epidemiological studies which suggest that carcinogenesis is a multistep process, it is interesting to speculate how as yet unidentified viral or cellular functions may contribute to the malignant BL phenotype.

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CHROMOSOMAL TRANSLOCATIONS INVOLVING THE MYC ONCOGENE ARE CHARACTERISTIC OF BL AND INDEPENDENT OF EBV ASSOCATION

In central Africa, where BL occurs with an annual incidence as high as 1.6 per 100,000 general population, most tumors are associated with EBV, as was demonstrated by the presence of viral markers (i.e., DNA or EB nuclear antigen [EBNA]) in the tumor cells. Only a low proportion--4%--of the tumors are free of any detectable viral fingerprints (Geser 1983). Conversely, in Europe or the United States, where BL occurs with a 20 fold lower incidence than in central Africa, only a minority of the cases--15%--are associated with EBV (Table 1). The inescapable conclusion is that the virus cannot be the only cause of BL. However, taking into consideration the fact that EBV has the capacity to immortalize human B lymphocytes, and is able to induce malignant lymphoma in nonhuman primates, it is likely that, at least in the so-called BL endemic areas, the virus is one of the factors involved in the causation of this tumor.

Table 1. BL/EBV ASSOCIATION IN DIFFERENT PARTS OF THE WORLD

	Incidence of BL	% of BL tumors associated with EBV	Reference
Central Africa	high	96%	Geser (1983)
North Africa	low-intermediate	90-95%	Ladjaj (1984)
Europe	low	15%	Lenoir and Philip unpublished
USA	low	10-25%	Andersson(1976)

Efforts to identify common features of BL from low and high incidence areas has brought into focus the cytogenetic rearrangements in the malignant cells. Three types of chromosomal translocations are repeatedly observed in BL: t(8;14), t(8;22), and t(2;8) (Bernheim 1981); all three types of translocation have been detected in cases presumed to originate from Europe, Japan, central Africa, the United States, and more recently from North Africa (Mark-Vendel 1983). The translocations are independent of the geographic origin of the patient, and are found in both EBV-positive and EBV-negative tumors. One conclusion of the International Agency for Research on Cancer's collaborative study of BL is that t(8;14), t(8;22, and t(2;8) can be considered as characteristic features of BL cells. In that study, all tumors examined carried one of the specified translocations. In 30% of the cases the translocation is the only cytogenetic anomaly detected in the malignant cells. The proportion of the tumors carrying each of the translocations is indicated in Table 2, and up to now seems to be valid for high as well as low incidence areas.

Table 2. CHROMOSOMAL TRANSLOCATIONS IN BURKITT'S LYMPHOMA[1]

	t(8;14)	t(8;22)	t(2;8)
47 cell lines analyzed[1]	36	7	4
Percent	76.5	15	8.5

[1]Based on the analysis of cell lines derived from BL patients from central Africa, north Africa, and Europe (IARC/BL project).

It has recently been shown that each BL translocation joins the cellular oncogene c-myc to a region encoding one of the immunoglobulin genes (see Leder 1983). This may lead to altered regulation of the c-myc gene. Possible models

and mechanisms for this event are discussed in detail by others at this conference. Since chromosomal translocations involving chromosome #8 are a characteristic feature of BL, one of the key features of BL cells is the presence of the "activated" c-myc gene. The significance of this activation can now be discussed in the light of the recent discoveries which describe the cooperation between cellular oncogenes in cellular transformation.

COOPERATION BETWEEN TWO ONCOGENES: THE TUMORIGENIC CONVERSION OF PRIMARY RAT EMBRYO FIBROBLASTS

With gene transfer techniques, it has been demonstrated by independent groups of investigators that mouse cell cultures such as NIH3T3 fibroblasts can be transformed into cells with a malignant phenotype following transfection with genomic DNA prepared from malignant tumors of human or animal origin. Untransfected NIH3T3 cells do not have a transformed phenotype, and as such are unable to form colonies in soft agar or to induce tumors following injection into nu/nu mice. The genes responsible for the transformation have been shown, in general, to be already identified cellular oncogenes which belong to the ras family (Weinberg 1984). These ras proto-oncogenes have been activated by point mutation which alters the amino acid sequence of the gene product (for example, the EJ gene of human bladder carcinoma; Tabin 1982).

When primary cells such as primary rat embryo fibroblasts (REFs) are used as recipient cells of oncogenes instead of the established NIH3T3 cells, different results are observed. The REFs carrying the transfected activated ras oncogene become morphologically altered but are unable to be grown indefinitely in culture and are unable to form tumors following injection into nu/nu mice or syngeneic rats (Land 1983). The interpretation of these results is that the ras oncogene is unable to fully transform the REFs, and that other factors such as additional oncogenes might be needed to fully transform these non-established cells. Cotransfection experiments with the use of dominant selectable

drug resistance markers demonstrated that concomitant introduction of both EJ-*ras* and viral *myc* oncogenes into REFs resulted in the rapid appearance of transformed foci (Land 1983a; Table 3). Cells from these foci were highly

Table 3. COMPLEMENTATION OF TRANSFORMATION BY COTRANSFECTION OF DIFFERENT ONCOGENES IN REFS

Transfected oncogene	% of colonies with transformed morphology (Ecogpt-selective medium)	Tumorigenicity in Nu/Nu mice (No. tumors/ no. injections
Control (gpt)	0	0/10
ras (EJ)	80	0/11
v-*myc*	0	0/7
pc-*myc* (murine c-myc) SV40 promoter)	0	0/6
v-*myc* + *ras* (EJ)	80	10/10
pc-*myc* + *ras* (EJ)	80	9/9
polyoma MT	15	0/6
polyoma LT	0	0/5
PyMT + *ras* (EJ)	10	0/5
PyLT + *ras* (EJ)	80	6/6

tumorigenic when injected into nude mice or syngeneic Fischer rats. This experimental evidence suggests that the ras and myc oncogenes can perform qualitatively distinct roles in the process of tumorigenesis and opens speculation about whether this observation may be generalized to other oncogenes. This work and work by others suggests that oncogenes such as c-myc or Ela of adenovirus and the large T of polyoma can cooperate with ras or ras-like genes (Table 3) to transform primary cells into tumorigenic cells (see Land 1983b).

Genes like Ela, polyoma large T, or myc can be considered to have immortalizing functions. They allow primary cells to be established as cell lines. However, inclusion of ras-type genes in the transfections leads to the appearance of tumorigenic foci within the immortalized cell populations. Could this model of cooperating oncogenesis be applied to other cell systems, and can we generalize it to human cancers such as lymphoma or leukemia?

COOPERATION BETWEEN ONCOGENES IN BURKITT'S LYMPHOMA

Numerous investigators have used gene transfer procedures to identify transforming DNA sequences in human cancers. These human cancers include both solid (Pulciani 1982) and hematopoietic tumors (Eva 1983). In most cases they were found to be ras oncogenes with the N-ras subtype being frequently isolated from lymphomas and leukemias. It appears also that activation of these genes is independent of tumor phenotype.

Interestingly, in the human promyelocytic leukemia cell line, HL-60, an activated N-ras oncogene was found to coexist with an altered myc gene, namely an amplified c-myc oncogene (Murray 1983). The hypothesis that two oncogenes that cooperate in making the cell malignant is provocative, especially given the demonstration that this cooperation is functional in turning REFs into malignant cells.

When Burkitt's lymphomas were tested for presence of transforming genes in the NIH3T3 assay, two types of results were obtained independently. In G. Cooper's laboratory at Harvard, all BL tested were found to contain an activated gene identified as the human homologue of the chicken B-lym gene (Diamond 1983). At the present time it is unclear whether the human B-lym gene is capable of cooperating with myc to render lymphoid cells malignant, and more work is needed for better characterization of this gene and its function.

In our laboratory one Burkitt's lymphoma cell line, designated Ramos, was found to contain an activated N-ras gene (Murray 1983). This BL cell carries a t(8;14) translocation such that the translocated myc gene coexists with a mutated transforming ras gene. This finding raises various questions. 1) Was the activated N-ras present in the original tumor, or was it activated during in vitro manipulation of the cells? The answer to this question requires that the search for transforming genes in BL be carried out with unmanipulated tumors rather than with established lines. 2) Could the activation of ras gene be related to the treatment of the patients? For this reason, tumor samples taken before any therapy must also be studied. 3) The Ramos cell line was established from an EBV-negative BL tumor. It is therefore important to establish whether EBV-positive tumors also contain ras oncogenes. One alternative possibility is that the cellular ras functon is not required in these tumors, but that the virus provides a ras-like function. A comparative study of EBV genome-positive and genome-negative tumors must be performed to clarify this issue.

In our recent experiments (G. Lenoir, in preparation), 14 BL lymphomas (tumor samples and cell lines, 6 of which were EBV-free) were tested for the presence of transforming genes by transfection into NIH3T3. Four out of the 14 DNA samples transform NIH3T3 cells. Two samples were from EBV-negative BL (one cell line and one tumor), and two were from EBV-positive cases (one cell line and one tumor.) One of the transfectants contains an activated Ki-ras allele.

Identification of the transforming genes in the three other cases is still in progress. Thus, transforming functions can be detected in BL, which raises the question whether cooperation with the myc oncogene occurred in making the lymphoid cell malignant.

CONCLUSION

Epidemiological studies as well as many experimental chemical- or radiation-induced carcinogenesis models have demonstrated that carcinogenesis is a process which involves multiple independent steps. At the cellular level, however, it was shown recently that the tumor cell phenotype can be understood as the activation of at least two sets of distinct genes. Both genes may be present within the same viral DNA, such as in polyoma virus or adenovirus. But cooperation between "activated" cellular proto-oncogenes may have the same end results. This demonstrates that oncogenes can have different qualitative functions, and allows categorization of oncogenes--both viral and cellular.

BL represents a special case in human cancer because at least two independent steps that are implicated in oncogenesis are found to be present. In all BL, the c-myc gene is altered, and likely activated. In some cases, ras genes are also found in activated form. In addition, the role of EBV in many of these tumors, though not understood, is likely to be an oncogenic one. These observations in BL not only provide a working hypothesis for approaching the molecular events which lead to cellular transformation, but it also sets the stage for further speculations. At what stage in tumorigenesis are the myc, ras, and EBV oncogenic functions critical? It is important to understand whether any of these functions are involved in premalignancy, malignancy, or in late progression.

The isolation of an activated ras gene following transfection of BL DNA into NIH3T3 cells indicates that the coexistence of altered myc and ras genes can be identified in these tumors.

If activated ras or ras-like genes are found consistently in BL, this would strengthen the suggestion that the cooperation between oncogenes observed in experimental rat cell systems reflects the events in human tumor appearance and progression.

REFERENCES

Andersson M, Klein G, Ziegler JL, Henle W (1976) Association of Epstein-Barr viral genomes with American Burkitt's lymphoma. Nature 260: 357-359.

Bernheim A, Berger R, Lenoir G (1981) Cytogenetic studies on African Burkitt's lymphoma cell lines: t(8;14), t(2;8) and t(8;22) translocations. Cancer Genetics and Cytogenetics 3:307-315.

Diamond A, Cooper GM, Ritz J, Lane M-A (1983) Identification and molecular cloning of the human Blym transforming gene activated in Burkitt's lymphomas. Nature 305: 112-116.

Eva A, Tronick SR, Gol RA, Pierce JH, Aaronson SA (1983) Transforming genes of human hematopoietic tumors: frequent detection of ras-related oncogenes whose activation appears to be independent of tumor phenotype. Proc. Natl. Acad. Sci. USA 80: 4926-4930.

Geser A, Lenoir GM, Anvret M, Bornkamm G, Klein G, Williams EH, Wright DH, De-The G (1983) Epstein-Barr virus markers in a series of Burkitt's lymphomas from the West Nile District, Uganda. Eur. J. Cancer Clin. Oncol. 19: 1393-1404.

Klein G (1981) The role of gene dosage and genetic transpositions in carcinogenesis. Nature 294: 313-318.

Ladjaj Y, Philip T, Lenoir GM, Tazerout FZ, Bendisari K, Boukheloua R, Brunat-Mentigny M, Aboulola M Abdominal Burkitt-type lymphomas in Algeria (A report of 49 cases). Brit. J. Cancer, in press.

Land H, Parada LF, Weinberg RA (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304: 596-602.

Land H, Parada LF, Weinberg RA (1983) Cellular oncogenes and multistep carcinogenesis. Science 222: 771-778.

Leder P, Battey J, Lenoir GM, Moulding C, Murphy W, Potter H, Stewart T, Taub R (1983) Translocations among antibody genes in human cancer. Science 222: 765-771.

Mark-Vendel E, Philip T, Ladjaj Y, Aboulola M, Lenoir GM (1983) Chromosomal translocations in Algerian Burkitt's lymphoma. Lancet, p. 788.

Murray MJ, Cunningham JM, Parada LF, Dautry F, Lebowitz P, Weinberg RA (1983) The HL-60 transforming sequence: a ras oncogene coexisting with altered myc genes in hematopoietic tumors. Cell 33: 749-757.

Pulciani S, Santos E, Lauver AV, Long LK, Aaronson SA, Barbacid M (1982) Oncogenes in solid human tumours. Nature 300: 539-542.

Tabin CJ, Bradley SM, Bargmann CI, Weinberg RA, Papageorge AG, Scolnick EM, Dhar R, Lowy DR, Chang EH (1982) Mechanisms of activation of a human oncogene. Nature 300: 143-149.

Weinberg RA (1984) Oncogenes and chemical carcinogenesis. In Biochemical Basis of Carcinogenesis, eds. H Greim, R Jung, M Kramer, H Marquardt, F Oesch. (NY: Raven Press), pp. 137-142.

Detection of an Adenovirus E1A-like Activity in Mammalian Cells

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INTRODUCTION

The E1A gene product of adenovirus is responsible for the activation of early viral transcription during a lytic infection (Jones & Shenk, 1979; Berk et al., 1979; Nevins, 1981). We have previously proposed that the E1A gene product mediates transcriptional activation through an indirect mechanism, possibly involving the inactivation of a cellular negative acting regulatory element (Nevins, 1981). This notion has been strengthened by the findings that a heterologous activator, the herpesvirus immediate early gene product, can fully supply E1A function for the activation of the adenovirus genes (Feldman et al., 1982; Imperiale et al., 1983). Furthermore, at least one cellular gene, that encoding a 70kd heat shock protein, is activated as a result of the action of the E1A protein (Nevins, 1982; Kao & Nevins, 1983). Thus, it is quite clear that the activation process mediated by E1A is not an adenovirus-specific mechanism but rather appears to be a more general mechanism of transcriptional control. We have questioned whether or not the control mediated by the E1A gene is also a control mechanism normally operating in the cell. To attempt to define such a cellular E1A-like activity, we have studied the control of the expression of the heat shock gene during normal cell growth. We have reasoned that changes in the control of this cellular gene, which is subject to E1A induction, might well reflect the activity of a cellular E1A type gene. We have therefore examined various cell lines for the level of expression of the heat shock gene under normal growth conditions. We indeed do find differences and more importantly, we find that those cells that have a high level of expression of the heat shock gene also permit early viral transcription in the absence of the E1A gene product.

RESULTS AND DISCUSSION

To study the control of the expression of the heat shock gene, we have employed both an antisera specific to the protein as well as a cDNA clone specific to the mRNA. Proteins were prepared from a number of human cell lines and assayed for the heat shock protein by probing Western blots with the specific antisera. The level of the protein in several human cell lines was found to vary considerably. For instance, HeLa cells were found to contain at least 50 times more heat shock protein than WI38 cells. The same low level as in the WI38 cell line was also found for a HEK (human embryonic kidney) cell line and the 143B cell line [a tk(-) derivative of an osteogenic cell line transformed by Kirsten sarcoma virus (Rhim et al., 1975)]. Several other cell lines derived from human tumors were found to contain levels of the protein approaching that found

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in HeLa cells. In fact, with one exception, HeLa cells possessed more of the protein than any other cell line examined. The one exception was the adenovirus transformed 293 cell line (Graham et al., 1977) that constitutively expresses the adenovirus E1A gene (Aiello et al., 1979). In the 293 cells we found three to five times more heat shock protein than in any other cell line, including HeLa.

To directly measure the level of expression of the heat shock gene in these cells, we have measured the amount of heat shock mRNA by Northern blot analysis. RNA prepared from various human cell lines was fractionated by gel electrophoresis and, after blotting, the heat shock mRNA was detected by hybridization with a specific cDNA clone (Kao & Nevins, 1983). The results of such an experiment demonstrated that the differences we had observed in heat shock protein levels were a direct reflection of the relative abundance of the mRNA. HeLa cells possessed at least 50 times more heat shock mRNA than did HEK cells, WI38 cells, or 143B cells. Finally, recent data measuring actual transcription rates in isolated nuclei indicates that the cell-specific control is due to regulation of transcription of the heat shock gene (Kao & Nevins, unpublished data).

We have now posed the question: Is the expression of the heat shock gene a reflection of a cellular regulatory function that is similar in nature to the adenovirus E1A activity? If so, then we might expect to find E1A-independent expression of early viral genes in those cells expressing the heat shock gene at a high level (i.e. HeLa cells). Various human cell lines were infected with the E1A deletion mutant d1312 (Jones & Shenk, 1979) and then subsequently assayed for early viral gene expression by measuring the formation of the 72kd protein product of the E2 gene (Feldman et al., 1982; Imperiale et al., 1983). The results of such an experiment clearly indicated that in those cells in which there was a high level of expression of the heat shock gene there was also expression of the adenovirus genes in the absence of E1A. Conversely, there was a strict requirement for the E1A gene, to activate early viral transcription, in cells that were not expressing the heat shock gene.

The preceding results suggested a strong correlation between the regulatory properties of the heat shock gene and an E1A independence of early adenovirus transcription. That is, the early adenovirus genes and the heat shock gene appear to be subject to the same cellular control, consistent with the fact that the E1A gene product is an inducer of the heat shock gene. However, to further substantiate this point, we have used a particular cell line whose properties can be altered by experimental manipulation. This particular system, the mouse F9 teratocarcinoma cell line, can be induced to differentiate in culture by treatment with retinoic acid and dibutyryl cyclic AMP (Strickland & Mahdavi, 1978; Strickland et al., 1980; Kuff & Fewell, 1980). By such a treatment, the phenotypic properties of the cells change markedly. We have used this system to ask if differentiation alters the cells with respect to transcriptional control of the heat shock gene and the early adenovirus genes. Once again, cells were infected with the E1A mutant d1312 and early viral gene expression assayed by measuring formation of the 72kd protein. The results were striking in that in the absence of E1A function there was considerable induction of the adenovirus E2 gene in the undifferentiated F9 cells; however, upon

differentiation there was a loss in the ability to express this gene resulting in a strict requirement for the E1A function. Thus, as in many of the human tumor cell lines, there appears to be an E1A-like activity in the F9 cells. Of added interest in this case is the finding that such an activity is lost upon differentiation.

That this phenotypic change does indeed reflect a change in an E1A-like function was suggested by the finding that the heat shock gene also appeared to be similarly controlled. For this analysis we have utilized the antibody to measure protein levels before and after differentiation. Probing RNA blots with the human cDNA clone failed to detect the mRNA, presumably due to a lack of sequence conservation at the nucleic acid level. Assay of the undifferentiated F9 cells yielded a significant level of the protein, indicating that the gene was indeed expressed in these cells. Upon differentiation, there was a marked decline in the abundance of the protein. Although the decrease in the protein levels could have been due to post-transcriptional or even post-translational events, based on the correlation between RNA levels and protein levels in the human cells, it is likely that this change in the F9 cells is due to a change in transcription of the gene.

What might be the normal role for a cellular E1A-like function? Several lines of evidence suggest a role for such a gene in modulating gene expression in relation to cellular growth control. For instance, almost without exception, those cell lines that we have studied that possess an E1A like activity are rapidly growing cell cultures derived from tumor sources. Cells with stricter growth regulation have little detectable E1A activity. Secondly, the cellular gene that is regulated by such an activity (the heat shock gene) appears to be cell cycle controlled (Kao et al., unpublished data). HeLa cells synchronized at the G1/S boundary by a thymidine-aphidicolin block (Heintz et al., 1983) were found to have very low amounts of the heat shock mRNA. Upon release into S phase, however, the mRNA rose in abundance and by G2 had increased at least 30 fold. Thus, we would suggest that the activity that normally controls the level of expression of this gene is an activity regulated by cell growth.

Finally, it is known that the adenovirus E1A gene, as well as the myc oncogene, have the ability to immortalize cultures of primary rat embryo fibroblasts (Ruley, 1983; Land et al , 1983). This result thus suggests that the E1A gene can, in some manner, confer a continuous growth potential on these normally growth restricted cells. Given the properties of the E1A mediated activation of transcription as well as the finding that the cellular gene that is E1A responsive (heat shock gene) appears to be regulated in relation to the cell cycle, we would propose that the role of a cellular E1A type activity is to provide a primary stimulus for transcription of genes required in cell growth. What might such an E1A-like activity be? As indicated above, the E1A gene and the myc oncogene share functional properties. Furthermore, the E1A gene and the myc gene actually share limited sequence homology (Ralston & Bishop, 1983). This opens the possibility that the myc gene may in fact be the activity that we have detected in these cells. This may indeed be the case since an analysis of myc RNA levels in the various cell lines described above yields the result that myc levels correlates with the presence of an E1A-like activity (Kao et al., unpublished data). And, particularly relevant is a recent re-

port that indicates that myc expression is controlled in differentiating F9 cells (Campisi et al., 1984). Of course, the data is thus far only correlative. However, the possibility remains and is supported by the available data, that the myc gene is a transcriptional activator that regulates the expression of a group of cellular genes during cell growth.

REFERENCES

Aiello, L, Guiloyle, R, Huebner, K, and Weinmann, R (1979) Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryo kidney cells (HEK-Ad5 or 293). Virology 94: 460-469.

Berk, A, Lee, F, Harrison, T, Williams, J, and Sharp, PA (1979) Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell 17: 935-944.

Campisi, J, Gray, HE, Pardee, AB, Dean, M, and Sonenshein, G (1984) Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell 36: 241-247.

Feldman, LT, Imperiale, MJ, and Nevins, JR (1982) Activation of early adenovirus transcription by the herpesvirus immediate early gene: evidence for a common cellular control factor. Proc. Nat'l. Acad. Sci. USA 79: 4952-4956.

Graham, FL, Smiley, J, Russell, WC, and Nairn, R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus 5. J. Gen. Virol. 36: 59-72.

Heintz, N, Sive, HL, and Roeder, RG (1983) Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. 3: 539-550.

Imperiale, MJ, Feldman, LT, and Nevins, JR (1983) Activation of gene expression by adenovirus and herpesvirus regulatory genes acting in trans and by a cis acting adenovirus enhancer element. Cell 35: 127-136.

Jones, N and Shenk, T (1979) An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Nat'l. Acad. Sci. USA 76: 3665-3669.

Kao, HT and Nevins, JR (1983) Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. Mol. Cell. Biol. 3: 2058-2065.

Kuff, EL and Fewell, JW (1980) Induction of neural-like cells in cultures of F9 teratocarcinoma treated with retinoic acid and dibutyryl cyclic adenosine monophosphate. Devel. Biol. 77: 103-115.

Land, H, Parada, LF, and Weinberg, RA (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating genes. Nature 304: 596-602.

Nevins, JR (1981) Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell 26: 213-220.

Nevins, JR (1982) Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. Cell 29: 913-919.

Ralston, R and Bishop, JM (1983) The protein products of the myc and myb oncogenes and adenovirus E1A are structurally related. Nature 306: 803-806.

Rhim, JS, Cho, HY, and Huebner, RJ (1975) Non-producer human cells induced by murine sarcoma virus. Int. J. Cancer 15: 23-29.

Ruley, HE (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304: 602-606.

Strickland, S and Mahdavi, M (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell 15: 393-403.

Strickland, S, Smith, KE, and Marotti, KR (1980) Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. Cell 21: 347-355.

Does the Large T Protein of Polyoma Virus Regulate the Expression of the Cellular myc Gene?

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Upon expression of the large T protein of polyoma virus from genes encoding only this protein (plt genes: intron-less gene in pPyLT1 plasmid)(Tyndall et al., 1981), hrt mutant NG18 (Schaffhausen et al., 1978)), changes were observed in the behaviour in culture of rodent embryo fibroblast cells in primary cultures (REF) (Rassoulzadegan et al., 1982, 1983): the cells grew in culture for an unlimited number of generations ("immortalization") and grew in the presence of low concentrations of serum (below 1% newborn calf serum). They maintained, however, the extended morphology, the low saturation density and the anchorage dependency characteristic of the normal fibroblast. Unlike the original REF cells, these "immortalized" lines could be transformed by transfer of the polyoma virus gene encoding only the middle T protein (pmt gene: plasmid pPyMT1, Treisman et al., 1981), thus demonstrating a two-step process in tumorigenic transformation. A similar two-step mechanism was suggested by Land et al. (1983) for the myc and ras families of cellular oncogenes: expression either of the polyoma plt or of v-myc and rearranged c-myc genes allowed transformation of REF cells with activated forms of the ras genes. These results suggest that both genes exert similar function(s) at an early step of the transformation of REF cells in culture. This class of oncogenes also includes the E1a genes of adenovirus 2, required for transformation by the E1b genes of the same virus and which can also cooperate with a ras oncogene in REF transformation (Van den Elsen et al., 1982, Ruley, 1983).

HYPOTHESES

Immortalization is an obvious requirement for the isolation of permanently transformed cells in culture. On the other hand, we cannot a priori consider that unlimited growth potential is necessarily acquired by a unique mechanism. The spontaneous variation giving rise to the 3T3 lines (Todaro and Green, 1963), infection with polyoma (Vogt and Dulbecco, 1963), adenoviruses (Van de Elsen et al., 1982) and other agents might in fact not act on the same targets at the molecular level. In this case, the fact that myc, E1a and plt cooperate with ras and pmt for the establishment of a transformed line could be coincidental (Hypothesis I). Alternatively, these three genes could independently act on the same cellular targets. It is known that both the plt and the myc products are nuclear proteins which bind strongly to DNA (Gaudray et al., 1981, Bunte et al., 1983, Galup et al., 1983, Hann et al., 1983, Clertant et al., 1984). The polyoma large T protein, as its SV40 equivalent (see Hand, 1981 for review), is thought to exert, via high-affinity recognition of genomic sites (Gaudray et al., 1981, Dilworth et al., 1984), a variety of regulatory functions on the viral genome: initiation of

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DNA replication, negative and positive control of gene expression (Francke and Eckhart, 1973, Clertant and Cuzin, 1980, Cogen, 1978, Fenton and Basilico, 1982, G. Khoury and M. Yaniv, personal communication). It is therefore conceivable that the myc and the large T proteins might regulate the expression of the same, or of overlapping subsets of cellular promotors (Hypothesis II). A third possible model (Hypothesis III) would be that the immortalization step corresponds primarily to changes in the regulation of the c-myc gene, induced by the large T protein. One of the possible ways of altering the regulation of c-myc expression is suggested by results of Kelly et al. (1983), indicating that the expression of c-myc in normal cells is restricted to defined periods of the cell cycle. Large T might then induce a change from cyclic regulation to constitutive expression throughout the cell cycle. One could further assume that regulation of c-myc expression in the cell cycle is dependent on cis-acting sequences, which bind an endogenous regulatory protein and/or the large T protein. Changes equivalent to those induced by large T binding might therefore also result from the rearrangements of non coding sequences observed in many tumor cells (see Robertson, 1983 for review) as well as in the viral gag-myc fusion gene.

IDENTITY OF THE PHENOTYPES INDUCED BY LARGE T AND BY THE ONCOGENIC FORMS OF myc.

As a first approach, we asked whether the similarity between plt and myc, previously demonstrated for their ability to cooperate with pmt and ras in inducing tumors (Land et al., 1983), could be extended to the other biological properties which were shown to depend on the activity(ies) of the polyoma large T protein. Results to be presented in detail elsewhere (E. Mougneau et al., manuscript in preparation) indicated that, indeed, two rearranged forms of the c-myc gene (Land et al., 1983) were as efficient as the polyoma plt gene in a series of biological assays previously used for the latter (Rassoulzadegan et al., 1982, 1983):

(i) colony formation by REF cells seeded at low density in high serum medium;
(ii) colony formation by established FR3T3 rat fibroblasts at low serum concentration;
(iii) complementation of cells of established lines expressing only the middle T protein (MTT lines) for the expression of transformed growth properties in low serum medium (focus formation, colony formation in suspension);
(iv) preliminary results suggest in addition that expression of either the plt or the rearranged myc genes allows phenotypic transformation upon exposure to tumor promoting agents (TPA) (G. Connan and M. Rassoulzadegan, personal communication).

Cells were picked from colonies produced by REF cells after transfer of myc sequences. They could be further propagated in culture, as expected from previous studies showing that the ability to divide at very low cell densities is acquired together with unlimited growth potential in culture ("immortalization") (Todaro and Green, 1963, Rassoulzadegan et al., 1983). By contrast with both 3T3 and plt-immortalized lines, however, these cultures grew unefficiently, continuously shedding off dead cells in the culture medium. Similarly, MTT cells complemented by the myc genes for focus formation in low serum medium were difficult to propagate in culture, again in clear contrast with comparable lines obtained after transfer of the large T genes (Rassoulzadegan et al., 1982). This observation is

reminding of that reported by Land et al. (1983) of a faster growth in nude mice of the plt-ras- than of the myc-ras-induced tumors.

Except for this slow growth in culture of the cells carrying the rearranged forms of myc, responses induced by these genes in different biological assays were similar to those induced by plt. This makes Hypothesis I somewhat unlikely. The specificity of the plt-myc phenotype is underlined by two a contrario observations. The spontaneous immortalization process leading to the establishment of the permanent 3T3 fibroblast lines (Todaro and Green, 1963), even though it makes the cells susceptible to transformation by the polyoma virus pmt gene (Treisman et al., 1981) and by ras genes (see Land et al., 1983 for review), does not confer serum-independence (Rassoulzadegan et al., 1982), at least without further changes accumulated during growth in culture. It was also observed that other DNA tumor viruses, such as Bovine Papilloma Virus Type 1 (BPV1), transform established fibroblast lines in culture, but do not appear to immortalize REF cells in the assays previously used for plt and myc (Rassoulzadegan et al., 1983 and B. Binétruy and G. Meneguzzi, personal communication). Furthermore, BPV1 does not confer the ability to grow at low serum concentrations on cultured rat fibroblast, nor on cells which were grown as tumors in the animal (Grisoni et al., 1984).

An additional interest of defining the phenotype induced by transfer of rearranged myc genes into normal rodent cells (REF, FR3T3) would be to provide biological assays and selective conditions for this family of genes. Further studies are however required to define the proper cell type for such a screening, as REF cells, or FR3T3 derivatives, may not pick up genomic DNA with a high enough efficiency in the Ca^{++} transfection assay.

DOES LARGE T REGULATE THE EXPRESSION OF CELLULAR (ONCO)GENES?

Experiments presently in progress in our laboratory should allow us to check whether, as hypothesized above (Hyp. II and III), binding of large T to defined nucleotide sequences of the host genome leads to modulation of the expression of cellular genes, possibly including known protooncogenes and more specifically (Hyp. III), the c-myc gene.

In a first series of experiments, cellular (mouse) sequences were cloned, and amplified in bacterial vectors, which are recognized in vitro by the large T protein with the same high affinity as the known binding sites in the origin of replication-promotor region of the viral genome (Gaudray et al., 1981, Dilworth et al., 1984). One such sequence has been so far characterized in detail and shown to be highly homologous to a region of the promotor of the large terminal repeats of Moloney Sarcoma Virus (Galup et al., 1983 and C. Galup, P. Gaudray and F. Cuzin, unpublished results). The mouse genomic sequences adjacent to the cloned fragments are being isolated from libraries in Lambda phages.

On the other hand, cloned fragments from cellular protooncogenes are being tested for the presence of sequences which could bind the large T protein with high affinity. It is of interest in this respect to notice that sequences which exhibit significant similarities with the large T binding sites of the polyoma genome are in several instances encountered near the coding sequences of cellular protooncogenes: for instance, immediately upstream of the first ATG codon

of the human c-myc gene (Colby et al., 1983, nucleotides 972 to 1022), the following sequence is found:

```
     *** ********    *** *  *### ##*****
(3') CGGAGGGCGAAACACACGGGGCGAGGTCGTCGGAGGGCGCTGCTAC
(5') GCCTCCCGCTTTGTGTGCCCCGCTCCAGCAGCCTCCCGCGACGATG(initiator)....
```

The underlined (5')G-Purine-GGC(3') repeats were considered as characteristic of the large T binding sites in polyoma DNA (Gaudray et al., 1981). Furthermore, one of the polyoma binding sites, also located upstream of the first ATG codon (Dilworth et al., 1984), corresponds to the sequence (Soeda et al., 1980, nucleotides 129 to 175, identical nucleotide blocks indicated by symbols):

```
    *** *  **** * **   *** *  * ******* ### ##
(3')CGGGGTTGGCGGAGAAGGGCGGAGTAAAGTCGGAGTGGTGGTAGTAC
(5')GCCCCAACCGCCTCTTCCCGCCTCATTTCAGCCTCACCACCATCATG(initiator)....
```

A second approach uses libraries of cDNAs representative of the mRNA population of cells expressing large T to search for probes corresponding to genes whose expression might be modulated by the viral protein. Nine independent cDNA clones were so far isolated, which correspond to mRNAs overrepresented in cells expressing the large T protein, in low serum medium, as compared with cells transformed by the middle T-only gene (MTT lines, Rassoulzadegan et al., 1982)) in the same medium. Four of these mRNAs were also expressed at higher levels in normal cells grown in high serum medium, suggesting that these genes might be involved in the control of cellular growth (N. Glaichenhaus, P. Masiakowski, P. Chambon and F. Cuzin, unpublished results).

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REFERENCES

Bunte, T, Greiser-Wilke, I & Moelling, K (1983) The transforming protein of the MC29-related virus CMII is a nuclear DNA-binding protein whereas MH2 codes for a cytoplasmic RNA-DNA-binding polyprotein. The EMBO J 2:1087-1092

Clertant, P & Cuzin, F (1980) Initiation of polyoma virus DNA replication in vitro and its dependence on the viral gene A protein. Nucl Acids Res 8:4377-4392

Clertant, P, Gaudray, P & F Cuzin (1984) Salt stable binding of the large T protein to DNA in polyoma virus chromatin complexes. The EMBO J, in press

Cogen, B (1978) Virus-specific early RNA in 3T6 cells infected by a tsA mutant of polyoma virus. Virology 85:222-230

Colby, WW, Chen, EY, Smith, DH & Levinson, AD (1983) Identification and nucleotide sequence of a human locus homologous to the v- myc oncogene of avian myelocytomatosis virus MC29. Nature 301:722-725

Dilworth, SM, Cowie, A, Kamen, RI & Griffin, BE (1984) DNA binding activity of polyoma virus large T-antigen Proc Natl Acad Sci USA, in press

Fenton, RG & Basilico, C (1982) Regulation of polyoma virus early transcription in transformed cells by large T-antigen. Virology 121:384-392

Francke, B & Eckhart, W (1973) Polyoma gene function required for viral DNA synthesis. Virology 55:127-135

Galup, C, Trejo-Avila, L, Mougneau, E, Lemieux, L, Gaudray, P, Rassoulzadegan, M & Cuzin, F (1983) The oncogenic function(s) of the large T protein of polyoma virus. INSERM, in press

Gaudray, P, Tyndall, C, Kamen, R & Cuzin, F (1981) The high affinity binding site on polyoma virus DNA for the viral large-T protein. Nucl Acids Res 9:5697-5710

Grisoni, M, Meneguzzi, G, de Lapeyrière, O, Binétruy, B, Rassoulzadegan, M & Cuzin, F (1984) The transformed phenotype in culture and tumorigenicity of Fischer rat fibroblast cells (FR3T3) transformed with Bovine Papilloma Virus Type 1. Virology, in press

Hand, R (1981) Functions of T antigens of SV40 and polyoma virus. Biochem Biophys Acta 651:1-24

Hann, SR, Abrams, HD, Rohrschneider, LR & Eisenman, RN (1983) Proteins encoded by v- myc and c- myc oncogenes: identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. Cell 34:789-798

Kelly, K, Cochran, BH, Stiles, CD & Leder, P (1983) Cell-specific regulation of the c- myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603-610

Land, H, Parada, LF & Weinberg, RA (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596-602

Rassoulzadegan, M, Cowie, A, Carr, A, Glaichenhaus, N, Kamen, R & Cuzin, F (1982) The roles of individual polyoma virus early proteins in oncogenic transformation. Nature 300:713-718

Rassoulzadegan, M, Naghashfar, Z, Cowie, A, Carr, A, Grisoni, M, Kamen, R & Cuzin, F (1983) Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cells. Proc Natl Acad Sci USA 80:4354-4358

Robertson, M (1983) Paradox and paradigm: the message and meaning of myc. Nature 306:733-736

Ruley, HE (1983) Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304:602-606

Schaffhausen, BS, Silver, JE & Benjamin, TL (1978) Tumor antigen(s) in cells productively infected by wild-type polyoma virus and mutant NG-18. Proc Natl Acad Sci USA 75:79-83

Soeda, E, Arrand, JR, Smolar, N, Walsh, JE & Griffin, BE (1980) Coding potential and regulatory signals of the polyoma virus genome. Nature 283:445-453.

Todaro, GJ & Green, H (1963) Quantitative studies on the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17:299-313

Treisman, R, Novak, U, Favaloro, J & Kamen, R (1981) Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. Nature 292:595-600

Tyndall, C, La Mantia, G, Thacker, CM, Favaloro, J & Kamen, R (1981) A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nucl Acids Res 9:6231-6250

Van den Elsen, PJ, de Pater, S, Houweling, A, van der Veer, J & van der Eb, A (1982) The relationship between region E1a and E1b of human adenoviruses in cell transformation. Gene 18:175-185

Vogt, M & Dulbecco, R (1963) Steps in the neoplastic transformation of hamster embryo cells by polyoma virus. Proc Natl Acad Sci USA 49:171-179.

Genome Structure and Host Range Restriction of the Lymphotropic Papovavirus (LPV): Identification of a Viral Lymphocyte Specific Enhancer Element

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INTRODUCTION

Lymphotropic Papovavirus (LPV) is a new member of the polyomavirus genus of the Papovaviridae (zur Hausen and Gissmann, 1979). It was isolated from an African Green Monkey B-lymphoblastoid line and subsequently adapted to the human B-lymphoma line BJA-B. LPV shows a highly restricted host range in vitro. Only continuously growing cells of primate B-lymphocyte origin, i.e. B-lymphoma lines and B-lymphoblastoid cell lines immortalised by Epstein Barr Virus (EBV) are permissive to productive infection by LPV particles (zur Hausen and Gissmann, 1979; Brade et al., 1981; Takemoto et al., 1982). Normal lymphocytes, even with mitogenic stimulation are not permissive to LPV.

Takemoto and Kanda (1984) reported transforming activity of LPV. The transformed hamster embryo cells contain LPV DNA, express LPV T-antigen and grow into highly malignant tumors when injected into newborn hamsters. However, attempts to transform lymphoid cells by LPV have failed so far.

We are interested in viral and host functions that determine the host range of LPV. Here we briefly summarize the organization of the LPV genome and present experimental results indicating that two separate mechanisms may restrict the LPV host range: a specific receptor and/or uncoating function present only in transformed and EBV immortalised B lymphocytes and a LPV enhancer element with specificity for B- and T-lymphocytes.

LPV GENOME STRUCTURE

The complete 5270 basepair (bp) sequence of the LPV genome has been determined (Pawlita et al., 1984). The genome organization is summarized in Fig. 1. Like the genomes of all other polyomaviruses, it contains an early and late coding region which are transcribed predominatly in the early and late phase of the lytic cycle respectively. The LPV early region can encode only two proteins, small and large T-antigen. The LPV late region encodes the three structural proteins VP1, VP2 and VP3.

Sequence comparison of the LPV proteins VP1, VP2 and large T-antigen with the corresponding proteins from SV40 and Polyoma (Py) showed that LPV and Py share 44%, LPV and SV40 42% and SV40 and Py 41% identical amino acids thus indicating that LPV, SV40 and Py are about equally related to each other (Pawlita et al., 1984).

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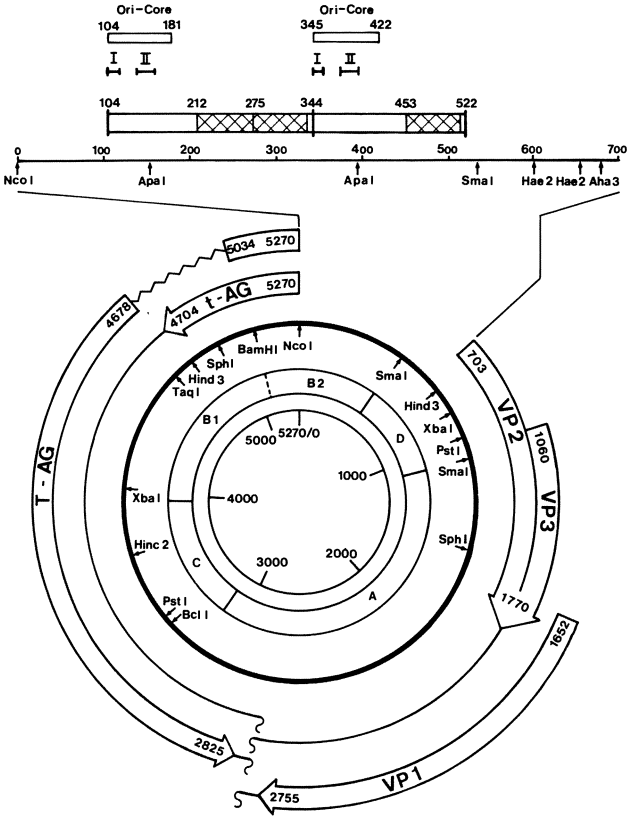

Fig. 1. Organisation of the LPV genome with postulated coding and control regions relative to a physical map of the virus. Numbering starts at the single Nco I restriction site coinciding with the early start codon and proceeds clockwise through the noncoding control region towards late and early coding region. The noncoding control region is depicted in larger scale at the top with repeated sequences symbolized by rectangular boxes; the 63 bp repeat probably containing the LPV enhancer element is hatched.

LYMPHOCYTE SPECIFIC ENHANCER ELEMENT OF LPV

The noncoding region in the LPV genome contains two sets of tandem repeats (Fig. 1). In the large 173 bp repeat (bp 104 to 344 and 345 to 522) the core of the origin of DNA replication is duplicated. In addition a 63 bp sequence is duplicated in tandem within the first copy of the large repeat (bp 212 to 274 and 275 to 337). The location of this 63 bp repeat is similar to that of the SV40 72 bp repeat which contains the SV40 enhancer element (Benoist and Chambon, 1981; Gruss et al., 1981; Banerji et al., 1981).

The LPV 63 bp and SV40 72 bp repeats show sequence homology only in that short region that has been defined as essential enhancer core in SV40 by Weiher et al. (1983) (Fig. 2). In addition the same region of the LPV 63 bp repeat has a 16 out of 21 bp homology with the lymphocyte specific mouse immunoglobulin heavy chain gene (mouse IgH) enhancer (Banerji et al., 1983) (Fig. 2). These sequence homologies led to the hypothesis that the LPV 63 bp repeat might contain an enhancer element with lymphocyte specificity.

Enhancement activity of the LPV 63 bp repeat was tested with the Chloramphenicol-acetyltransferase (CAT) assay (Gorman et al., 1982). In this assay, a bacterial gene encoding the enzyme CAT is linked to

```
SV40              GTCAGTTAGGGTGTGGAAAGT
                        * * *****  **
LPV               AAAGAGGAAGCTGTGGTTAGA
                  ***   * ********** **
Mouse IgH         AAACTTGTAGCTGTGGTTTGA
Core consensus                GTGGTTTG
                                 AAA
```

Fig. 2. DNA sequence comparison of the SV40 enhancer core region (Weiher et al., 1983) within the SV40 72bp repeat with the homologous region within the LPV 63 bp repeat (Pawlita et al., 1984) and a homologous region within the mouse immunoglobulin heavy chain gene enhancer element (Banerji et al., 1983). The enhancer core consensus sequence of Weiher et al. (1983) is part of all 3 sequences.

several regulatory signals from the early transcription unit of SV40. The resulting plasmid pA10 CAT2 includes a portion of the early SV40 promoter from which the SV40 enhancer sequence has been deleted (Fig. 3). The addition of enhancer elements to pA10 CAT2 results in efficient CAT gene expression when plasmid DNA is introduced into eukaryotic cells by transfection. The amount of gene product (CAT) in cell extracts is measured by its in vitro conversion of chloramphenicol to an acetylated form.

The plasmid pLPV1 CATs was constructed by cloning the 241 bp Hae III fragment (bp 159 to 399) into the Bgl II site of pA10 CAT2 in the sense orientation (relative to its position in the LPV genome, Fig. 1). The plasmids pSV2 CAT (Gorman et al., 1982; Fig. 3) and pIGH1 CAT (not shown in Fig. 3) contain the SV40 and mouse IgH enhancer elements respectively in similar position. Cells were transfected with DNA from these plasmids using DEAE-Dextran (McCutchan and Pagano, 1968) and 48 hours later cell extracts were assayed for CAT activity as desribed (Gorman et al., 1982). Results are summarized in Table 1.

Table 1. Tissue Specifity of LPV, SV40 and Mouse IgH Enhancers

Cell Line	Cell Type	Origin	CAT Activity		
			LPV	SV40	Mouse IgH
BJA-B	B-lymphoma	human	+	+	+
Molt 4	T-lymphoma	human	+	+	-
HELA	epithelial, cervix carcinoma	human	-	+	ND
HEF	embryonic	hamster	-	+	-

[1]ND = not determined

The LPV fragment in pLPV1 CATs has enhancing activity in the CAT assay. The enhancing activity is restricted to cells of B and T lymphocyte origin. In contrast to the lymphocyte specificity of the LPV enhancer element, the SV40 enhancer element shows no tissue specificity.

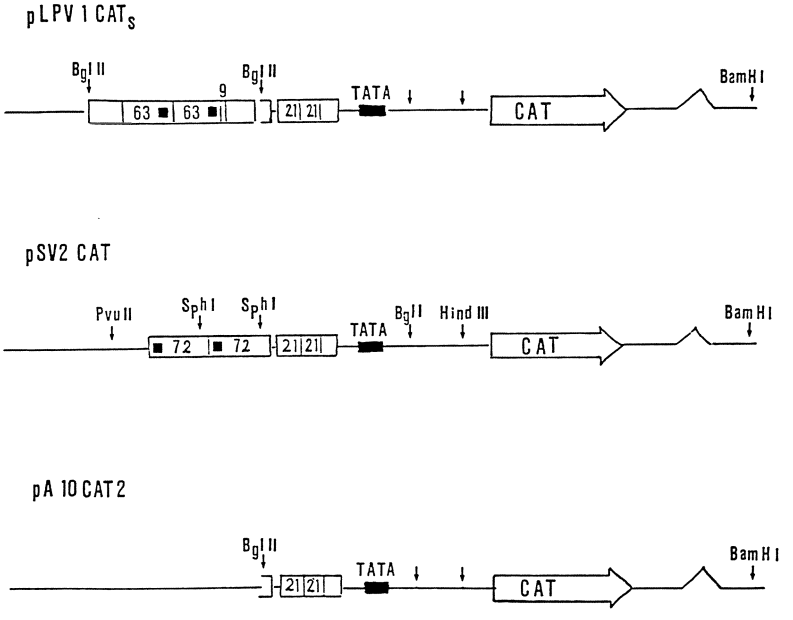

Fig. 3. Structure of CAT expression plasmids used to measure enhancer activity. pLPV1 CATs contains the 241 bp LPV Hae III fragment (bp 159 to 399) cloned into the Bgl II site of pA10 CAT 2 (Gorman et al., 1982) and pSV2 CAT (Gorman et al., 1982) contains the SV40 enhancer element in similar position. Black squares denote location of the enhancer core sequences within the 63 bp and 72 bp repeat respectively. The 21 bp repeats (boxed) and TATA-box are part of the SV40 early promoter that is used in the expression of the gene coding for the bacterial enzyme Chloramphenicol-acetyltransferase (CAT).

LPV SPECIFIC RECEPTOR IN TRANSFORMED B-LYMPHOCYTES ?

Since the "host range" of the LPV enhancer element includes T-lymphoma cells it is wider than the host range of the virus, which is restricted to transformed/immortalized B-lymphocytes. This indicates that a LPV specific cell receptor or a specific penetration/uncoating mechanism may be present in these cells.

We tested this hypothesis by transfection of T-Lymphoma cells with purified LPV DNA using DEAE-Dextran. Expression of late LPV antigens (capsid proteins) 48 hours after transfection and infection was measured by indirect immunofluorescence using a rabbit serum raised against purified LPV particles. The T-lymphoma line Molt 4 and also mitogen stimulated peripheral blood lymphocytes are not productively infected by LPV particles but these cells express late LPV antigens when transfected with purified DNA (Table 2). These results strengthen the hypothesis that B-lymphoma cells and B-lymhoblastoid cells immortalised by EBV but not mitogen stimulated lymphocytes nor T-lymphoma cells express a function (receptor ?) that allows adsorption of LPV to the cell membrane,followed by penetration and uncoating of the viral DNA.

Table 2. Expression of LPV Capsid Antigens

Cell Line	Cell Type	Infection	Transfection
BJA-B	B-lymphoma	1.6 %[1]	1.25 %
Molt-4	T-lymphoma	0 %	0.4 %
PBL + PWM	mitogen stimulated lymphocytes	0 %	1.1 %
PBL + PHA	mitogen stimulated lymphocytes	0 %	0.75 %

[1]percentage of immunofluorescence positive cells 48 hrs. after infection with LPV and transfection with purified viral DNA respectively stained with a rabbit serum raised against purified LPV particles.

ACKNOWLEDGEMENTS

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REFERENCES

Banerji J, Olson L and Schaffner W (1983) A Lymphocyte-specific Cellular Enhancer Is Located Downstream of the Joining Region in Immunoglobulin Heavy Chain Genes. Cell 33: 729-749

Banerji J, Ruscon and Schaffmer W (1981) Expression of a ß-Globin Gene Is Enhanced by Remote SV40 DNA Sequences. Cell 27: 299-308

Benoist C and Chambon P (1981) In vivo Sequence Requirements of the SV40 Early Promoter Region. Nature 290: 304-310

Brade L, Vogl W, Gissmann L and zur Hausen H (1981) Propagation of B-Lymphotropic Papovavirus (LPV) in Human B-Lymphoma Cells and Characterization of Its DNA. Virology 114: 228-235

Gorman CM, Moffat LF and Howard BH (1982) Recombinant Genomes Which Express Chloramphenicol Acetyltransferase in Mammalian Cells. Mol. Cell. Biol. 2: 1044-1051

Gruss P, Dhar R and Khoury G (1981) Simian Virus 40 Tandem Repeated Sequences as an Element of the Early Promoter. Proc. Natl. Acad. Sci. USA 78: 943-947

McCutchan JH and Pagano JS (1968) Enhancement of the Infectivity of Simian Virus 40 Deoxyribonucleic Acid With Diethylaminoethyl-Dextran. J. Natl. Cancer Inst. 41: 351-357

Pawlita M, Clad A and zur Hausen H (1984) Complete DNA Sequence of Lymphotropic Papovavirus (LPV): Prototype of a New Species of the Polyomavirus Genus. submitted for publication

Takemoto KK, Furuno A, Kato K and Yoshiike K (1982) Biological and Biochemical Studies of African Green Monkey Lymphotropic Papovavirus. J. Virol. 42: 502-509

Takemoto KK and Kanda T (1984) Lymphotropic Papovavirus Transformation of Hamster Embryo Cells. J. Virol. in press

Weiher H, König M and Gruss P (1983) Multiple Point Mutations Affecting the Simian Virus 40 Enhancer. Science 219: 626-631

zur Hausen H and Gissmann L (1979) Lymphotropic Papovavirus Isolated from African Green Monkey and Human Cells. Med. Microbiol. Immunol. 167: 137-153

Tlym-I, a Stage Specific Transforming Gene Shares Homology with MHC I Region Genes

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We previously reported the identification of five different stage specific transforming genes activated in T and B-lymphoid neoplasms (Lane, 1982). These genes are evolutionarily well-conserved between mouse and man and are expressed in multiple neoplasms representative of particular stages of normal lymphoid cellular differentiation.

The first of these genes to be characterized, Blym-1, was isolated from chicken bursal lymphomas and was found to share substantial amino acid homology with the transferrin family proteins (Goubin, 1983). This gene was used as a probe to isolate human Blym-1 from African and American Burkitt's lymphomas, tumors which represent an intermediate stage of B-lymphocyte differentiation (Diamond, 1983). The predicted amino acid sequence of human Blym-1 also shares substantial homology with transferrin and transferrin related proteins (Diamond, personal communication). Utilizing antisera prepared against synthetic peptides representative of the amino or carboxy terminals of chicken Blym-1, it has been determined that the bulk of this protein can be localized to the nucleus (Cooper, personal communication).

A second stage-specific gene, Tlym-I has now been isolated and characterized. This gene has been found to be activated in eight mouse T-cell lymphomas and three human T-cell lymphomas (Lane, 1982), based upon its sensitivity to inactivation with restriction endonucleases. Tlym-I was isolated from the mouse T cell line S49 which was derived from a mineral oil induced T lymphoma from a BALB/c mouse.

Tlym-I was isolated by sib selection and transfection of a transforming gene-enriched λ Charon 30 library containing cell DNA inserts in the 8kb range. A single pnage was isolated containing a cellular insert of 8.7kb which was biologically active. To facilitate further mapping a 4.7kb Hind-Bam fragment containing an EcoR1 site was subcloned into PBR 322. This fragment which contains the transforming region of the gene was chosen because digestion with EcoR1 was previously shown to inactivate the transforming activity of Tlym-I (Lane, 1982; Lane, 1984).

Hybridization of a flanking sequence probe to BALB/c liver DNA and to S49 tumor DNA indicated that activation of this gene did not occur as a result of gross rearrangements or deletions in cell DNA. Hybridization of a coding sequence probe to human DNA under conditions of slightly relaxed stringency indicated that this sequence was conserved between mouse and human species (Lane, 1984).

Hybridization of the 4.7kb fragment containing the transforming region to lymphoid cellular RNA detected major message species of 0.6, 0.7 and 1.6kb in both T and B cells and in addition, a message of 1.8kb in RNA from a T-suppressor clone. These message sizes were of interest to us because of a report by Peter Rigby and his colleagues concerning their isolation of cDNA clones by subtractive RNA hybridization from SV40 transformed cells. One group of genes referred to as Set 1 genes shared extensive sequence homology with MHC I genes from the TL/QA region. Set 1 genes used as probes detected messenger RNAs of 0.6, 0.7 and 1.6kb,

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and appeared to possess a novel repeat element having a structure similar to a transposon, as it was flanked by direct repeat sequences (Brickell, 1983).

Because of the similarity of RNA messenger sizes identified by both Tlym-I and Set 1 genes, and because Tlym-I had been isolated from a thymic lymphoma it was of interest to determine whether Tlym-1 did encode a gene within the MHC I region and it was of particular interest to determine whether our gene encoded an altered TL or QA product.

Tlym-I was found to hybridize to pMHC1 (Evans, 1982),described by Seidman and coworkers as a probe crossreactive with most MHC I region genes; to 64-c, reported by Rigby's group to contain exons four, five and six of the Set 1 gene, and to 64-E which contained their Set 1 repeat sequence. These experiments suggest that Tlym-I shares homology with Class 1 MHC genes.

As Tlym-I contained a Cla I site, some further analysis was possible based upon the report by Steinmetz et al. (1981) defining thirteen clusters containing 36 genes within the BALB/c MHC I region. From their reported analysis of ClaI sites within the gene clusters, we have been able to rule out all but nine genes mapping to four clusters. Clusters one and six map to QA regions, while clusters three and five map to TL regions as determined by these authors. If the genes contained in the thirteen clusters constitute all of the genes encoded within the MHC I region then this retrospective analysis further localizes Tlym-I to the QA/TL region of the MHC I complex.

Genes in the TL/QA region encode cell surface expressed proteins in the range of 40-45 thousand daltons, which have been found to be associated with β2 microglobulin. TL antigens are expressed in a highly stage specific manner and have been found only on thymus cells. TL negative strains in which thymic leukemias develop often express a novel TL on their surface. At present, the biological role of the TL antigens is unclear.

QA1 antigens have been detected on approximately two thirds of all $Thy1^+$ cells and are found on one class of helper T-cells as well as a feedback suppressor cell. QA2 is also found on a majority of $Thy1^+$ cells and is expressed on some but not all T cell leukemias. Proliferation to the mitogens ConA and PhA can be blocked if T cells are pretreated with antiserum directed against the QA2 antigen. QA3 is also expressed predominantly on Thy-1 positive cells and behaves as does QA2 in mitogen studies.

The antigens QA4 and QA5 have a different tissue distribution and appear not to be present on thymic cells. These antigens are present on Ig^- cells from spleen and lymph nodes and are additionally expressed on B cell blasts following lipopolysaccharide stimulation (reviewed in Flaherty, 1980).

At the nucleic acid level, the sequences encoding TL and QA do not exhibit extensive polymorphism and in some cases share homology of 80% or more, while the genes encoding H-2 seem to be more extensively polymorphic. For these reasons it will be of use to distinguish TL products from QA products utilizing serologic reagents.

In summary, it would appear that TL antigens, expressed uniquely in the thymus, may be involved in either homing or differentiation, while QA antigens also expressed as distinctive tissue specific antigens, may be involved in cell growth and/or division. Tlym-I may represent an altered form of a gene encoding one of these proteins, and is a biologically active transforming gene which alters both the morphology and the growth characteristics of NIH 3T3 cells. Therefore it is of great interest to determine which of these two classes of molecules Tlym-I encodes and what specific alterations contribute to the activation of Tlym-I in T-cell lymphomas.

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REFERENCES

Brickell PM, Latchman DS, Murphy D, Willison K, Rigby PWJ (1983) Activation of a Qa/TLa class I major histocompatibility antigen gene is a general feature of oncogenesis in the mouse. Nature 306:756-760

Cooper GM, Neiman PE. Personal communication

Diamond A, Cooper GM, Ritz J, Lane MA (1983) Identification and molecular cloning of the human Blym transforming gene activated in Burkitt's lymphomas. Nature 305:112-116

Diamond A, Cooper GM. Personal communication

Evans GA, Margulies DH, Camerini-Otero RD, Ozato K, Seidman JG (1982) Structure and expression of a mouse major histocompatibility antigen gene H-2L^{d}. Proc Natl Acad Sci USA 79:1994-1998

Flaherty L (1980) TLA-region antigens. In: The role of the major histocompatibility complex in immunology. Garland STPM Press, New York, p 33

Goubin G, Goldman DS, Luce J, Neiman PE, Cooper GM (1983) Molecular cloning and nucleotide sequence of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. Nature 302:114-119

Lane MA, Sainten A, Cooper GM (1982) Stage-specific transforming genes of human and mouse B- and T-lymphocyte neoplasms. Cell 28:873-880

Lane MA, Sainten A, Doherty KM, Cooper GM (1984) Isolation and characterization of a stage specific transforming gene, Tlym-1, from T-cell lymphomas. Proc Natl Acad Sci USA (in press)

Steinmetz M, Winoto A, Minard K, Hood L (1981) Clusters of genes encoding mouse transplantation antigens. Cell 28:489-498

Structural and Functional Analysis of ras and Blym Oncogenes

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INTRODUCTION

The transforming activity of tumor DNAs, detected by transfection of NIH 3T3 cells, has resulted in the identification of a variety of transforming genes which are activated in human neoplasms. Activated oncogenes of the ras family have been detected in approximately 20% of human neoplasms, including carcinomas, sarcomas, neuroblastomas, leukemia, and lymphomas (reviewed in Cooper and Lane, 1984). Thus, ras genes can apparently contribute to the development of neoplasms originating from a wide variety of differentiated cell types. Since they are detected in only a fraction of individual tumors, however, ras gene activation does not appear to be essential for development of any particular type of neoplasm.

In contrast, other transforming genes, such as Blym-1 (Goubin et al, 1983; Diamond et al, 1983) and Tlym-I (Lane et al, 1982, 1984) are activated highly reproducibly and specifically in neoplasms of particular differentiated cell types. Thus, Blym-1 has been detected as a biologically active transforming gene in all chicken B cell lymphomas and all human Burkitt's lymphomas investigated, but it has so far been observed as an activated transforming gene only in surface immunoglobulin-positive B cell lymphomas.

ras Gene Products

Activation of the transforming activity of ras genes in human neoplasms has been shown to result from amino acid substitutions at two positions in the ras gene products. Since these mutations affect the structure, and hence the function of ras proteins, we have investigated the comparative properties of normal and transforming ras gene products.

Studies of viral ras proteins have indicated that they are localized to the inner face of the plasma membrane (Willingham et al, 1980; Furth et al, 1982) and are modified by acylation (Sefton et al, 1982). The only established biochemical activity of ras proteins is guanine nucleotide binding (Scolnick et al, 1979; Furth et al, 1982).

Comparative analysis of the proteins encoded by normal human ras genes and by ras genes activated in human carcinomas indicated that the normal and transforming proteins were similarly localized to the plasma membrane and similarly modified by post-translational additon of lipid (Finkel et al, 1984). Furthermore, normal and transforming ras proteins did not differ in either affinity or specificity of nucleotide binding (Finkel et al, 1984). Both normal and activated ras proteins bound dGTP with Kd's of 1-2 X 10^{-8} M and were specific for GDP and GTP binding. These results indicated that mutations which activated ras genes in human tumors did not alter the intrinsic biochemical properties of the ras gene product.

To further investigate the physiological function of ras gene products, we have attempted to identify other cellular proteins with which ras proteins might interact. These experiments (Finkel and Cooper, 1984) indicated that ras proteins form a molecular complex with transferrin receptor which is detectable

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by co-immunoprecipitation. In addition, the complex between ras proteins and transferrin receptor is dissociated by transferrin. These findings suggest that ras proteins may function in conjunction with transferrin receptor in the regulation of cell growth, possibly by transduction of growth signals mediated by transferrin binding.

Blym Gene Products

A biologically active Blym transforming gene was first isolated from a chicken B cell lymphoma (Goubin et al, 1983). The nucleotide sequence of chicken Blym-1 indicated that this gene encoded a small protein of 65 amino acids (Goubin et al, 1983). Interestingly, a computer-assisted homology search revealed that the chicken Blym-1 protein was partially homologous (36%) to the amino-terminal region of transferrin family proteins (Goubin et al, 1983).

The chicken Blym-1 gene was used as a probe to isolate a related transforming gene (human Blym-1) which was activated in human Burkitt's lymphomas (Diamond et al, 1983). Nucleotide sequencing of human Blym-1 indicates that it encodes a small protein (58 amino acids). The chicken and human Blym-1 amino acid sequences share 33% identity, indicating that they are related but divergent proteins (Diamond et al, in preparation). However, the human Blym-1 amino acid sequence retains the transferrin homology observed with chicken Blym-1. Given the overall divergence between the chicken and human gene products, this observation suggests that the transferrin-homologous region is important to Blym function. In view of the interaction between ras proteins and transferrin receptor, the homology of Blym proteins to transferrin suggests the possibility that both ras and Blym oncogenes may function in a common pathway related to transferrin and its cell surface receptor.

REFERENCES

Cooper, GM, Lane MA (1984) Cellular transforming genes and oncogenesis. Biochim. Biophys. Acta Rev. on Cancer, in press.

Diamond A, Cooper GM, Ritz J, Lane MA (1983) Identification and molecular cloning of the human Blym transforming gene activated in Burkitt's lymphomas. Nature 305: 112-116.

Finkel T, Cooper GM (1984) Detection of a molecular complex between ras proteins and transferrin receptor. Cell, in press.

Finkel T, Der CJ, Cooper GM (1984) Activation of ras genes in human tumors does not affect subcellular localization, post-translational modification or guanine nucleotide binding properties of p21. Cell, in press.

Furth, ME, Davis LJ, Fleurdelys B, and Scolnick EM (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. J. Virol. 43: 294-304.

Goubin G, Goldman DS, Luce J, Neiman PE, Cooper GM (1983) Molecular cloning and nucleotide sequence of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. Nature 302: 114-119.

Lane MA, Sainten A, Cooper GM (1982) Stage-specific transforming genes of human and mouse B- and T-lymphocyte neoplasms. Cell 28: 873-880.

Lane MA, Sainten A, Doherty KM, Cooper GM (1984) Isolation and characterization of a stage specific transforming gene, Tlym-I, from T cell lymphomas. Proc. Natl. Acad. Sci. USA, in press.

Scolnick, EM, Papageorge AG, Shih TY (1979) Guanine nucleotide- binding activity as an assay for src protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. USA 76: 5355-5359.

Sefton BM, Trowbridge IS, Cooper JA, Scolnick EM (1982) The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell 31: 465-474.

Willingham MC, Pastan I, Shih TY, Scolnick EM (1980) Localization of the src gene product of the Harvey strain of MSV to plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell 19, 1005-10014.

Structural Organization of Mouse c-myb Locus and the Mechanism of its Rearrangement in ABPL-2 Tumor Line Induced by Pristane and Abelson Murine Leukemia Virus

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INTRODUCTION

Abelson murine leukemia virus (A-MuLV) is a replication-defective transforming retrovirus that arose by recombination of nondefective helper virus (M-MuLV) and cellular sequences present within the normal mouse genome. The latter sequences, termed abl, appear to code for the transforming properties of the virus (for review see Rosenberg and Baltimore, 1980). This virus induces in adult BALB/c mice a variety of lymphoid neoplasms predominantly of the pre-B cell series (ABLS tumors). However, when the mice are previously injected with pristane, which induces intraperitonal granulomatous tissue, this virus also rapidly induces plasmacytomas (ABPC tumors) and occasionally, a morphological subset of lymphosarcomas characterized by plasmacytoid cytoplasm but with very little immunoglobulin production (ABPL tumors) (Potter et al.,1978). Our preliminary experiments indicated that ABPC and ABLS tumors produced abundant amounts of infectious A-MuLV particles while most ABPL tumors, in striking contrast, did not. In an effort to understand the molecular mechanisms involved in the genesis of these tumors, a detailed study of the expression of abl, myc and myb oncogenes was undertaken (Mushinski et al., 1983). These studies demonstrated that: 1) ABLS and ABPC tumors contain integrated A-MuLV proviral genome in the cellular DNAs and express abundant quantities of A-MuLV RNAs. In contrast, ABPL tumors, with the exception of ABPL-3, do not contain the A-MuLV proviral genome and do not express detectable levels of A-MuLV RNA. 2) All three tumor types expressed a 2.4 kb myc RNA. The band intensities of myc RNA in the three classes of tumors varied considerably with the ABLS's generally containing the lowest amount of myc RNA. None of the ABLS or ABPL tumors were found to contain rearrangements in myc locus while ABPC's generally exhibited rearrangements in this locus. 3) The ABLS and ABPC tumors do not exhibit any rearrangements in myb locus and do not express any abnormal sized mRNAs. On the other hand, all ABPL tumors show rearrangements in the c-myb locus and express elevated levels of myb RNA. In addition a majority of them contain an abnormal sized mRNA which readily hybridized with the v-myb probe. These experiments appeared to indicate that DNA rearrangements in the c-myb locus are responsible for the enhanced expression and appearance of abnormal sized c-myb encoded mRNAs in these cells. In order to understand the molecular mechanisms by which these DNA rearrangements occur, we undertook molecular cloning of normal c-myb sequences from mouse embryonic DNA and the rearranged c-myb sequences from the ABPL-2 tumor line.

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STRUCTURAL ORGANIZATION OF MOUSE c-myb LOCUS

Restriction of normal mouse liver DNA with Eco RI and hybridization with a v-myb specific probe showed the presence of three bands in the genomic DNA which cross hybridized to the probe. They were 4.2 kbp, 1.6 kbp and 0.5 kbp long (data not shown). On the other hand, hybridization of ABPL-2 DNA with the same v-myb probe showed the presence of an additional band which was 7.5 kbp long in addition to the three DNA bands seen in normal liver DNA. The larger band appeared to be a rearranged version of the 4.2 kbp germline fragment, since this fragment hybridized with diminished intensity compared to the germline.

Following these studies, a c-myb clone was isolated from a partial Eco RI library of mouse embryonic DNA cloned in Charon 4A vector. This clone, which had a 17 kbp insert, contained all three Eco RI fragments (4.2, 1.6 and 0.5 kbp long) which cross hybridized with the v-myb probe. A restriction map of this c-myb clone is shown in Fig. 1. The black boxes indicate the approximate location of the v-myb hybridizing regions present in this clone. To orient the mouse sequences with respect to the v-myb gene, the myb related Eco RI fragments of 4.2, 1.6 and 0.5 kbp were used as probes and hybridized with various restriction digests of the v-myb clone. These results (not presented) indicated that 4.2 kbp Eco RI fragment hybridized to the 5' end of the v-myb clone, while the 0.5 kbp Eco RI fragment hybridized to the 3' end of the v-myb sequences. The 1.6 kbp Eco RI fragment hybridized to the middle region of v-myb sequences, thus orienting the molecule. This orientation was further confirmed by partial nucleotide sequence analysis of this clone.

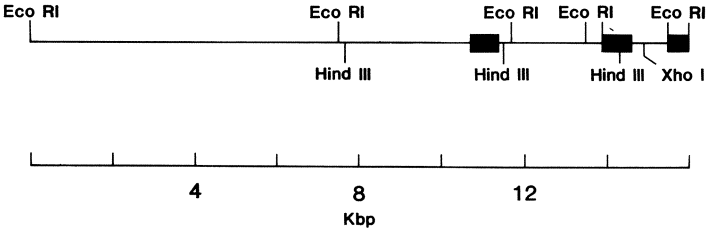

Fig. 1. Restriction enzyme map of a c-myb (mouse) DNA clone. v-myb related regions are indicated by black boxes. The positions of homologous regions were deduced by restriction enzyme digestion and Southern blotting analysis.

STRUCTURE OF myb LOCUS IN ABPL-2 CELLS

In order to study the altered myb locus in ABPL-2 tumor, the DNA from the tumor was digested with Eco RI and the 7.5 kbp DNA band was cloned in λgt Wes B vector. An Eco RI insert from one such clone was isolated and compared with 4.2 kbp c-myb Eco RI fragment by heteroduplex analysis. The results of such an analysis are shown in Fig. 2. These results clearly indicate that the two DNA fragments shared a homology of 0.9 kbp and 3.3 kbp at either ends and the rearranged myb-fragment from ABPL-2 tumor contained a large insertion of approximately 3.0 kbp towards one end of the fragment.

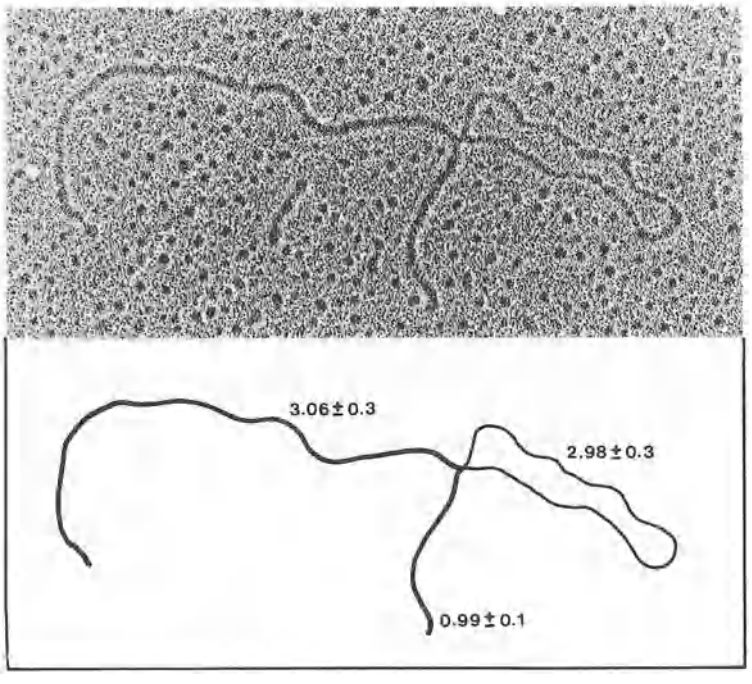

Fig. 2. Electron micrograph of a heteroduplex formed between the 4.2 kbp Eco RI fragment of normal mouse c-myb DNA and the 7.5 kbp Eco RI fragment of ABPL-2 DNA. A diagramatic representation of the heteroduplex is shown below the electron micrograph. Contour lengths are given in kbp.

In order to characterize the nature of the inserted DNA fragment, the DNA was digested with various restriction enzymes and hybridized with a Mo-MuLV LTR-specific probe. These results indicate that the inserted piece of DNA had at least two LTRs derived from Mo-MuLV. Restriction mapping and nucleotide sequence analysis have indicated that the inserted DNA has a similar nucleotide sequence to that of Mo-MuLV with a large deletion in the polymerase and envelope regions. This insertion of the proviral genome appears to have occurred towards the 5' end of the v-myb related sequence (Fig.3).

The enhanced transcription of the c-myb locus therefore appears to be due to the enhancer effect of LTR sequences on the c-myb locus, while the altered size of c-myb DNA fragment in ABPL-2 tumor is due to the insertion of a defective Mo-MuLV proviral genome upstream to the myb oncogene related sequences in these tumors. This situation is analogous to that found in avian leukosis virus induced bursal lymhomas (Hayward et al. 1981; Neel et al., 1981; Payne et al. 1982).

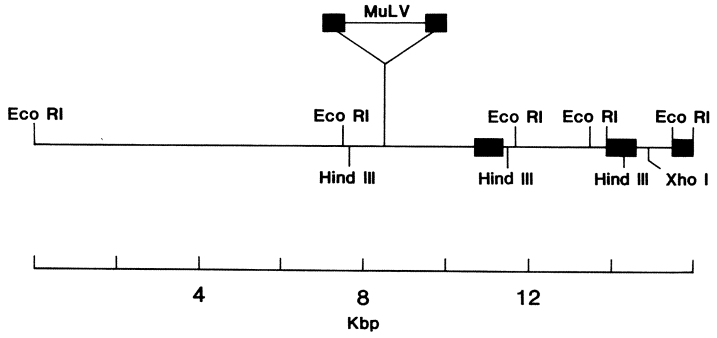

Fig. 3. A schematic representation of the derivation of the rearranged c-myb DNA from normal mouse c-myb, by insertion of a proviral genome. The MuLV proviral genome inserted has suffered large deletion in pol and env regions and hence in only 3.0 kbp long.

SUMMARY

The results presented above demonstrate that the rearrangement of c-myb locus observed earlier by Mushinski et al. (1983) in ABPL-2 tumor cells is due to the insertion of a proviral genome upstream to the myb oncogene related sequences . The retroviral LTRs are known to contain enhancer elements (for review see Varmus, 1982) and presumably the insertion of these LTRs lead to the altered rates of expression of myb-encoded mRNAs in these cells. ABPL tumors also seem to produce structurally altered mRNAs from the rearranged c-myb locus. Whether these altered myb mRNAs synthesize myb proteins that are structurally different leading to an altered biochemical function which in turn is responsible for the oncogenic activity requires further experimentation.

REFERENCES

Hayward WS, Neel BG, Astrin SM (1981) Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) 290: 475-480

Mushinski JF, Potter M, Bauer SR, Reddy EP (1983) DNA rearrangement and altered RNA expression of the c-myb oncogene in mouse plasmacytoid lymphosarcomas. Science 220: 795-798

Neel BG, Hayward WS, Robinson H, Fang J, Astrin SM (1981) Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new mRNAs: Oncogenesis by promoter insertion. Cell 23: 323-334

Payne GS, Bishop JM, Varmus HE (1982) Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature 295: 209-214

Potter M, Reddy EP, Wivel NA (1978) Immunoglobulin production by lymphosarcomas induced by Abelson virus in mice. Natl Cancer Inst Monograph 48: 311-320

Rosenberg N, Baltimore D (1980) Abelson Murine Leukemia Virus. In: Klein G (ed) Viral Oncology, Raven, New York, p 187-203

Varmus HE (1982) Form and function of retroviral proviruses. Science 216: 812-820

Disruption and Activation of the c-myb Locus by M-MuLV Insertion in Plasmacytoid Lymphosarcomas Induced by Pristane and Abelson Viruses

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INTRODUCTION

The c-myb proto-oncogene is the cellular homolog of the avian myeloblastosis virus oncogene (v-myb) (Roussel et al., 1979; Souza et al., 1980a; Bergman et al., 1981). The boundaries of c-myb are not yet known; however, studies have shown that the v-myb sequence is transduced from a portion of the coding region of c-myb into the avian myeloblastosis virus (AMV) (Klempnauer and Bishop 1983). AMV is a retrovirus that causes myeloblastic or monocytic leukemia in chickens and transforms myelomonocytic hematopoietic cells in culture (Moscovici 1975). The v-myb sequence is thought to be essential for the oncogenic potential of AMV (Duesberg et al., 1980; Souza 1980a; Gonda et al., 1981) and it appears that only certain target cells are responsive to the v-myb gene product (Moscovici 1975).

Our laboratory has previously examined three types of tumors termed plasmacytomas (ABPC's), lymphosarcomas (ABLS's) and plasmacytoid lymphosarcomas (ABPL's) that arise in BALB/c mice treated with pristane and Abelson virus (Mushinski et al., 1983). Abelson virus contains two retroviral elements, a replication defective transforming A-MuLV and a transmissable Moloney leukemia virus, M-MuLV. Both units have the same 5' and 3' ends of the genome, but in A-MuLV the central portion of the retroviral gene is replaced by the transforming v-abl sequence (Goff et al., 1980; Shields et al. 1979). All ABLS's and ABPC's contain integrated A-MuLV proviral genomes and synthesize abundant v-abl RNA while ABPL's do not. Instead, the ABPL tumors have undergone rearrangements in the c-myb locus resulting in the synthesis of abnormal mRNA transcripts.

In order to understand the possible involvement of the altered c-myb locus in the ABPL tumorigenesis and to elucidate the mechanism by which this alteration arose, we have cloned and analyzed the rearranged portion of the c-myb locus from four independently derived ABPL's.

ACTIVATION OF C-MYB EXPRESSION IN ABPL'S

Figure 1 shows analysis of blots of the ABPL RNA's after hybridization with an avian v-myb probe that was isolated as a 1.3 kbp KpnI-XbaI fragment from chicken AMV proviral clone (Souza et al., 1980b; Rushlow et al., 1982). Particularly dramatic elevations in the amount of myb RNA are found in ABPL-1 and ABPL-2. In addition, the tumors also contain myb RNA's that are larger than the 3.8- and 4.2- kb myb RNA that are present in thymus. The size of these ABPL-specific myb RNA's varies among the tumors but are usually around 5.0 kb. In

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ABPL122, elevated amounts of 3.8-, 4.2-, 5.0- and 7.5- kb myb RNA are found (data not shown). Hence, we can conclude that c-myb expression is activated as well as altered in ABPL's.

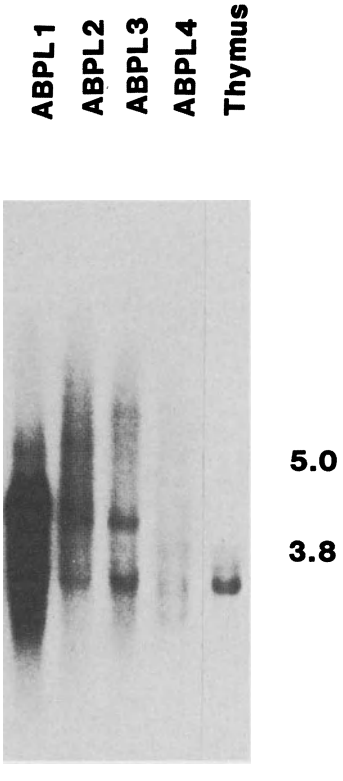


Fig. 1. Blot analysis of 5 μg of polyadenylated RNA's from thymus and ABPL's. The conditions used are as previously described (Mushinski et al.,1983)

REARRANGEMENT IN C-MYB LOCUS

The c-myb locus of the ABPL's was next examined at the DNA level. The Southern blot analysis of EcoRI digested normal BALB/c liver and ABPL DNA's are shown in Fig. 2. The DNA's were probed with the v-myb sequence described above. In addition to the 4.3 and 1.6 kbp myb-hybridizing bands, each of the four ABPL's contain an additional larger band of varying size that is not found in normal BALB/c liver DNA. An EcoRI-XbaI fragment which represents the 3' half of the v-myb hybridized only to the 1.6 kbp band (data not shown). This indicates that the 4.3 kbp EcoRI fragment contains the 5' v-myb region and that this fragment is altered in the ABPL's giving rise to the larger EcoRI fragment. Presumably, it is this rearrangement in the c-myb locus in ABPL's that results in the synthesis of larger myb RNA transcripts.

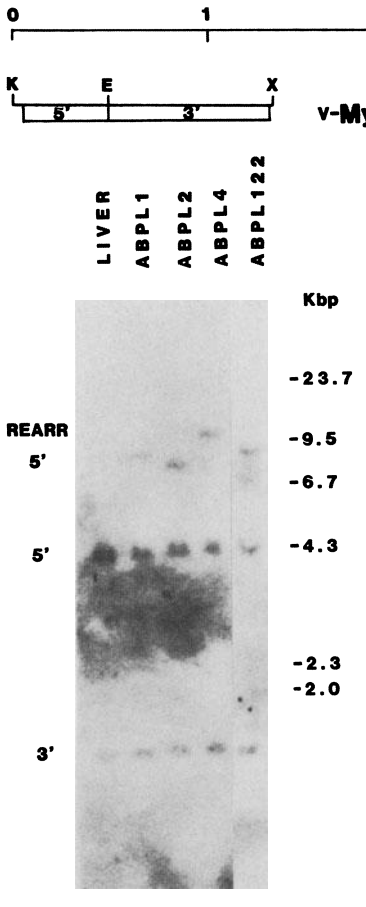


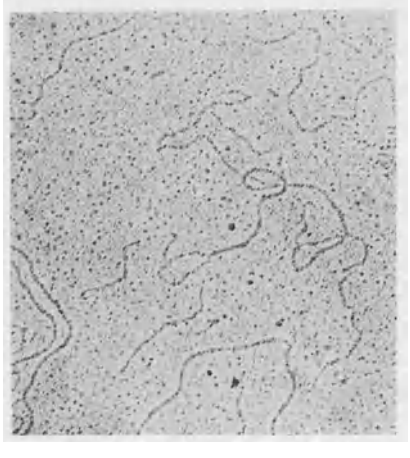
Fig. 2. Southern blot analysis of EcoRI digests of 15 μg of genomic DNA from BALB/c liver and ABPL's. The diagram of the v-myb probe is shown above the blot. The various hybridizing bands are designated as 5' and 3' based on their hybridization to the 5' or 3' v-myb sequences (see text).

RESTRICTION ANALYSIS OF THE ABPL ALTERED C-MYB CLONES

In order to study the altered myb locus in ABPL's, sequences that are homologous to v-myb were isolated from the genome of ABPL's using λ phage vectors. Four different recombinant λ clones were selected from libraries of EcoRI digested genomic DNA's from four ABPL's. The position of sequences homologous to v-myb in the altered c-myb fragment in each of the four clones was determined by hybridizing, with the v-myb probe, appropriate restriction digests of the four recombinant clones. The v-myb sequence can be localized to a 1.0 kbp XbaI fragment flanked on the 3' side by approximately 1.0 kbp and on the 5' side by varying lengths of sequence that are non-homologous to avian v-myb. The sizes of the EcoRI inserts correlate with the ABPL altered myb bands as seen in Fig. 2.

Our previous results have shown that among the various types of tumors that develop in BALB/c mice treated with pristane and A-MuLV, only ABPL's lack A-MuLV proviral genome. It was then suggested that the alterations in the c-myb locus might involve a "hit and run" mechanism whereby an A-MuLV proviral genome is initially integrated, but subsequently excised. In order to determine if the ABPL myb locus is

altered by the presence of a highly deleted A-MuLV provirus containing only long terminal repeat (LTR) sequences, the four clones were examined for the presence of retroviral LTR sequences. Interestingly, each of the four clones contain two LTR sequences that are spaced approximately 2.3 kb to 5.4 kbp apart. The similarity of restriction sites such as PstI, XhoI, and ClaI in the sequences that are found between the LTR's suggests that a common viral element has inserted into the c-myb locus. The restriction sites between the LTR's in ABPL myb clones do not correlate with those of A-MuLV sequence (Srinivasan et al., 1981). Instead, the pattern is strikingly similar to M-MuLV the helper virus associated with A-MuLV in the preparation of Abelson virus used in the induction of ABPL tumors. Further Southern blot analysis with probes isolated from different regions of M-MuLV (data not shown) and heteroduplex mapping (Fig. 3) confirms the integration of a M-MuLV sequence in a 5' → 3' orientation upstream from myb sequences that are homologous to the v-myb sequence. The variations in the length of the myb-EcoRI fragments in the ABPL tumors are due to internal deletions in the M-MuLV inserts. This point was confirmed by heteroduplex mapping of the ABPL rearranged myb clones with a M-MuLV proviral insert in which a distinct single stranded deletion loop in the middle region of the M-MuLV was seen. The rest of the ABPL proviral sequences appeared to be completely homologous to the M-MuLV sequence without evidence of substitution loops.



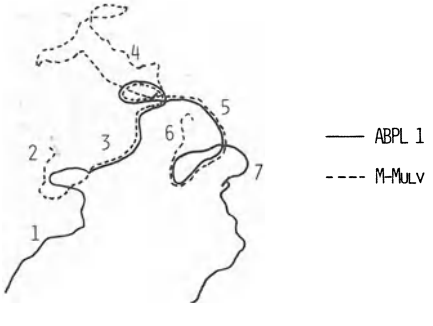


Fig. 3. Heteroduplex analsis of the M-MuLV EcoRI proviral insert isolated from infected rat cell with the cloned EcoRI myb fragments from ABPL1. The 10.9 kbp EcoRI fragment of M-MuLV proviral insert and the plasmid subclone of the 8.7 kbp EcoRI fragment of ABPL1 were digested with EcoRI, denatured, reannealed under stringent conditions and spread for electron microscopy.
1: ABPL cellular sequence; 2: 1.4 kb rat cellular sequence flanking the M-MuLV 5' LTR; 3: duplex region of ABPL sequence hybridized with the 5' portion of M-MuLV sequence; 4: deletion loop of M-MuLV sequence; 5: duplex region of ABPL sequence with the 3' portion of M-MuLV sequence; 6: 0.7 kb rat cellular sequence flanking the M-MuLV 3' LTR; 7: ABPL cellular sequence containing sequences homologous to the v-myb gene.

DISCUSSION

The data presented here indicate M-MuLV and not A-MuLV insertion is the critical event in the pathogenesis of ABPL tumors. The "hit and run" mechanism originally proposed to explain the pathogenesis of ABPL tumors was based on the notion that A-MuLV inserted temporarily and that v-abl transcription in the cells has been essential for the development of the tumors. While A-MuLV insertion may have occurred elsewhere in the genome, the evidence presented here clearly shows the M-MuLV insertion is directly related to the increased myb transcription. It is still possible that Abelson virus infection in some way was indirectly involved in the pathogenesis of the ABPL tumors. If the M-MuLV insertion near myb is the critical event, it should be possible to induce tumors of this type in BALB/c mice by injecting pristane and M-MuLV.

In the ABPL tumors an incomplete provirus that contains both LTR regions appears to activate myb transcription. The situation is analagous to the findings of Payne et al. (1982) who found that insertion of ALV proviral sequences of varying distances 5' or 3' to c-myc resulted in increased c-myc transcription. The presence of both LTR's of M-MuLV upstream of myb sequences suggests that promoter insertion is not the likely mechanism. It has been argued by Cullen et al. (1984) that when the 5' LTR if present in an insert the 3' LTR is unable to efficiently promote downstream transcription.

SUMMARY

Plasmacytoid lymphosarcoma cells (ABPL's) are a morphological subset of early B cell tumors that arise about 100 days after infection of pristane primed BALB/c mice with Abelson virus. These tumors are characterized by unusually abundant and abnormally large myb RNA transcripts and by rearrangements within the c-myb genome. Molecular cloning of the rearranged myb region of four ABPL's shows that the rearrangement is due to the integration of a deleted Moloney murine leukemia virus upstream from the 5' most c-myb exon that contains v-myb sequences. Different amounts of internal sequence have been deleted from the viruses inserted in the different ABPL's, which accounts for the different-sized DNA fragment. The disruption of the c-myb locus presumably is responsible for the intense activation of myb RNA transcription.

REFERENCES

Bergmann DG, Souza LM, Baluda MA (1981) Vertebrate DNAs contain nucleotide sequences related to the transforming gene of avian myeloblastosis virus. J Virol 40:450-455

Cullen BR, Lomedico PT, Ju G (1984) Transcriptional interference in avian retroviruses - implications for promoter insertion model of leukemogenesis. Nature 307:241-245

Duesberg PH, Bister K, Moscovici C (1980) Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus particle. Proc Natl Acad Sci USA 77:5120-5124

Goff SP, Gilboa E, Witte ON, Baltimore D (1980) Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell 22:777-785

Gonda TJ, Sheiness, DK, Fanshier L, Bishop JM, Moscovici C, Moscovici MG (1981) The genome and the intracellular RNAs of avian myeloblastosis virus. Cell 23:279-290

Jaehner D, Stuhlman H, Jaenisch R (1980) Conformation of free and integrated Moloney leukemia virus proviral DNA in preleukemic and leukemic BALB/Mo mice. Virology 101:111-123

Klempnauer K-H, Bishop JM (1983) Transduction of c-myb into avian myeloblastosis virus: locating points of recombination within the cellular gene. J Virol 48:565-572

Moscovici C (1975) Leukemic transformation with avian myeloblastosis virus: present status. Curr Topics Microbiol Immunol 71:79-101

Mushinski JF, Potter M, Bauer SR, Reddy EP (1983) DNA rearrangement and altered RNA expression of the c-myb oncogene in mouse plasmacytoid lymphosarcomas. Science 220:795-798

Payne GS, Bishop JM, Varmus HE (1982) Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature 295:209-214

Roussel M, Saule S, Lagrou C, Rommens C, Beug H, Graf T, Stehelin D (1979) Three types of viral oncogenes of cellular origin specific for hematopoeitic cell transformation. Nature 281:452-455

Rushlow KE, Lautenberger JA, Papas TS, Baluda MA, Perbal B, Chirikjian JG, Reddy EP (1982) Nucleotide sequence of the transforming gene of avian myeloblastosis virus. Science 216:1421-1423

Shields A, Goff S, Paskind M, Otto G, Baltimore D (1979) Structure of the Abelson murine leukemia virus genone. Cell 18:955-962

Souza LM, Strommer JN, Hilyard RL, Komaromy MC, Baluda MA (1980a) Cellular sequences are present in the presumptive avian myeloblastosis virus genome. Proc Natl Acad Sci USA 77:5177-5181

Souza LM, Briskin MJ, Hillyard RL, Baluda MA (1980b) Identification of the avian myeloblastosis virus genome. II Restriction endonuclease analysis of DNA from proviral recombinants and leukemic myeloblast clones. J Virol 36:325-336

Srinivasan A, Reddy EP, Aaronson SA (1981) Abelson murine leukemia virus: molecular cloning of infectious integrated proviral DNA. Proc Natl Acad Sci USA 78:2077-2081

Follicular Hyperplasia in the Prelymphomatous Avian Bursa: Relationship to the Incidence of B-Cell Lymphomas

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INTRODUCTION

The bursa of Fabricius is the site of primary tumor development in chickens susceptible to avian leukosis virus (ALV)-induced B-cell lymphomas. These lymphomas first appear from 8 to 12 weeks after virus infection and eventually metastasize to the spleen, liver and kidneys, resulting in death of the animal (Purchase and Burmester, 1978). The tumor cells are characterized by both cytoplasmic and cell surface immunoglobulin of the IgM isotype (Cooper et al., 1974).

Molecular characterization of the tumor cell has revealed that the cellular locus c-myc has been interrupted by the insertion of ALV DNA sequences (Hayward et al., 1981; Payne et al., 1982). The insertion of these viral sequences positions a long terminal repeat (LTR), possessing the properties of both a transcriptional promoter and enhancer, within this normal cellular gene. The presence of the viral LTR is responsible for an increase in the expression of c-myc which can be detected as elevated levels of c-myc RNA (Hayward et al., 1981; Payne et al., 1982).

Transfection studies have identified a second genetic locus important in lymphoma development. This locus, designated B-lym, produces morphological transformation of NIH 3T3 fibroblasts following DNA-mediated transfer of tumor cell DNA (Cooper and Neiman 1980, 1981; Goubin et al., 1983). The relationship between the activation of c-myc, the role of B-lym, and the development of the lymphoma are currently not known.

Considerable data indicate that tumor development is a multistep process which is stochastic in nature (Foulds, 1969; Shabad, 1973). The development of the ALV-induced B-cell lymphoma is thought to begin with the appearance, soon after virus infection, of a small number of preneoplastic lesions within the bursa (Cooper et al., 1968; Neiman et al., 1980). This histopathological feature, referred to as focal follicular hyperplasia, is characterized by an accumulation of large, pyroninophilic lymphoblasts within individual follicles. If these hyperplastic follicles represent a preneoplastic stage in tumor development, they would contain a population of cells from which the primary tumor could subsequently develop.

Currently, the hypothesis that hyperplasia is involved in the subsequent development of bursal lymphomas is supported by three observations. i) hyperplasia is absent from uninfected control animals, ii) hyperplasia occurs within the target organ where

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primary bursal lymphomas develop, iii) cells within hyperplastic follicles are histologically similar to those seen in the tumor (Cooper et al., 1968; Neiman et al., 1980). The analysis described in this study was designed to assess whether the frequency of hyperplasia is correlated with the incidence of primary lymphoma. By analyzing the incidence of hyperplasia in two lines of chickens that differ in their susceptibility to ALV-induced B cell lymphomas, data evaluating the frequency of hyperplasia necessary for, or associated with, neoplasia have been obtained.

TWO LINES OF CHICKENS THAT RESPOND DIFFERENTLY TO INFECTION BY AVIAN LEUKOSIS VIRUS.

The SC and FP line of chickens have been developed by Hyline International (Dallas Center, Iowa). The SC line is C/O, gs^-, chf^-, and V^-, while the FP line is C/E, gs^+, chf^+, and V^-. Both lines, therefore, are susceptible to infection by the RAV-1 strain of ALV and neither produce the endogenous avian retrovirus. In three different tumor trials, one day old chicks were infected i.v. with 2×10^5 IU of RAV-1 and observed for 24 weeks for the development of bursal tumors. The results demonstrate that approximately 50% (27/50) of the SC animals developed primary bursal lymphomas while no tumors (0/36) were detected in the FP animals (Baba and Humphries, 1984a). Histopathological analysis of the tumors in the SC animals revealed the cells to be large, lymphoblastoid and pyroninophilic in character. Disseminated disease was routinely observed, with the liver and spleen most commonly involved.

The animals were further examined for the presence of infectious virus (Table 1).

Table 1. Analysis of viremia in Hyline SC and Hyline FP chickens

Weeks Post-infection	SC	(%)	FP	(%)
1	27/30	(90) [a]	7/41	(17)
2	20/20	(100)	6/20	(30)
2½	30/33	(91)	19/40	(48)
4	5/12	(42)	5/12	(42)
5	2/21	(10)	10/32	(31)
10	0/8	(0)	0/9	(0)
24	0/10	(0)	1/20	(5)

One day old chicks were infected i.v. with 2×10^5 IU of RAV-1. Plasma was obtained at different times after infection and assayed for the presence of infectious virus. The data are taken from multiple experiments (from Baba and Humphries, 1984b).

[a]Number of animals with greater than 5×10^2 IU/ml of plasma/ number of animals tested.

Plasma was obtained from animals at different times after infection and assayed on chicken embryo fibroblasts. Cells were transferred twice after infection and the culture medium was harvested and assayed for the presence of reverse transcriptase. One hundred percent of the SC animals developed viremia within two weeks of infection. Three weeks later, only 10% of these animals were positive for infectious virus. Virus was not detected in any SC animals examined at later times. Analysis of the FP animals revealed that infectious virus was detected in only 50% of the animals that received RAV-1. Plasma samples obtained at two and four weeks after infection were titrated to compare the levels of viremia in both types of animals. No difference in the level of circulating virus was detected. Titers ranged from approximately 5 x 10^3 to 5 x 10^5 IU/ml.

Infected birds were analyzed further by determining the extent to which virus integration occurred in target and nontarget tissue. DNA was isolated and purified from red blood cells and bursal cells obtained from chickens at different times after infection, and assayed for integrated virus sequences.

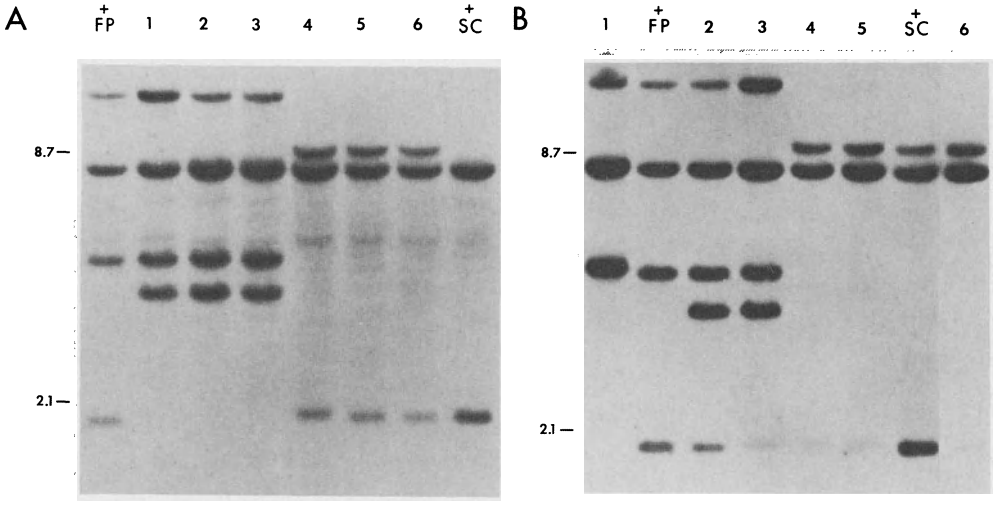


Fig. 1. Detection of the Eco RI specific 2.1 kb DNA fragment indicative of RAV-1 infection. DNA samples, obtained 2 weeks post infection, were prepared from bursal cells (A) or red blood cells (B), digested by Eco RI endonuclease, and analyzed by electrophoresis, Southern DNA transfer, and hybridization to a 5' viral DNA probe. Ten micrograms of DNA obtained from three infected FP animals (1-3) and three infected SC animals (4-6) were analyzed. DNA from RAV-1-infected FP, (FP+), or SC, (SC+), chicken embryo fibroblasts was used as a positive control. The 2.1 kb RAV-1 DNA fragment and the 8.7 kb Eco RI-specific junction of ev-1 are indicated (from Baba and Humphries, 1984b).

This experiment demonstrated that both lines of chickens contained integrated virus DNA (Table 2).

Table 2. Presence of the RAV-1 specific Eco RI 2.1 kb DNA fragment in bursal and RBC DNA obtained from infected FP and SC chickens

Weeks Post-Infection	SC Bursa	(%)	SC RBC	(%)	FP Bursa	(%)	FP RBC	(%)
1	13/15	(87)[a]	13/15	(87)	1/15	(7)	3/15	(20)
2½	9/12	(75)	7/8	(88)	3/13	(23)	3/8	(38)
5	7/27	(26)	14/27	(52)	5/26	(19)	8/26	(31)
7½	0/5		0/5		0/5		0/5	
10	0/5		0/5		1/5		1/5	
24	N.A.[b]		0/21		N.A.		0/12	

One day old chicks were infected i.v. with 2 x 10^5 IU of RAV-1. DNA was prepared from red blood cells and bursal cells and analyzed for the presence of the 2.1 kb Eco RI DNA fragment specific for RAV-1 infection. Animals were considered negative if they contained less than approximately 0.1 copy of the 2.1 kb fragment/genome (from Baba and Humphries, 1984b).

[a] Number of positive animals/number of animals tested.

[b] N.A. = not available. Bursal tissue, which normally begins involution in the chicken at 14 to 16 weeks of age, was absent from these animals.

Approximately 90% of the SC animals contained the 2.1 kb DNA fragment in both target (bursal) and non-target (erythrocyte) cellular DNA. Within the FP population, approximately 40% of the individuals had detectable levels of RAV-1 sequences in red blood cell DNA, while only 25% were positive for the 2.1 kb fragment following analysis of the bursal cell DNA. Consistent with the analysis of viremia in these animals, fifty-two of fifty-three individuals from both lines were apparently free of integrated viral sequences when examined after five weeks of infection.

These results differ from those of other studies (Kung, et al., 1982; Robinson, et al., 1980). Analysis of integrated viral DNA in Regional Poultry Research Laboratory lines 6_3 and $15I_5$ x 7_2 showed no loss of viral DNA eight weeks after infection. Similarly, viremia persisted for several months after infection of susceptible chickens with RAV-1. It is conceivable that the difference between the results presented here and those of other studies is due to differences in the immunological status of the different flocks.

The results obtained from these experiments establish that the SC and FP animals differ significantly in their response to infection by RAV-1. Following infection, 100% of the SC animals develop viremia, 90% show evidence of integrated viral DNA, and 50% develop B-cell lymphomas. In contrast, 50% of the FP animals develop viremia, 40% possess detectable levels of integrated viral DNA, and none of the 36 animals examined developed tumors. It is important to point out that among those FP animals that did contain virus in their plasma and integrated viral DNA in bursal

cell or red blood cell DNA, the levels of these markers of virus infection were equivalent to those observed in the infected SC animals.

INCIDENCE OF FOLLICULAR HYPERPLASIA IN THE HYLINE SC AND FP CHICKENS

The striking difference in the incidence of lymphoma development observed between the SC and FP animals described above provided an experimental system in which it was possible to evaluate the association of follicular hyperplasia with the development of the B-cell lymphoma. Hyperplastic follicles within the bursa are identified by histological analysis of the tissue. The entire organ is removed, fixed in neutral buffered formalin, serially sectioned, and stained with methyl green pyronin. A typical example of follicular hyperplasia is presented in Fig. 2. The cells within the follicles are large, lymphoblastoid and pyroninophilic. Further, the distinction between the medullary and cortical compartments of the follicle is not observed. Microscopic analysis of the tissue sections, at 400 micron intervals, permits the examination of each of the approximately 10^5 follicles within the tissue, on average, only once.

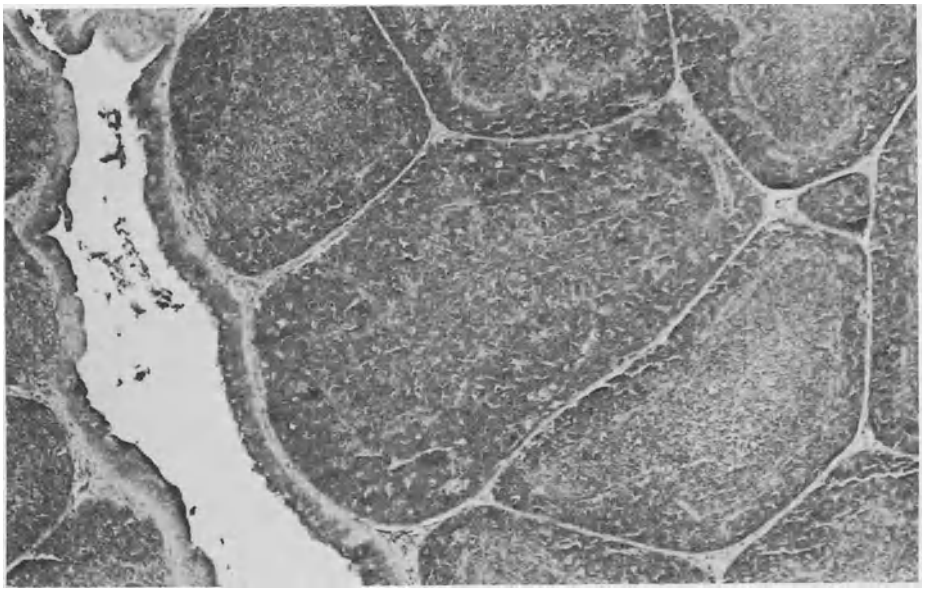
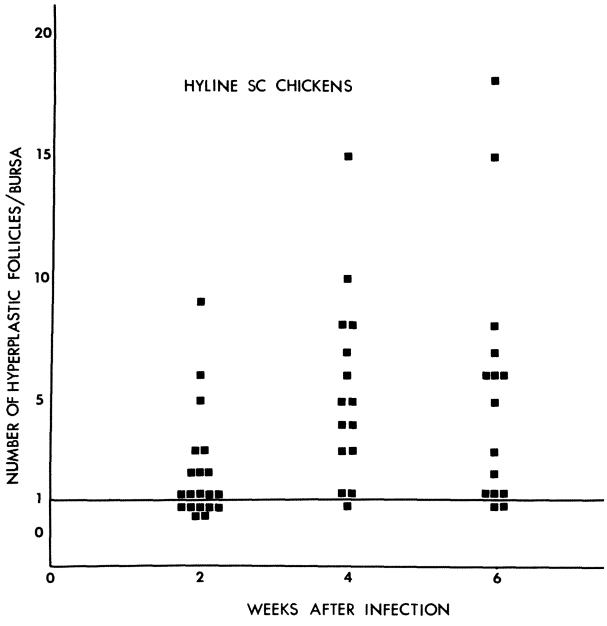


Figure. 2. Histological analysis of a bursa from a RAV-1 infected SC chicken. Several normal follicles are seen adjacent to the hyperplastic follicle. MGP. (Magnification 190X)

Forty-four FP chicks and fifty SC chicks were infected with RAV-1 one day after hatching. Several animals were sacrificed at two, four and six weeks after infection and the incidence of hyperplasia within each bursa determined. Analysis of the incidence of hyperplasia in the bursas of FP and SC animals revealed the FP animals developed significantly less hyperplasia than the SC animals (Fig. 3).



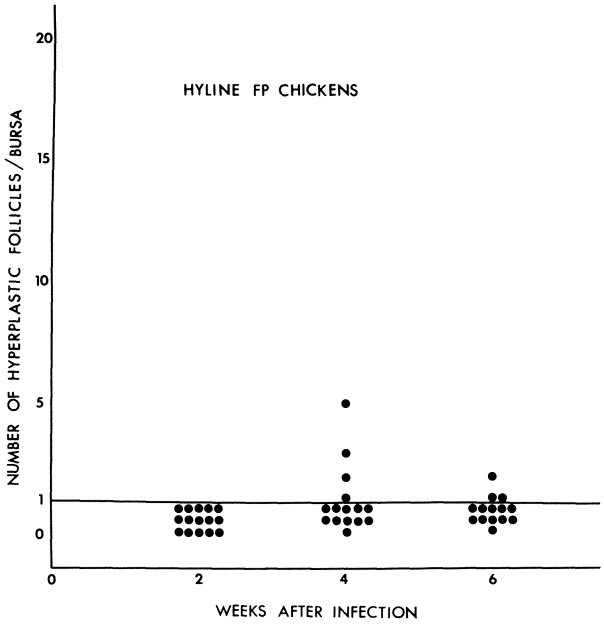


Fig. 3. The incidence of hyperplasia in SC and FP bursas following infection by RAV-1.

SC and FP chickens were infected i.v. with 2 x 10^5 IU of RAV-1. Animals were sacrificed at different times after infection and the complete bursa examined histologically using methyl green pyronin staining for hyperplastic follicles as described. Each symbol represents the number of hyperplastic follicles observed in one bursa. (from Baba and Humphries, 1984c).

The experiment demonstrated that only seven of the forty-four FP (16%) animals developed hyperplasia compared with forty of the fifty SC (80%) animals. Further, among those animals that developed hyperplasia, the SC animals contained, on average, twice as many hyperplastic follicles as the FP animals (5.6 and 2.5 hyperplastic follicles per bursa). Moreover, the comparison at any specific time after infection revealed that approximately one-half of the SC animals contained more hyperplastic follicles than detected in any of the FP animals. For example, at six weeks after infection, eight of the fifteen SC bursas analyzed contained five or more hyperplastic follicles while no FP bursa contained more than three.

CONCLUSION

We have examined the quantitative relationship between hyperplasia and lymphoma development by comparing the incidence of follicular hyperplasia observed in chicken lines that are resistant or susceptible to ALV-induced lymphomas. Our data support the following two conclusions. First, the frequency of lymphoma development correlates with the incidence of follicular hyperplasia. Animals susceptible to ALV-induced lymphomas exhibited significantly more hyperplasia than resistant animals. Second, hyperplasia itself is not sufficient for tumorigenesis. Although a correlation between the frequency of hyperplasia and the development of a lymphoma exists, each hyperplastic follicle does not progress to form a tumor. Furthermore, molecular analysis of SC animals has revealed no evidence for the occurrence of multiple tumors within a single animal. Therefore, even in animals that develop tumors, the majority of hyperplastic follicles do not become neoplastic.

Our observations indicate that, compared with FP chickens, the greater incidence of hyperplasia within the SC line of chickens is related to the increased number of these chickens that develop tumors. At present, the identification of hyperplastic follicles prevents their subsequent progression into a tumor. It is reasonable however, that the more hyperplasia within a single animal, the greater the probability of a tumor developing within the animal. These observations provide support for the hypothesis that the cells within the hyperplastic follicles represent a pool of cells from which a lymphoma develops. Therefore, within any animal, the greater the number of cells within the pool (i.e. the greater the number of hyperplastic follicles), the greater the probability that this preneoplastic stage of the disease will progress to frank neoplasia.

Two distinct genetic loci, c-*myc* and B-*lym*, have been implicated in the development of the bursal lymphoma (Hayward *et al.*, 1981;

Payne et al., 1982; Goubin et al., 1983). It has been demonstrated that integration of viral sequences is responsible for activation of the c-myc locus. Since our data demonstrate that most viral integration occurs within the first four weeks of infection, c-myc activation is probably an early event. It is of interest to note that we also observe the greatest incidence of hyperplasia within this time period. It has been suggested that the expression of c-myc results in cell proliferation but not transformation (Land, et al., 1983). It is therefore possible that ALV activation of c-myc, while responsible for the appearance of the large, lymphoblastoid cells located within follicular hyperplasia, is itself insufficient for the production of neoplasia and requires the function of additional genetic loci such as B-lym.

ACKNOWLEDGEMENTS

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REFERENCES

Baba, T.W. and Humphries, E.H. (1984a). Differential response to avian leukosis virus infection exhibited by two chicken lines. Virology (in press).

Baba, T.W. and Humphries, E.H. (1984b). Avian leukosis virus infection: Analysis of viremia and DNA integration in susceptible and resistant chicken lines. J. Virol. (in press).

Baba, T.W. and Humphries, E.H. (1984c). Follicular hyperplasia correlates with the subsequent development of Avian leukosis virus-induced bursal lymphoma. (Submitted).

Cooper, G.M. and Neiman, P.E. (1980). Transforming genes of neoplasms induced by avian lymphoid leukosis viruses. Nature (London) 287: 656-659.

Cooper, G.M. and Neiman, P.E. (1981). Two distinct candidate transforming genes of lymphoid leukosis virus-induced neoplasms. Nature (London) 292: 857-858.

Cooper, M.D., Payne, L.E., Dent, P.B., Burmester, B.R., and Good, R.A. (1968). Pathogenesis of avian lymphoid leukosis. I Histogenesis. J. Natl. Can. Inst. 41: 374-389.

Cooper, M.D., Purchase, H.G., Bockman, D.E., and Gathings, W.E. (1974). Studies on the nature of the abnormality of B-cell differentiation in avian lymphoid leukosis: Production of heterogeneous IgM by tumor cells. J. Immunol. 113: 1210-1222.

Foulds, L. (1969). Neoplastic Development. Academic Press, New York. (Vol. I).

Goubin, G., Goldman, D.S., Luce, J., Neiman, P.E., and Cooper, G.M. (1983). Molecular cloning and nucleotide sequence of a transforming gene detected by transfection of chicken B-cell lymphoma DNA. Nature (London) 302: 114-119.

Hayward, W.S., Neel, B.G., and Astrin, S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 290: 475-480.

Kung, Y.-K.T., Fadly, A.M., Crittenden, L.B., and Kung, H.-J. (1982). Avian lymphoid leukosis virus infection and DNA integration in the preleukotic bursal tissues: a comparative study of susceptible and resistant lines. Virology 119: 411-421.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304: 596-602.

Neiman, P.E., Jordan, L., Weiss, R.A., and Payne, L.N. (1980). Malignant lymphoma of the Bursa of Fabricius: Analysis of early transformation. in Viruses in Naturally Occurring Cancers (eds. Essex, M., Todaro, G., and zur Hausen, H.) 519-528. Cold Spring Harbor Laboratory.

Payne, G.S., Bishop, J.M. and Varmus, H.E. (1982). Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature 295: 209-214.

Purchase, H.G. and Burmester, B.R. (1978) in "Diseases of Poultry". (eds. Hofstad, M.S., Calnek, B.W., Helmboldt, D.F., Reid, W.M., and Yoder, H.M.) Iowa State University Press, Iowa. pp. 418-468.

Robinson, H.L., Pearson, M.N., DeSimone, D.W., Tsichlis, P.N., and Coffin, J.M. (1980). Subgroup-E avian leukosis virus-associated disease in chickens. Cold Spring Harbor Symp. Quant. Biol. 44: 1133-1142.

Shabad, L.M. (1973). Precancerous morphologic lesions. J. Natl. Can. Inst. 50: 1421-1428.

Multiple Steps in the Transformation of Normal B Cells Towards Neoplasia

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Our current knowledge of the generation, stimulation and half life of normal B cells in the immune system predicts at least three functionally distinct ways for transformation to the neoplastic state.

GROWTH REGULATION OF NORMAL B CELLS

Three steps can be distinguished in the activation of a B cell from its resting state to proliferation into a clone of mature immunoglobulin (Ig)-secreting cells. In the first step, antigen-nonspecific soluble B cell growth and maturation factors are made, when helper T cells recognize antigen presented on accessory (A) cells. In the second step B cells are excited from their resting state to become susceptible to the action of B cell growth factors. In the third step the B cell growth and maturation factors produced in the first step regulate the cell cycle of B cells excited in the second step. Each step has variations, i.e. can be effected in different ways.

In the first step of T cell-dependent B cell responses, when helper T cells recognize antigen presented by A cells in the context of class II-major histocompatibility complex (MHC) antigens, α factors such as interleukin-1 and others are produced by A cells, (macrophages, dendritic cells) while B cell growth and maturation factors are produced by helper T cells. Interferon-γ, a costimulator of B cell maturation, is also made in the interaction of A and T cells. Helper T cells can also produce T cell growth factor (interleukin-2) and colony-stimulating factors that can indirectly contribute to a B cell response by expanding the number of helper T cells and A cells that produce the B cell specific factors.

The first step of T cell-dependent B cell responses is the activation of A cells to α-factor production. The production of B cell growth and maturation factors is apparently not needed in these responses and suggests that such T cell independent antigens have properties that mimic the action of the factors. In the second step of T cell-dependent B cell responses helper T cells recognize antigen bound to surface Ig on resting B cells. They do so in conjunction with class II MHC molecules present on the B cells.

T cell-independent antigens of type 1 (example: hapten on polysaccharides) appear to excite resting B cells by crosslinking surface Ig on B cells by their repetitive haptenic ligands. Class II MHC molecules are apparently not involved in this type of excitation from the resting state. Type 1 T-independent antigens, at high concentrations, are polyclonal activators while type 2 are not. Immobilized Ig-specific antibodies, in this classification,

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appear to be a special case in which the binding properties to Ig would classify them as type 2 T-independent activators although they activate polyclonally. Excitation leads to the appearance of receptors for B cell growth factors and for transferrin.

In the third step of T cell-dependent B cell responses α factors and B cell growth factors act together early in the G1-phase of each cell cycle to stimulate the excited B cells through the late G1-phase, S-phase, G2-phase and mitosis. When α and/or B cell growth factors are removed the cells are arrested in growth after mitosis. Growth arrest also occurs when transferrin is removed. Even in the continued presence of the factors and of transferrin, however, B cells will cease to divide after 10-15 divisions and die. The second step of B cell activation has so far not been separated experimentally from the third step. Biochemical reactions that occur early after B cell activation can, therefore, not yet be interpreted in their function of regulating excitation and cell cycle of B cells. (A list of references pertinent to the regulation of growth of normal B cells can be found in Melchers, Corbel and Leptin, 1984.)

STEPS TOWARDS B CELL NEOPLASIA

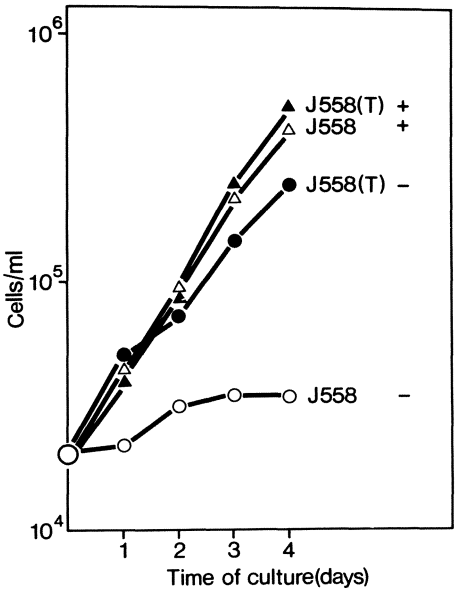
Malignant B cells appear to have acquired the expanded, if not unlimited, capacity for self-renewal and for uninduced (i.e. constitutive) replication. Since most normal B cells in the body turn over with a half life of one or a few days (Brahim and Osmond, 1973), malignant B cells must also have acquired the capacity to be long-lived, avoiding the normal rapid turnover. I would, therefore, like to order transforming events in B cells into three functionally distinct categories.

1) Transforming events that render B cells constitutive for replication: Since normal B cells require transferrin, α factors and B cell growth factors for replication, and since the production of two of these factors normally has to be induced by the cooperation of A cells and T cells, constitutive production of any of these factors and independence of B cells from these factors can be considered transforming steps toward malignant, uncontrolled growth of B cells. The system, but not the B cells, can be considered transformed when either A cells or T cells produce α-factors and/or B cell growth factor constitutively. More direct steps towards B cell malignancy should be attained when B cells themselves gain the capacity to produce α factors and/or B cell growth factors, particularly when they do so constitutively. I would expect all of these transformed states to go undetected in an in vitro test for prolonged or immortal growth of B cells as long as these B cells did not acquire the double capacity to produce, by themselves, α-factors and B cell growth factors, and as long as transferrin is present in the tissue culture medium. As long as the transformed B cells produce only α-factors or only B cell growth factors, they should remain dependent for replication on contributions by host T respectively A cells.

One such example of the dependence of the transformed B cell on the host is the α factor-dependent in vitro replication of in vivo growing myeloma cells (Namba and Hanaoka, 1972; Corbel and Melchers, 1983). The in vivo host environment for this α factor-dependent growth of myelomas is likely to be a granuloma that contains activated macrophages and that is generated when mineral

oil is injected into the peritoneal cavity. Plasmacytomas arise in oil-granulomatous tissue (Cancro and Potter, 1976) and require cells and factors from the oil granuloma for growth (Potter, Pumphrey and Walters, 1972).

FIGURE 1



Replication of J558 myeloma cells in vitro. The original line (J558) only grows in the presence (+) but not in the absence (-) of 10% α factors generated in high density (>$5x10^6$ cells/ml) culture supernatants of P388D1 macrophages incubated for 3 days. After transfection (T) with medium conditioned for two days with an influenza virus-specific T cell clone grown for 3 days in IL-2 containing medium (Gerhard, Hackett and Melchers, 1983) J558(T) cells now grow in the presence (+) as well as in the absence (-) of α factors. Transfection of J558 cells was done as follows: the cells were washed in serum-free medium, thereafter attached to COSTAR 25 cm^2 tissue culture flasks (COSTAR 205, Broadway, Cambridge, Mass., USA) at $2.5x10^7$ cells per flask by incubation for 30 minutes, the supernatant medium removed. Thereafter 5 ml 30% polyethyleneglycol (MW 6000, Fluka, Buochs, Switzerland) in serum-free medium was added for one minute, removed and replaced by 10 ml 5% polyethyleneglycol in T cell-conditioned medium (see above). After 6 hrs the polyethyleneglycol-containing medium was removed and replaced by full tissue culture medium (Iscove and Melchers, 1978). Cells were counted under the microscope.

The α-factor-dependent in vitro growth of certain plasmacytomas can be transformed to α factor independence. Thus, plasmacytoma cells that are rendered susceptible for uptake of large molecules by polyethyleneglycol shock will begin to grow α factor-independently after they are exposed to media conditioned with killer or helper T cell lines grown in T cell growth factors, or with B cell lymphomas and plasmacytomas (Figure 1). The α factor-independent phenotype remains stable for several months. The active transforming principle can be pelleted at 100,000 x g through 2.2M sucrose and is currently being analyzed in detail.

In summary, a B cell will become fully transformed and growing independently of its environment when it has acquired the capacity to constitutively produce transferrin, α factors and B cell growth factor.

Tranformation of a B cell would also be achieved by changes in the cell that make it independent of the action of α and B cell growth factors. I would envisage that structural changes in the receptors

for these factors, and in intracellular sites of signalling for proliferation could lead functionally to constitutive proliferation of B cells even in the absence of factors. Reactions of excitation as well as reactions controlling the cell cycle could be changed, and one such change alone may again not be sufficient for full independence from the host environment of excitation and factor action.

It follows that consecutive changes to full transformation, i.e. independence from transferrin, α-factors and B cell growth factors could be a combination of changes to constitutive production of some factors, and changes in the receptors and intracellular signalling structures for the other factors.

2) Transforming events that block terminal differentiation: Normal B cells increase their rate of Ig synthesis and secretion and develop more and more endoplasmic reticulum with each division (Melchers and Andersson, 1973). As they continue to mature in this way they loose their capacity to divide so that they finally die. This terminal differentiation or maturation is most evident when resting B cells are exposed to maturation factors: the B cells mature to blast-like Ig secreting cells without dividing. At the same time they loose their capacity to be induced to proliferation (Melchers et al. 1980).

Events that induce blocks in the reactions leading to terminal maturation could therefore be transforming steps since they would prevent B cells from dying. A series of IgM-producing plasmacytomas that appear arrested at different stages of maturation to high-rate IgM-secreting plasma cells may,in part, be transformed due to such blocks (Andersson et al. 1974). The ability of B cells transformed in these ways to grow continuously in vivo should depend on the host's environmental activities of A cells and T cells producing α and B cell growth factors. Again, in vitro, they should go undetected.

3) Transforming events that prolong the life of a B cell: Since 5% to 10% of all B cells are newly generated each day, the same number of B cells must die even when they are not stimulated, i.e. when they are resting cells. Little is known of the properties of a B cell that render it long-lived. One factor that may prolong the life of a B cell in the body is the chemo-attraction by the appropriate foreign antigen presented on A cells. The adherence to an antigen-primed layer of A cells may simply prevent its further migration to the site of removal from the body. It predicts selection of eventually transformed B cells by long-lasting antigens, a process that has long been proposed to account for the high frequency of plasmacytomas producing Ig molecules with specificities for ubiquitous bacterial antigens (Potter, 1977).

Memory for a past exposure to antigen appears to increase the life expectancy of a B cell in the immune system. A memory B cell can recirculate (see Sprent, 1977), due to an altered surface membrane phenotype that allows the migration from blood to lymph to blood (Reichert et al., 1983). Such phenotypes could, therefore contribute to the total process of malignant transformation that includes metastase, particularly when their normal expression would be deregulated.

MULTIPLE STEPS IN TRANSFORMATION

None of the transforming events alone appears to be sufficient for full transformation to in vivo B cell malignancy. Cells that are transformed to constitutive replication will still be removed from the body at the rate of turnover of a normal cell, i.e. within a few days. Since most activated, normal as well as malignant, B cells divide approximately once a day there seems hardly a chance for clonal expansion of cells tranformed by events of the first category before they are eliminated by normal turnover. This is also true for cells transformed by events of the second category.

Even when the host environment of A and T cells is strongly activated for long periods of times, B cells transformed events of category 1 and/or 2 will still be removed by normal rapid turnover, unless they can change to become long-lived. B cells transformed by events of category 3 will, again, die the normal death of an activated plasmablast and plasma cell, unless they can change to constitutive proliferation and/or to a block in terminal differentiation.

It follows that multiple steps must be involved in the process of transformation of a single B cell to neoplasia: one (or two) that induce constitutive replication, one that blocks terminal differentiation, and one that prolongs the life of a B cell in the body.

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REFERENCES

Andersson J, Buxbaum J, Citronbaum R, Douglas S, Forni L, Melchers G, Pernis B and Scott D (1974) IgM producing tumors in the Balb/c mouse - a model for B cell maturation J Exp Med 140:742-763

Brahim F and Osmond DG (1973) The migration of lymphocytes from bone marrow to popliteal lymph nodes demonstrated by selective bone marrow labelling with (^{3}H) thymidine in vivo Anat Rec 175:737-745

Cancro M and Potter M (1976) The requirement of an adherent substratum for the growth of developing plasmacytoma cells in vivo J Exp Med 144:1554-1566

Corbel C and Melchers F (1983) The synergism of accessory cells and of soluble α factors derived from them in the activation of B cells to proliferation Immunol Rev 78: in press

Gerhard W, Hackett C and Melchers F (1983) The recognition specificity of a murine helper T cell hemagglutinin of influenza virus A/PR8/34 J Immunol 130:2379-2385

Iscove NN and Melchers F (1978) Complete replacement of serum by albumin, transferrin and soybean lipid in cultures of lipopolysaccharide reactive B lymphocytes J Exp Med 147:923-933

Melchers F and Andersson J (1973) Synthesis, surface deposition and secretion of immunoglobulin M in bone marrow-derived lymphocytes before and after mitogenic stimulation Transplant Rev 14:76-130

Melchers F, Andersson J, Lernhardt W and Schreier MH (1980) H-2 unrestricted polyclonal maturation without replication of small B cells induced by antigen-activated T cell help factors Eur J Immunol 10:679-685
Melchers F, Corbel C and Leptin M (1984) What does it take to stimulate a B cell? Progr Immunol V in press
Namba Y and Hanaoka M (1972) Immunocytology of cultured IgM-forming cells of mouse. I. Requirement of phagocytic cell factor for the growth of IgM-forming tumor cells in tissue culture J Immunol 109:1193-1200
Potter M, Pumphrey JG and Walters JL (1972) Growth of primary plasmacytomas in the mineral oil-conditioned peritoneal environment J Natl Cancer Inst 49:305-308
Potter M (1977) Antigen binding myeloma proteins of mice Adv Immunol 25:141-210
Reichert RA, Gallatin WM, Weisman IL and Butcher EC (1983) Germinal center B cells lack homing receptors necessary for normal lymphocyte recirculation J Exp Med 157:813-827
Sprent J (1977) Recirculating lymphocytes. In: The lymphocyte - Structure and Function, Part I, ed. JJ Marchalonis, Marcel Dekker Inc New York and Basel 5:43-111

Transferrin Receptors Regulate Proliferation of Normal and Malignant B Cells

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INTRODUCTION

Proliferation of normal and malignant lymphoid cells is dependent on the serum glycoprotein transferrin (Barnes and Sato, 1980). Interestingly, although serum transferrin concentrations remain consistently high (in the 4 mg/ml range), resting lymphocytes do not possess detectable transferrin receptors and do not respond to transferrin (Tormey et al. 1972; Dillner-Centerlind et al., 1979; Larrick and Cresswell, 1979), suggesting that induction and maintenance of transferrin receptor expression plays a critical role in the control of lymphoid cell proliferation. In this paper we show (1) the sequence of receptor mediated events leading to growth factor induction of transferrin receptor expression on B cells, which is followed by entry into S phase, and (2) inhibition of cell proliferation by transferrin receptor blockade, even in the presence of the uninhibited interaction of growth factor and its receptor.

Although normal lymphoid cells require the interaction of growth factors and growth factor receptors for the induction of transferrin receptor expression, proliferating malignant cells express transferrin receptors, on which they depend for growth, but frequently lack exogenous growth factor requirements. The data suggest a model for malignant transformation in which constitutive expression of the transferrin receptor permits cell proliferation in the absence of homeostatic control by growth factors.

RESULTS

Regulation of Transferrin Receptor Induction in Normal B Cells Requires Two Signals

We have previously demonstrated that mitogen-induced T cell proliferation depends on transferrin receptor induction, and that this induction requires two signals: mitogen (for induction of IL-2 receptors) and IL-1 or TPA (for stimulation of IL-2 production) (Neckers and Cossman, 1983). Only after IL-2 interacts with its receptor do transferrin receptors appear.

It is clear that transferrin receptor induction on B cells following mitogen stimulation has similar requirements (Fig. 1). B cell transferrin receptor induction depends on mitogen stimulation (Cowan Staphylococcus aureus, CSA) and the addition of B cell growth factor (BCGF) - containing T cell conditioned medium. Addition of either agent separately does not result in transferrin receptor induction.

Transferrin Receptor Acquisition is Required for Normal B Cell Proliferation

Prolonged elevation in thymidine incorporation and actual cell growth occurs only if transferrin receptors are present (Fig. 1). Lack of transferrin receptors due to improper signalling (i.e., CSA or BCGF alone), or blockade of the transferrin receptor with an antibody that blocks transferrin binding (42/6)(Trowbridge and Lopez,

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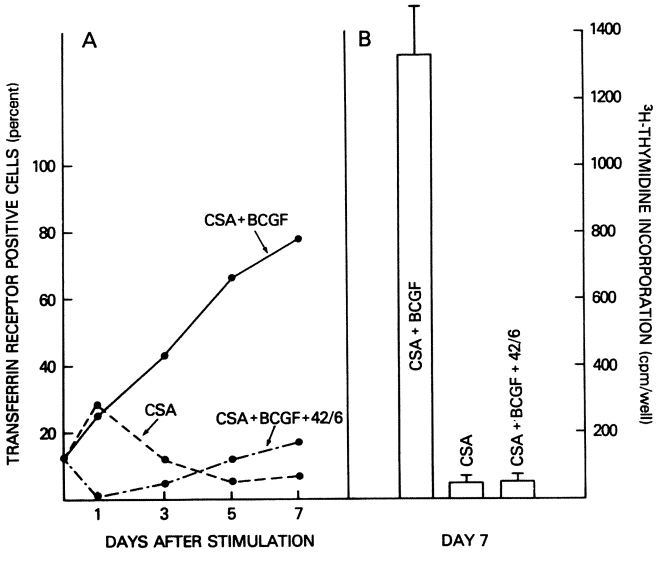

Fig. 1. Mitogen-induced B cell proliferation requires transferrin receptor expression. Transferrin receptors are induced on B cells only in the presence of CSA and BCGF. These receptors can be blocked by the monoclonal anti-transferrin receptor antibody, 42/6, and DNA synthesis is greatly inhibited. Incomplete signalling (CSA or BCGF alone) cannot sustain prolonged DNA synthesis in the absence of transferrin receptors.

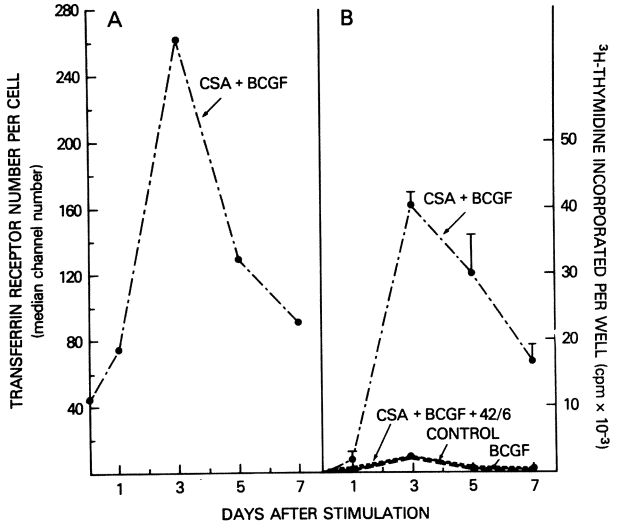

Fig. 2. Transferrin receptor number correlates with the rate of DNA synthesis. As a cell population's mean transferrin receptor number increases so does the mean rate of DNA synthesis. The reverse also holds true. Transferrin receptor number is quantified by determining the mean fluorescence intensity of a cell population following fluorescent tagging of the receptors by a monoclonal anti-transferrin receptor antibody (OKT9) and flow cytometric analysis (for details see Neckers and Cossman, 1983).

1982), correlates with very low levels of DNA synthesis. This is true even in the presence of excess BCGF.

Relative transferrin receptor number per cell (determined by flow cytometric analysis; see Neckers and Cossman, 1983 for details) also correlates quite closely with the amount of thymidine incorporated by a cell population (Fig. 2). That is, during the course of B cell proliferation, the mean number of transferrin receptors on a cell population varies as does the mean rate of DNA synthesis of that same population. Together with the strict dependence of replicative DNA synthesis on the presence of functional transferrin receptors, these data imply a close linkage between DNA synthesis rate and transferrin receptor number.

Observation of the time course of transferrin receptor acquisition and onset of DNA synthesis suggests that transferrin receptor appearance on stimulated B cells is a late G_1 event (Fig. 3). These data, together with those obtained in the study of T cell activation (Neckers and Cossman, 1983), point to transferrin receptor appearance on the cell surface and the ligand/receptor interaction as one of the last surface receptor-dependent events necessary for S phase entry.

Malignant B Cells Need Transferrin Receptors to Grow

Transferrin receptor expression in malignant B cells is not cell cycle dependent In other words, the receptors are present on transformed cells throughout the cell cycle (data not shown). We added anti-receptor antibody (42/6) to two Burkitt's cell lines, Raji and Daudi (Fig. 4). In both cases, blockade of the transferrin receptor resulted in markedly reduced cell growth. These cells have no extrinsic requirement for other growth factors, although they may synthesize their own (Blazar, et al., 1983; Gordon et al., in press) They are nonetheless dependent on transferrin for growth (Gordon et al., in press) and, as we have demonstrated, their growth depends on continued expression of transferrin receptors. Such transferrin receptor expression may be constitutive, or may be dependent on autocrine growth factors.

Mean Cellular Transferrin Receptor Number Varies With Cell Growth Rate

Another way of showing the strict dependence of malignant cell growth on transferrin receptors is to correlate growth rate with transferrin receptor number. One finds that cells express more transferrin receptors when they grow quickly and fewer transferrin receptors when they grow more slowly (Table 1). Raji cells were grown either in RPMI 1640, which supports their rapid growth, or in MEM, which supports a much slower growth rate. The cells growing in MEM demonstrate a 40% reduction in mean transferrin receptor number, while their doubling time is increased more than three-fold.

DISCUSSION

Normal resting B cells do not express transferrin receptors. The induction of transferrin receptor expression in normal B cells is a multistep, tightly regulated process. Antigen or mitogen stimulation, production of growth factors, and acquisition of functional growth factor receptors are required for the induction of transferrin receptors in normal B cells (Fig. 1). Proliferating malignant B cells, however, express transferrin receptors constitutively. These cells show high transferrin receptor number per cell, and express receptor throughout the cell cycle. In both normal and malignant cells, blockade of the transferrin receptor prevents cell proliferation, even in the presence of growth factors (Figs. 1 and 3).

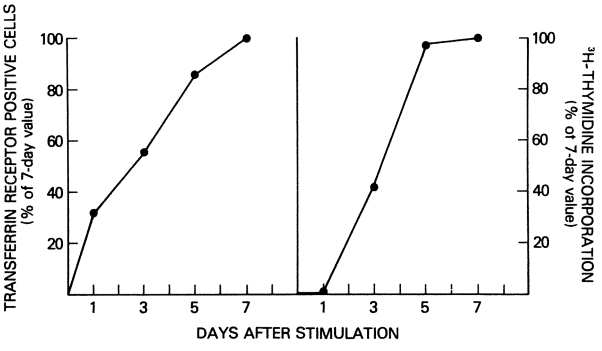

Fig. 3. Mitogen-induced appearance of transferrin receptors and onset of DNA synthesis have a similar time course. OKT9 staining and flow cytometric analysis allows the determination of that fraction of a cell population possessing detectable transferrin receptors. Transferrin receptor acquisition occurs just prior to onset of DNA synthesis.

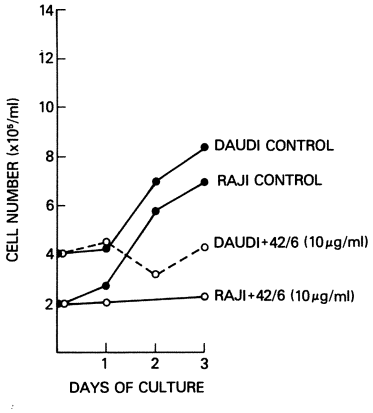

Fig. 4. Transferrin receptors are required for the growth of malignant B cells. Monoclonal antibody 42/6 (10μg/ml) was added at the initiation of the cultures. Cell counts were made using a hemocytometer on a daily basis thereafter.

Furthermore, in T cells, a system in which a monoclonal antibody is available to the growth factor receptor (Leonard et al., 1982), once transferrin receptor is expressed on normal activated T cells, blockade of the TCGF (IL-2) receptor has no ability to inhibit short-term proliferation (Neckers and Cossman, 1983; Neckers and Cossman, in press). This suggests that a functional transferrin receptor provides, of the previously described surface molecules (Howard and Paul, 1983), the most immediate stimulus for entry into S phase and cell proliferation.

It appears, therefore, that the transferrin receptor plays a critical role as a surface sine qua non of cell proliferation, and that growth factors, and growth factor receptors play a critical role in regulating the timely appearance of transferrin receptors. This model predicts that leukemogenesis, still transferrin receptor-dependent, could occur if the biochemical sequelae of growth factor/growth factor receptor interaction were constitutively expressed. Figure 5a depicts our scheme of growth factor-controlled induction of transferrin receptor expression in normal B cells, in contrast to Fig. 5b, which depicts several models of unregulated transferrin receptor expression in malignant cells. The model for normal cells follows those of Muraguchi et al. (1984) and Howard and Paul (1983), but we have extended their findings by placing transferrin receptor expression in the time sequence leading to proliferation. Transferrin receptor induction on normal B cells is dependent on prior exposure to at least two signals, which can be provided by mitogen and a BCGF-containing T cell supernatant. In a process analogous to T cell activation, mitogen presumably makes B cells responsive to BCGF, by BCGF receptor induction (Muraguchi et al., 1984). Interaction of BCGF with its receptor then results in transferrin receptor expression in late G_1.

B cells are not unique in their dependence on transferrin for growth (Barnes and Sato, 1980), and regulation of transferrin receptor expresssion appears to be critical in preventing uncontrolled proliferation. This suggests that transferrin receptor expression would have to be regulated in non-lymphoid cell lineages, as well as in lymphoid cells, by specific growth signals. Our data suggest that these growth signals move cells out of G_0 into G_1, where the cell becomes responsive to tissue-specific growth factors, such as BCGF. Only following this sequence of events, in which there are at least two regulating steps, e.g. acquisition of responsiveness to cell specific growth factor and stimulation of growth factor production, are transferrin receptors induced.

In malignant B cells transferrin receptors are constitutively expressed throughout the cell cycle, but are still required for cell proliferation. Figure 5b depicts three possible routes to constitutive transferrin receptor expression. In panel 1 overproduction of growth factor leads, following interaction with the growth factor receptor, to transferrin receptor induction. The inappropriately produced growth factor could be autocrine, or supplied from another cell. In panel 2 the growth factor receptor itself is constitutively expressed and activated in the absence of appropriate homeostatic controls, resulting in constitutive expression of the transferrin receptor. Panel 3 shows constitutive expression of the transferrin receptor in the absence of growth factor or growth factor receptor expression.

Embryogenesis, wound healing, and the immune response represent normal physiologic events requiring controlled induction of cell proliferation. In each of these events one would expect growth factors to induce and regulate transferrin receptor expression and cell division. Aberrant control of growth factors or growth factor receptors could lead to inappropriate expression of the transferrin receptor and increased likelihood of cell immortalization. These speculations suggest functional connections between the recently described proto-oncogene-encoded and/or homologous growth factors (Waterfield et al., 1983; Robbins et al., 1983) and growth factor receptors (Downward et al., 1984; Privalsky et al., 1984), deregulation of the transferrin receptor and malignant transformation.

Table 1. Transferrin receptor number correlates with cell growth in Raji Cells

Growth Medium	Transferrin Receptor[a] Density per Cell	^{3}H-Thymidine[b] Incorporation	Doubling[c] Time
RPMI 1640	306	96,000	24
MEM	180	18,000	86

[a] mean fluorescence intensity as determined by flow cytometry following labelling with monoclonal anti-transferrin receptor antibody (for method see Neckers and Cossman, 1983).

[b] mean log phase cpm incorporated by 5 X 10^{5} cells following a 4-hour pulse with 1 uCi ^{3}H-thymidine.

[c] mean doubling time measured in hours.

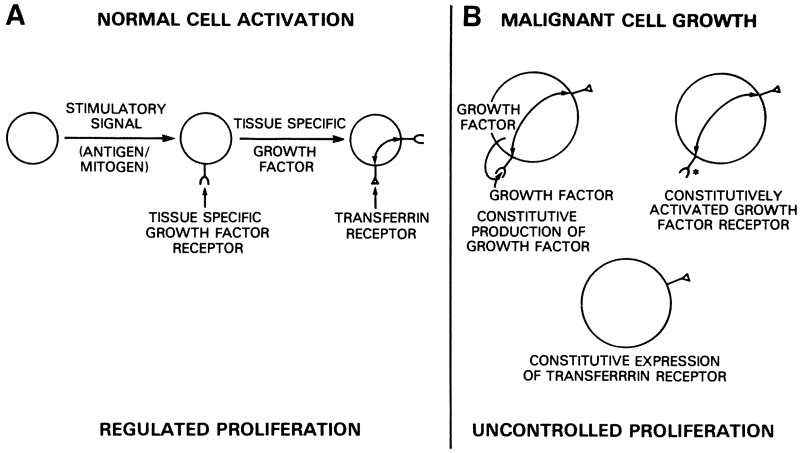

Fig. 5. Regulation of transferrin receptor expression in normal activated and malignant cells. In normal cells, transferrin receptor expression is regulated by interaction of tissue specific growth factors with their receptors, which in turn must be synthesized during the initial stages of cellular activation. In malignant cells, homeostatic regulation of transferrin receptor expression is lost, either by uncontrolled production of growth factor receptor and/or growth factor, or by deregulated constitutive expression.

REFERENCES

Barnes, D, Sato, G (1980) Serum-free cell culture: a unifying approach. Cell 22: 649-655

Blazar, BA, Sutton, LM, Strome, M (1983) Self-stimulating growth factor production by B-cell lines derived from Burkitt's lymphomas and other lines transformed in vitro by Epstein-Barr Virus. Cancer Research 43: 4562-4568

Dillner-Centerlind, ML, Hammarstrom, S, Perlmann, P (1979) Transferrin can replace serum for in vitro growth of mitogen-stimulated T lymphocytes. Eur. J. Immunol. 9: 942-948

Downward, J, Yarden, Y, Mayes, E, Scrace, G, Totty, N, Stockwell, P, Ullrich, A, Schlessinger, J, Waterfield, MD (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature(London) 307: 521-527

Gordon, J, Ley, SC, Melamed, MD, Aman, P, Hughes-Jones, NP (to be published) Soluble factor requirements for the autostimulatory growth of B-lymphoblasts immortalized by Epstein-Barr Virus. J. Exp. Med.

Howard, M, Paul, WE (1983) Regulation of B cell growth and differentiation by soluble factors. Ann. Rev. Immunol. 1: 307-333

Larrick, JW, Cresswell, P (1979) Modulation of cell surface iron transferrin receptors by cellular density and state of activation. J. Supramol. Struct. 11: 579-586

Leonard, WJ, Depper, JM, Uchiyama, T, Smith, KA, Waldmann, TA, Greene, WC (1982) A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. Nature(London) 300: 267-269.

Muraguchi, A, Kehrl, JH, Butler, JL, Fauci, AS (1984) Sequential requirements for cell cycle progression of resting human B cells after activation by anti-Ig J. Immunol. 132: 176-180

Neckers, LM, Cossman, J (1983) Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc. Natl. Acad. Sci. USA 80: 3494-3498

Neckers, LM, Cossman, J (to be published) Long-term maintainance of transferrin receptors on activated T cells requires interleukin 2 and its receptor and is in turn required for maintainance of interleukin 2 receptor expression. In: Goldstein, A (ed) GWUMC Annual Spring Symposia Series, 1983

Privalsky, ML, Ralston, R, Bishop, JM (1984) The membrane glycoprotein encoded by the retroviral oncogene v-erb-B is structurally related to tyrosine-specific protein kinases. Proc. Natl. Acad. Sci. USA 81: 704-707

Robbins, KC, Antoniades, HN, Devare, SG, Hunkapiller, MW, Aaronson, SA (1983) Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. Nature(London) 305: 605-608

Tormey, DC, Imrie, RC, Mueller, GC (1972) Identification of transferrin as a lymphocyte growth promoter in human serum. Exp. Cell Res. 74: 163-169

Trowbridge, IS, Lopez, F (1982) Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth in vitro

Waterfield, MD, Scrace, GT, Whittle, N, Stroobant, P, Johnsson, A, Wasteson, A, Westermark, B, Heldin, CH, Huang, JS, Deuel, TF (1983) Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature(London) 304: 35-39

Cloned Neoplastic B Cells Release a Growth Factor Which Augments Lymphokine-Mediated Proliferation of Normal B Cells

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Several types of neoplastic cells secrete growth factors which bind to the producer cells and stimulate their growth (autocrine factors) (Todaro 1980; Kaplan 1982; Bishop 1983). As a first step in identifying a putative B cell-derived autocrine growth factor, we have examined a series of neoplastic B cell clones which were derived from the in vitro-adapted BCL_1 cell line and were selected for their lack of spontaneous IgM secretion (Brooks 1983). Based on other autocrine models, it was predicted that a putative growth factor might act during G_1 of the cell cycle. Thus, BCL_1 cells were synchronized at the G_1/S border using either a triple thymidine block or the α DNA polymerase inhibitor, aphidicolin. The cells were washed free of the blocking agent, cultured and the supernatants (SN) were harvested at various times. These SN were evaluated for their ability to sustain the proliferation of anti-Ig-stimulated normal splenic B cells.

Although we did not find B cell growth factor (BCGF) activity in culture SN of asynchronously growing BCL_1 clones, activity was consistently present in the SN harvested approximately 24 hours after release of the BCL_1 cells from the G_1/S block. In 14 experiments, the SN from synchronized BCL_1 cells gave a mean increase in tritiated thymidine uptake of 2.1-fold. In the same experiments, T cell-derived BCGF [BCGF I, M.W. 18,000 (Howard 1982)] present in the SN of phorbol myristate acetate-induced EL-4 thymoma cells gave a mean increase of 5.7-fold. The BCL_1-derived growth factor also synergized with human IL-1 and EL-4 SN in the maintenance of anti-Ig-induced B cell proliferation. This synergy was apparent when tritiated thymidine uptake was assessed 5-8 days after stimulation; in 5 experiments, the mean augmentation of proliferation by the BCL_1-derived BCGF was 3.14-fold. In contrast, BCL_1 SN did not augment dextran sulfate-initiated growth of normal B cells. This assay measures the activity of a second T cell-derived BCGF [BCGF II (Swain 1983)] of 55,000 M.W. None of the SN tested had any activity in this assay.

The BCL_1 SN was assessed for growth activity on T lymphocytes. No evidence for IL-2 or IL-1 was obtained: Thus, these SN did not: 1) support the growth of IL-2-dependent HT-2 cells (Kappler 1981); 2) synergize with Concanavalin A in the stimulation of thymocyte proliferation [an assay for IL-1 (Lachman 1977)]; and 3) substitute for IL-1 in an IL-1-dependent BCGF assay (Howard 1983).

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The molecular weight of the BCL_1-derived BCGF was 4500 daltons as determined by fractionation of SN on G-50 gel filtration columns. In some fractionations a second, smaller peak of activity was present and had a M.W. of 1450. The BCL_1-derived BCGF is therefore considerably smaller than all previously described lymphokines and has a M.W. comparable to that of a number of peptide hormones such as epidermal growth factor, and the neuroendocrine-peptides ACTH and ß-endorphin.

In order to determine whether BCL_1 cells synthesized such peptide hormones, we probed BCL_1-derived mRNA with cloned DNA specific for the pro-opiomelanocortin gene (POMC) which encodes a polyprotein which is subsequently cleaved into several peptides including ACTH and ß-endorphin (Mains 1977). Poly(A)$^+$ mRNA from BCL_1 cells showed positive hybridization with the POMC probe. When compared by Northern blotting to the POMC mRNA derived from the murine pituitary tumor line, AtT-20 (which secretes ACTH), the BCL_1 mRNA was approximately 200 bases larger. Several other B cell tumors such as a sIg-negative clone (sIg$^-$) of BCL_1 cells (Bl.4.23), the LPS-inducible WEHI-231 (Boyd 1981) cells and a hybridoma between the *in vivo*-derived BCL_1 cells and X63 cells as well as X63 itself, lacked POMC mRNA. EL-4 thymoma cells were also negative.

The above results suggest a correlation between the presence of POMC mRNA and the production of growth factor. Furthermore, clones of BCL_1 cells which secrete the growth factor in a cell-cycle-dependent manner also show cell-cycle regulated expression of the POMC gene. Preliminary data indicate that SN containing growth factor also contain an anti-ACTH-reactive molecule. Furthermore, SN from the sIg$^-$ BCL_1 clone (Bl.4.23) lack both BCGF activity and an ACTH-like molecule; Bl.4.23 cells do not synthesize POMC mRNA.

Experiments are in progress to determine 1) the nature of the molecule encoded by the POMC gene in BCL_1 cells, 2) the relationship between the BCL_1-derived BCGF and ACTH, and 3) whether BCL_1-derived BCGF and/or ACTH are autocrine growth factors for BCL_1.

REFERENCES

Bishop JM (1983) Cellular oncogenes and retroviruses. Ann Rev Biochem 52:301.

Boyd AW, Goding JW, Schrader JW (1981) The regulation of growth and differentiation of a murine B cell lymphoma. J Immunol 126:2461.

Brooks K, Yuan D, Uhr JW, Krammer PH, Vitetta ES (1983) Lymphokine-induced IgM secretion by clones of neoplastic B cells (BCL_1). Nature 302:825.

Howard M, Farrar J, Hilfiker M, Johnson B, Takatsu K, Hamaoka T, Paul WE (1982) Identification of a T cell-derived B cell growth factor distinct from interleukin-2. J Exp Med 155:914.

Howard M, Mizel SB, Lackman L, Ansel J, Johnson B, Paul WE (1983) Role of interleukin-1 in anti-immunoglobulin-induced B cell proliferation. J Exp Med 157:1529.

Kaplan PL, Anderson M, Ozanne B (1982) Transforming growth factor(s) production enables cells to grow in the absence of serum: An autocrine system. Proc Natl Acad Sci (USA) 79:485.

Kappler JW, Skidmore B, White J, Marrack (1981) Antigen-inducible, H-2 restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. J Exp Med 153:1198.

Lachman LB, Hacker MP, Blyden GT, Handshumacher RE (1977) Preparation of lymphocyte-activating factor from continuous murine macrophage cell lines. Cell Immunol 34:416.

Mains RE, Eipper BA, Ling N (1977) Common precursor to corticotropins and endorphins. Proc Natl Acad Sci (USA) 74:3014.

Swain SL, Howard M, Kappler J, Marrack P, Watson J, Booth R, Wetzel GD, Dutton RW (1983) Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. J Exp Med 158:822.

Todaro GJ, Fryling C, Delarco JE (1980) Transforming growth factors produced by certain human tumor cells: Polypeptides that interact with epidermal growth factor receptors. Proc Natl Acad Sci (USA) 77:5258.

The Action of an Lyb2.1-Specific Monoclonal Antibody in Soluble or Immobilized Form on Resting and Activated B Cells

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INTRODUCTION

Lyb2, a 45,000 dalton glycoprotein differentiation antigen is present on all murine B lymphocytes (Sato and Boyse, 1976, Tung et al., 1977). The Lyb2.1 allele is present on B cells of many different inbred strains of mice including $DBA/_2$ and $CBA/_J$, and absent on many others including $C57BL/_{6J}$ and $CBA/_N$. A monoclonal antibody specific for Lyb2 has been shown to inhibit the T cell dependent in vitro responses of B cells to sheep erythrocytes (SRC) (Yakura et al., 1981). Another monoclonal antibody specific for the Lyb2.1 allele has been found to have the same properties. In addition this monoclonal antibody changes the morphology of B cells to blasts and induces proliferation of activated B cells expressing the proper Lyb2 allele (Subbarao and Mosier, 1983). Only a subset of B cells appears to be involved since B cell-deficient $CBA/_N$ x $DBA/_2$ F1 male cells do not show these effects while the corresponding normal female cells do. These findings have suggested that Lyb2 is a membrane glycoprotein that participates in murine B cell activation, maybe even as a receptor for B cell growth factors.

B cell growth factors do not stimulate resting, but only activated B cells to proliferation (Melchers et al., 1980). If, therefore, the Lyb2.1-specific monoclonal antibody mimics the action of B cell growth factors it could be expected to have the same differential action on resting and activated B cells.

MATERIALS AND METHODS

Animals: $DBA/_2$, $CBA/_J$ and $C57BL/_{6J}$ $nu/_{nu}$ mice were obtained from the Institut für Biologisch-Medizinische Forschung A.G., Füllinsdorf, Switzerland. $CBA/_N$ x $DBA/_2$ F1 mice were bred in the Basel Institute for Immunology by E. Ackerley. All animals were between 6 and 12 weeks old.

Tissue culture, cells and reagents: Iscove's modified Dulbecco's medium containing $5x10^{-5}$M 2-mercaptoethanol, and human transferrin, bovine serum albumin and soybean lipids as serum replacements (Iscove and Melchers, 1978) was used for all tissue cultures. Small, resting B cells were enriched from preparations of spleen cells by velocity sedimentation at 1xg (Miller and Phillips, 1969) as described previously (Andersson, Lafleur and Melchers 1974). In order to obtain activated cells they were then cultured at $5x10^5$ cells/ml in a CO_2-incubator at 10% CO_2 in air, 37°C with lipoprotein (Melchers, Braun and Galanos, 1975) (25 µg/ml) or lipopolysaccharide (LPS, Andersson, Sjöberg and Möller, 1972) (50

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μg/ml) in Costar culture flasks, 75 cm^2 (Costar 3575, Cambridge, Mass., U.S.A.) for 2 days. The activated, large B blasts were thereafter enriched from nonactivated cells again by velocity sedimentation as described previously (Andersson, Coutinho and Melchers 1979). In the experiments that tested the action of the Lyb2.1-specific monoclonal antibody, small cells were cultured at $3x10^5$ cells/ml, activated B blasts at $5x10^4$ cells/ml in Costar 96 well tissue culture clusters (Costar 3596), 0.2 ml per culture. Lipoprotein was prepared from Escherichia coli as described by Braun and coworkers (Braun and Rehn, 1969; Braun and Sieglin, 1970). LPS was a gift from Drs. C. Galanos and O. Lüderitz, Max Planck Institut für Immunbiologie, Freiburg i.Br., West Germany.

Tissue culture media conditioned by Concanavalin A-activated T cell hybridomas 91.4.7, containing B cell replication and maturation factors were prepared as described (BRMF, Melchers et al., 1982) and used at 25% in the presence of 5mM α-methyl-mannoside (Sigma Chem. Co., St. Louis, Mo., U.S.A.).

Purified Lyb2.1-specific monoclonal antibody 10.1.D2 was prepared as described previously (Subbarao and Mosier, 1983) and used in cultures as indicated in the Results and Discussion section. The monoclonal antibody was also coupled to CNBr-activated Sepharose (Pharmacia A.B., Uppsala, Sweden) under sterile conditions in quantitites indicated in the Results and Discussion section. Over 95% of the antibody was found attached to the beads after the coupling procedure. Purified μ-heavy (H) chain specific antibody AK15 (Leptin et al., 1984) was similarly coupled to CNBr-activated Sepharose at 2.5 mg per ml packed Sepharose beads. Both preparations of coupled monoclonal antibodies were used at 0.2% bead suspension in culture.

Assays for B cell activation: Proliferation was determined either by incorporation of ^{3}H-thymidine (1 μCi per culture, 2 Ci/mMol, Amersham, U.K.) for 2 hours after 48 hours of stimulation as described previously (Andersson, Lafleur and Melchers, 1974), or by counting the number of cells in culture that included the visual observation of large activated blasts with an inverted microscope.

RESULTS AND DISCUSSION

The Action of Lyb2.1-Specific Monoclonal Antibody in B Cells.

1) Small, resting cells: Small, resting cells were prepared from spleens of $DBA/_2$, $CBA/_J$, $C57BL/_{6J}nu/_{nu}$ and $CBA/_N$ x $DBA/_2$ F1 ♀ mice that have normal B cells, and from $CBA/_N$ x $DBA/_2$ F_1 ♂ mice that bear an X-linked immunodeficiency of B cells. They were then cultured for 2 days in the presence of Lyb2.1-specific monoclonal antibody. The results in Table 1 show that neither the soluble nor the immobilized antibody could stimulate proliferation in small B cells even of the appropriate Lyb2 allele. Large, apparently nondividing, blast-like cells appeared in the presence of soluble but not of immobilized Lyb2-specific antibody at concentrations above 10 μg/ml, as they did in the presence of BRMF. The possible maturation of small cells to plasmablasts and plasma cells without divisions, induced by the Lyb2-specific antibody would be analogous to the action of BRMF with small cells (Melchers et al., 1980) and is presently under further investigation.

It should be noted that, in contrast to previous findings (Huber and Melchers, 1979) our present preparation of lipoprotein stimu-

TABLE 1 STIMULATION OF SMALL RESTING AND ACTIVATED B CELLS BY Lyb2.1-SPECIFIC MONOCLONAL ANTIBODY

a) Response of small cells from

Addition to culture[d]		DBA/2 a)	DBA/2 b)	CBA/J a)	CBA/J b)	C57BL/6J nu/nu a)	C57BL/6J nu/nu b)	CBA/N x DBA/2 F1♀ a)	CBA/N x DBA/2 F1♀ b)	CBA/N x DBA/2 F1♂ a)	CBA/N x DBA/2 F1♂ b)
none		0.5	20(1)	0.7	20(1)	0.9	25(2)	0.5	20(1)	0.3	15(1)
Lipoprotein		24	60(60)	30	50(50)	32	65(50)	41	65(50)	30	55(45)
LPS		26	60(50)	31	55(50)	30	60(55)	27	65(50)	5	25(5)
BRMF		2	20(30)	3	20(15)	2	25(20)	2	60(20)	1	20(5)
immobilized μ H-specific anti-body		18	55(40)	35	45(50)	37	40(55)	35	60(50)	3	20(5)
soluble Lyb2.1-specific anti-body (μg/ml)	100	2	20(21)	2	20(20)	3	20(2)	2	25(25)	1	15(3)
	10	1	20(14)	2	20(20)	2	20(2)	2	20(20)	1	15(2)
	1	1	15(3)	2	20(2)	2	25(2)	1	20(10)	1	15(2)
	0.1	0.5	20(1)	2	20(1)	2	20(2)	0.5	20(3)	0.5	15(1)
immobilized Lyb2.1-specific anti-body (μg/ml beads)	2500	1	20(1)	2	20(1)	2	25(1)	1	20(1)	1	15(1)
	250	1	20(1)	3	20(1)	2	20(1)	1	20(1)	1	15(1)
	25	0.5	20(1)	2	20(1)	2	25(2)	1	25(1)	1	15(1)

b) Response of lipoprotein-activated blasts from

Addition to culture[d]		DBA/2 c)	DBA/2 b)	CBA/J c)	CBA/J b)	C57BL/6J nu/nu c)	C57BL/6J nu/nu b)	CBA/N x DBA/2F1♀ c)	CBA/N x DBA/2F1♀ b)	CBA/N x DBA/2 F1♂ c)	CBA/N x DBA/2 F1♂ b)
none		2	5	2	5	3	5	2	5	2	5
Lipoprotein		25	20	35	20	30	25	27	25	31	25
LPS		18	15	20	30	22	20	24	25	5	25
BRMF		19	20	22	20	24	20	26	20	5	25
immobilized μ H-specific antibody		21	20	25	20	18	25	22	30	3	25
soluble Lyb2.1-specific antibody (μg/ml)	100	18	25	25	20	3	5	27	25	3	5
	10	14	20	20	20	3	5	21	20	2	5
	1	6	10	7	10	2	5	11	8	2	5
	0.1	2	5	2	3	3	5	4	5	2	5
immobilized Lyb2.1-specific antibody (μg/ml beads)	2500	2	5	2	3	3	5	2	5	2	5
	250	2	5	2	4	2	5	2	5	2	5
	25	2	5	2	3	3	5	2	5	2	5

a) + c) Uptake of ^{3}H-thymidine as described in Materials and Methods section; a) 10^{-3} x ^{3}Hcpm/$6x10^4$ cultured small cells c) 10^{-3} x ^{3}Hcpm/$1x10^4$ cultured blasts

b) 10^{-3} x cells/ml. In brackets are given the % of cells that appeared as blasts upon visual inspection (only for cultures with small cells).

d) For further details see the Materials and Methods.

lated resting B cells of all strains, including the B cell-deficient F1 ♀ cells, equally well, while the same F1 ♂ cells showed the expected reduced reactivity to LPS and to μ-H-chain-specific immobilized antibody. We, therefore, activated small cells of the different strains of mice with lipoprotein for 2 days to obtain equal numbers of activated replicating B blasts.

2) Activated B blasts: Activated B blasts, enriched by velocity sedimentation, need the stimulatory action of BRMF, mitogens or μ-H-chain-specific antibody to proceed through the next cell cycle and divide (Melchers et al., 1982), as can be seen from the data in Table 1. The B cell-deficient F1 ♂ blasts responded poorly to the stimulatory action of LPS, BRMF and μ H-chain specific antibody. Soluble Lyb2.1-specific monoclonal antibody, in concentrations above 10 μg/ml, now stimulated the activated B blasts as well as the polyclonally acting LPS, BRMF and μ H-chain specific antibody did, but only when the B blasts expressed to appropriate Lyb2.1 allele. This, as well as the deficient response of F1 ♂ blasts to the Lyb2-specific antibody, confirms earlier observations (Subbarao and Mosier, 1983). The immobilized Lyb2-specific antibody, on the other hand, did not stimulate even the appropriate B blasts. This, together with the earlier observations that the monovalent F_{ab}-fragments of the antibody are even better stimulators of activated B cells (Subbarao and Mosier, manuscript submitted), may indicate that the antibody has to be taken up by the cells to be stimulatory. This, again, seems to agree with the requirement for some growth factors that apparently have to be taken up by the stimulated cells.

Taken together, the results of the experiments in Table 1 show striking similarity of action of Lyb2-specific antibody and BRMF with B cells. They further strengthen our suspicion that Lyb2 is, in fact, a receptor for B cell replication factors.

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REFERENCES

Andersson J, Coutinho A and Melchers F (1979) Mitogen-activated B-cell blasts reactive to more than one mitogen. J Exp Med 149:553-564

Andersson J, Lafleur L and Melchers F (1974) IgM in bone marrow-derived lymphocytes. Synthesis, surface deposition turnover and carbohydrate composition in unstimulated mouse B cells. Eur J Immunol 4:170-180

Andersson J, Sjöberg O and Möller G (1972) Mitogens as probes for immunocyte activation and cellular cooperation. Transplant Rev 11:131-177

Braun V and Rehn K (1969) Chemical characterization, spatial distribution and function of lipoprotein (murine-lipoprotein) from E.coli cell walls. The specific effect of trypsin on the membrane structure. Eur J Biochem 10:426-438

Braun V and Sieglin V (1970) The covalent murein-lipoprotein structure of the Escherichia coli cell wall. The attachment of lipoprotein on the murein. Eur J Biochem 13:336-346

Huber B and Melchers F (1979) Frequencies of mitogen-reactive B cells in the mouse. Lipopolysaccharide-, lipoprotein- and Nocardia-mitogen-reactive B cells in $CBA/_N$ mice. Eur J Immunol 9:827-829

Iscove NN and Melchers F (1978) Complete replacement of serum by albumin, transferrin and soybean lipid in cultures of lipopolysaccharide-activated B lymphocytes. J Exp Med 147:923-933

Leptin M, Potash MJ, Grützmann R, Heusser Ch, Shulman M, Köhler G and Melchers F (1984) Monoclonal antibodies specific for murine IgM. I. Characterization of antigenic determinants on the four constant domains of the μ heavy chain. Eur J Immunol in press

Melchers F, Andersson J, Corbel C, Leptin M, Lernhardt W, Gerhard W and Farthen J (1982) Regulation of B lymphocyte replication and maturation. J Cell Biochem 19:315-332

Melchers F, Andersson J, Lernhardt W and Schreier MH (1980) H-2 unrestricted polyclonal maturation without replication of small B cells induced by antigen-activated T cell help factors. Eur J Immunol 10:679-685

Melchers F, Braun V and Galanos C (1975) The lipoprotein of the outer membrane of Escherichia coli: a B lymphocyte mitogen. J Exp Med 142:473-482

Miller RG and Phillips RA (1969) Separation of cells by velocity sedimentation. J Cell Physiol 73:191

Sato H and Boyse EA (1976) A new alloantigen expressed selectively on B cells: the Lyb-2 system. Immunogenetics 3:565-575

Subbarao B and Mosier DE (1983) Induction of B lymphocyte proliferation by monoclonal anti Lyb2 antibody. J Immunology 130:2033-2037

Tung J-S, Michaelson J, Sato H, Vitetta ES and Boyse EA (1977) Properties of the Lyb2 molecule. Immunogenetics 5:485-488

Yakura H, Shen F-W, Kaemmer M and Boyse EA (1981) Lyb2 system of mouse B cells. Evidence for a role in the generation of antibody-forming cells. J Exp Med 153:129-135

T Cell Factors Regulating B Cell Activation and Differentiation

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INTRODUCTION

Concepts involved in B cell neoplasia require a better comprehension of normal B cell activation and differentiation. Advances along these lines have been accelerated through the use of factors derived from T cell sources. T-B collaborations, previously attributed to cell to cell interaction, are mediated by these factors. T cells activated specifically or non-specifically in vitro secrete factors which induce proliferation of preactivated B cells (B cell growth factor -- BCGF) or differentiation (BCDF, TRF). Within these two broad categories there is evidence that subgroups of these factors exist, possibly acting on different B cell subpopulations (Swain et al. 1983; Okada et al. 1983; Isakson et al. 1982) making T-B interactions more complex than previously thought.

An inherent problem with many of the earlier studies has been the use of supernatants from activated heterogenous T cell populations or tumor lines activated with mitogens (Muragushi et al. 1981; Nakanishi et al. 1983; Howard et al. 1982). Frequently multiple factors as well as contaminating mitogen in the supernatant are present, making data interpretation more difficult and dissection of specific activities dependent on different assay systems. We have generated human T cell hybridomas in an effort to derive monoclonal T cell factors free of lectins. By using this technique we describe three factors which act on human B cells and induce maturation: B cell proliferation factor (BCPF) distinct from conventional BCGF, BCDF and an isotype specfic helper factor. In addition, within the BCDFs described we show evidence for considerable heterogeneity.

METHODS

Generation of T-T Hybridomas

Three methods were used to generate human T cell hybrids. The first two methods were as previously described (Mayer et al. 1982). Briefly, T cell lines were mutagenized with ethylmethanesulfonate and rendered HGPRT deficient. Lectin activated T cells were fused with these cell lines using PEG 1000mw (Sigma) and plated in HAT medium for 17-60 days. Growth positive wells were expanded and tested for activity in our B cell assay systems. Alternatively, HLA B27 positive activated T cells were fused with non-mutagenized T cell lines in an attempt to avoid the innate toxicity of aminopterin and thymidine (Foung et al. 1982). These fusions were cloned on soft agar and clones were selected for HLA B27 positivity. Such positive clones were expanded and supernat-

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ants tested as above. A third method, derived from the concept of the selectable surface marker, was devised for more rapid generation of testable hybridomas.[1] Fusion procedure with OKT4 negative non-mutagenized T cell lines was performed as above. OKT4 positive hybrids were selected after 2 days in culture by an indirect rosetting technique (Mayer et al. 1982). T/B cell separation was achieved by rosetting technique using neuraminidase treated sRBC and Ficoll-Hypaque density gradient centrifugation. All cells were maintained in RPMI 1640, 10% FCS, 1% penicillin, streptomycin and 2mM glutamine. In some experiments pokeweed mitogen (PWM - Gibco) was added at a 1/100 dilution. Cells from patients with chronic lymphocytic leukemia (CLL) were obtained from leukapheresis samples.

B Cell Growth and Proliferation Assay

A standard assay for BCGF using an affinity purified $F(ab)'_2$ goat α human μ antibody (10ng/ml - kindly provided by Dr. C. Y. Wang) was employed (Muraguchi et al. 1982; Howard et al. 1982). A "standard" BCGF preparation (partially purified by column chromatography) was kindly provided by Dr. C. Y. Wang. The assay for BCPF is as follows: supernatants were added at onset of culture to 1×10^5 non T cells with or without the addition of α human μ antibody (10ng/ml). ^3H thymidine ($2\mu Ci$) was added at 72 hours and cultures were incubated for an additional 18 hours prior to harvest. Results were recorded as mean cpm of triplicate cultures.

B Cell Differentiation Assay

PB non T cells were cultured in the presence or absence of T cell hybridoma supernatants (usually 10%) for 6 days. A reverse hemolytic plaque assay was performed for total Ig or specific isotypes using appropriate developing antisera (Gronowicz et al. 1976). Assay for differentiation of the B lymphoblastoid line, CESS, was performed as described (Muraguchi et al. 1982).

RESULTS

B Cell Proliferation Factor (BCPF)

We have previously described T cell hybridoma supernatants which induce B cell proliferation without differentation when added at the onset of culture to PB or tonsil non T cells (Mayer et al. 1982). Since freshly separated B cells may have been pre-activated in vivo we sought to determine the relationship of these factors to "standard" BCGF. As seen in Table 1, in a standard BCGF assay, addition of the BCGF preparation on Day 3 to non T cells preactivated with anti-μ antibody from Day 0, resulted in a greater than 50 fold increase in ^3H-thymidine incorporation on Day 6. In contrast, addition of test T cell hybridoma supernatant MOW1 to similarly preactivated cells, on Day 3, did not result in an increase in ^3H-thymidine uptake on Day 6. However as noted previously, MOW1 supernatant co-cultured with or without $\alpha\mu$ antibody increased ^3H-thymidine incorporation on Day 3 and this response dropped off to background levels by Day 6. There was only a minimal increase in proliferation on Day 3 when BCGF was added at the onset of culture and this was presumably due to circulating preactivated cells.

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1. Mayer L., Fu S.M., Kunkel, H.G. Rapid generation of human T cell hybridomas. Submitted for publication.

TABLE 1

ABILITY OF HYBRIDOMA SUPERNATANTS TO INDUCE B CELL PROLIFERATION

Relationship to BCGF

SUPERNATANT[a]	αμ[b]	DAY SUP. ADDED TO CULTURE	CPM[c]	
			DAY 3	DAY 6
0	-	-	193	190
0	+	-	186	408
BCGF[d]	-	3	-	575
"	+	3	-	10874
"	-	0	625	126
MOW1	-	0	4530	424
"	+	0	3868	401
"	+	3	-	378
MOW1 + BCGF	-	0 3	-	976

[a] T cell hybridoma MOW1 supernatant was added at 10% final concentration to 1 x 10^5 non T cells in triplicate 0.1 ml cultures

[b] $F(ab)'_2$ goat αhuman IgM (10 ng/ml) added at onset of culture

[c] Mean value (^{3}H-thymidine incorporation) of triplicate non T cell cultures

[d] Partially purified BCGF from PHA supernatant added at 50 μg/ml

From these data it appears that MOW1 (BCPF) is distinct from conventional BCGF and may act at an earlier stage of B cell activation, since there was no synergy noted with the addition of anti-μ antibody. In fact BCPF seems to induce proliferation of B cells much like αμ antibody and may be an in vivo counterpart of αμ. To test this possibility MOW1 was substituted for αμ on Day 0 and BCGF preparation was added on Day 3. As noted on the last line of Table 1, only a modest increase in ^{3}H-thymidine uptake was seen on Day 6. The lack of synergy in this case might indicate that the concentration of BCPF was too high or may reflect activation via a completely different pathway. Further evidence for the distinction between BCPF and BCGF is shown in Table 2.

TABLE 2

EFFECT OF BCPF OR BCGF ON LEUKEMIC B CELLS

FACTOR	MEAN CPM[a]	
	Day 3	Day 4
-	242	216
MOW1 (BCPF)	4251	6278
BCGF[b]	426	273

[a] Hybridoma supernatants were added at 10% at onset of culture to 1 x 10^5 CLL cells in triplicate cultures. Results are mean CPM at Day 3 or 4

[b] BCGF as described in Table 1, added at onset of culture

In this case BCPF or BCGF was added to monoclonal leukemic B cells at the onset of culture. ^{3}H-thymidine incorporation was measured on Days 3 and 4. The BCPF supernatant induced a marked increase in proliferation on both days (18 and 29 fold, respectively) whereas no change was seen with BCGF. Furthermore, experiments with "resting," 4F2 Θ, B cells obtained by Percoll gradient centrifugation demonstrated that BCPF induced proliferation in these cells while no effect was seen with BCGF (data not shown).

Thus it appears that BCPF does act on an earlier resting stage of B cell maturation, acting in a non-specific polyclonal fashion. Indeed it may be responsible for the polyclonal responses seen with specific antigen activation.

B Cell Differentiation Factors

Supernatants from several T-T hybridomas demonstrated the ability to induce PB or tonsil B cells to differentiation to Ig secreting cells (Tables 3, 4, and 5).

TABLE 3

PFC RESPONSE OF PB NON T CELLS TO HYBRIDOMA SUPTERNATANT MOP1L

CELLS	PFC/WELL[a]			
	MEDIUM CONTROL	MOP1L	EBV	+T+PWM
SH non T	490	9760	3620	22140
JB non T	470	3360	9280	6120
LP non T	120	2950	1120	4280
LV non T	245	6750	5280	4620

[a] 1×10^{6} non T cells were cultured with either medium alone, 10% MOP1L supernatant, EBV derived from marmoset line B958, or 1×10^{6} autologous T cells and PWM (1/100 dilution). A reverse hemolytic plaque assay for total Ig was performed on Day 6. Results are the means of duplicate plates.

In many cases the degree of differentiation was comparable to or greater than that seen with the more common differentiation stimuli, i.e., EBV infection or autologous T cells and PWM (Table 3). However what appeared striking was the heterogeneity in the degree of differentiation seen with supernatants from the various hybridomas. Table 4 demonstrates such findings.

TABLE 4

HETEROGENEITY OF BCDFS

CELLS		PFC/WELL[a]			
		MEDIUM CONTROL	MOP1L	RAC[b]	MTP[c]
#1	non T	115	4020	6820	3860
#2	non T	95	1430	2235	185
#3	non T	90	430	2430	160
#4	non T	180	380	610	1000

[a] As described in Table 3

[b] Supernatant from T cell clone with B cell differentiation activity

[c] Fusion between MOLT4, T cell line, and PWM stimulated tonsil T cells

In representative experiments PB non T cells from 4 normal volunteers gave different patterns of response to 3 different BCDF preparations. #1 differentiated similarly and significantly to all BCDF preparations. This pattern represents 80-90% of normal controls. However, #2 demonstrated response to only MOP1L and RAC (supernatant from an IL-2 dependent T cell clone demonstrating BCDF activity), but only a 2-fold increase in PFC with the addition of MTP supernatant to the culture. #3 has a dominant response to RAC supernatant whereas #4 responds primarily to MTP with poorer or no response to the other supernatants. These findings are not likely to be due to BCDF concentration differences in the supernatants since the conditions for each experiment are comparable. Furthermore these differences are more striking in cells from patients with acquired agammaglobulinemia and CLL where defects in B cell subpopulations may exist (data not shown). Patterns of response to the BCDF preparations appear to persist over time, i.e., MTP or RAC non-responders remain non-responders on retesting. In these cases differences in B cell subpopulations, even in normal controls may exist to account for the findings. Studies along these lines are currently in progress.

Further evidence for BCDF heterogeneity cames from co-stimulation experiments performed with either preactivating agents, Ag or available lymphokines. Hybridoma supernatant MOP1L demonstrated synergy with formalin fixed staphylococcus aureus organisms and antigen (PPD) whereas no such synergy was noted with supernatant RAC (data not shown). Of interest was a marked enhancement of B cell differentiation noted with co-stimulation of purified B cells with MOP1L or MTP supernatant and lectin-free IL-2 (10-20 fold increase - data not shown). There was no increase in proliferation in these cultures as measured by ^{3}H-thymidine incorporation. This appears to be indicative of the presence of IL-2 receptors on activated B cells as described by Waldmann et al. (personal communication). Exhaustive depletion of T cells did not abrogate this effect. Synergy with IL-2 was not demonstrated with either RAC or J2S1 supernatants.

In addition, our factors appear to be distinct from those described in the literature (Irigoyen et al. 1981; Muraguchi et al. 1982) in that there is no BCDF activity demonstrated when factors are co-cultured

with the CESS line nor is PWM a requisite for activity. Therefore it appears that there are numerous factors which comprise the category of BCDF. Each factor may act on a distinct subpopulation of B cells or stage of B cell differentiation. There is evidence for such a premise with BCGF (Swain et al. 1982; Okada et al. 1983) as well as with BCDF (Isakson et al. 1982).

Isotype Specific BCDF

As a more defined subgroup of BCDF we have recently described a factor that acts on B cells to induce IgA production and secretion (Table 5 and Mayer et al. 1982). As seen in Table 5, addition of factor J1.3 to tonsil or PB B cells results in a marked increase in IgA secretion approaching that seen with autologous T cells and PWM. There is no significant increase in other isotypes.

TABLE 5

INDUCTION OF IMMUNOGLOBULIN SECRETION BY HYBRID SUPERNATANTS

		Media Control			Supernatant+			Autologous T Cells		
	B Cells	G	A	M*	G	A	M	G	A	M
	Tonsil B_1	0	0	100	200	700	0	0	100	800
	Tonsil B_2	0	300	0	0	2400	0		-	
T Cell Hybridoma J1.3	Tonsil B_3	20	0	0	90	600	20		-	
	Tonsil B_4	20	0	0	10	260	0++		-	
									+ T + PWM	
	PB non-T	0	0	0	100	700	0	3420	880	1220
	PB MNC	0	0	0	0	500	0		-	
	Tonsil B_1	40	0	90	520	80	450	190	30	20
T Cell Hybridoma J2S1			GAM			GAM			GAM	
	Tonsil B_2		50			1170			-	
	Tonsil B_2		50			170+++			-	
									+T + PWM	
	PB non-T		100			700			1600	
Hybrids K1	Tonsil B		0			0			-	
K8	Tonsil B		0			0			-	
Parent line Jurkat 3	Tonsil B		0			0			-	

* PFC/10^6 cells at initiation of culture. Ig isotypes measured by reverse plaque assay at day 6.

\+ Supernatant at 1/3 dilution.

++ Supernatant at 1/100 dilution.

+++ Supernatant at 1/200 dilution.

Evidence that this factor acts directly on B cells was obtained from cultures of factor with monoclonal leukemic B cells bearing sIgA. Virtually complete differentiation was noted (Mayer et al. 1982).

To determine whether the supernatant was able to induce an Ig class switch or whether it acted on post switch IgA committed B cells, J1.3 supernatant was added to either unseparated, sIgA depleted, or negatively selected sIgM positive tonsil B cells. As can be seen in Table 6, depletion of sIgA bearing cells completely abrogated response to J1.3 supernatant. No IgA secretion was noted when supernatant was co-cultured with sIgM-positive cells. These combined findings suggest that a switch was not effected by the supernatant.

TABLE 6

DEPLETION OF sIgA BEARING B CELLS
ABROGATES IgA SPECIFIC HELPER FACTOR (J1.3) EFFECT

CELLS	PFC/WELL[a]								
	MEDIUM CONTROL			J1.3 SUP.			+T+PWM		
	G	A	M	G	A	M	G	A	M
TONSIL B	80	0	120	40	990	0	10120	2120	11680
sIgA DEPLETED[b] TONSIL B	110	0	150	80	40	110	9690	440	6280
sIgM ⊕[c] TONSIL B	20	0	80	60	0	210	5280	4180	7120

[a] As per Table 3 - reverse plaque assay performed with isotype specific developing antiserum (Cappel - 1/100 dilution)

[b] sIgA ⊕ B cells depleted by direct rosetting technique using $F(ab)'_2$ sheep α human IgA coated ox RBC (<0.4% sIgA ⊕ cells)

[c] sIgM ⊕ B cells negatively selected by directly rosetting out sIgG and sIgA bearing cells as above (<0.1% sIgA ⊕ <0.3% sIgG ⊕)

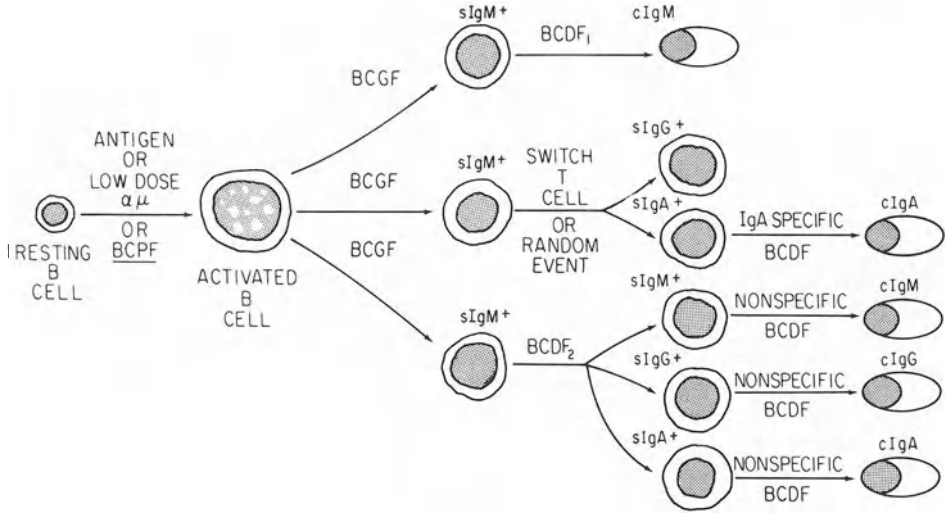
CONCLUSIONS

We have described three categories of T cell factors which act on B cells in distinct stages of differentiation. BCPF appears to exert its effect on "resting" B cells to induce them to proliferate, and does not synergize with anti-μ antibody. Thus this activity is distinct from conventional BCGF (Howard et al. 1982; Muraguchi et al. 1982). What is apparent, however, is that BCPF is distinct from the subgroups of BCGF described by Swain et al. (1983) and Okada et al. (1983), since BCPF is not active in any BCGF assay system. Since specific antigenic stimulation of T cells in vivo may give rise to BCPF production, this factor might account for polyclonal responses in these systems. A possible schema for BCPF activity is shown in Figure 1. Here BCPF bypasses the requirement for BCGF or, alternatively, may be the in vivo counterpart of anti-μ antibody which preactivates B cells to become sensitive to BCGF. The difference between these two possibilities may be concentration dependent.

We have also demonstrated heterogeneity of BCDFs. This has been suggested by ourselves and others (Isakson et al. 1982) but in a more isotype restricted fashion. However, even within the polyclonal BCDFs we note differences in response dependent upon the assay system, source of cells, or costimulus.

Thus it appears likely that distinct BCDFs might act on subpopulations of B cells. That these differences are noted even in the normal population might reflect the fact that the state of activation of circulating B cells varies from individual to individual. Alternatively certain subpopulations of B cells in one individual may not be required for normal B cell differentiation in others. Figure 1 is a schema depicting the described as well as some projected BCDFs. Each arrow may eventually be subdivided into a series of steps in differentiation. Studies isolating B cell subpopulations by monoclonal antibodies will certainly help to define this more precisely.

FIGURE 1



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REFERENCES

Foung SKH, Sasaki DT, Grumet FC and Engleman EG (1982) Production of functional human T-T hybridomas in selection medium lacking aminopterin and thymidine. Proc Natl Acad Sci USA 79:7484.

Gronowicz E, Coutinho A and Melchers F (1976) A plaque assay for all cells secreting Ig of a given type or class. Eur J Immunol 6:588.

Howard M, Garrar J, Hilfiker M, Johnson B et al. (1982) Identification of a T cell derived B cell growth factor distinct from interleukin 2. J Exp Med 155:194.

Irigoyen O, Rizzolo PV, Thomas Y and Chess L (1981) Generation of functional human T cell hybrids. J Exp Med 154:1827.

Isakson PC, Pure E, Vitetta ES, and Krammer PV (1982) T cell derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. J Exp Med 155:734.

Mayer L, Fu SM and Kunkel HG (1983) Differentiation of leukemic B cells by B cell differentiation factors produced by human T-T hybridomas. Fed Proc 42(4):834.

Mayer L, Fu SM, and Kunkel HG (1982) Human T cell hybridomas secreting factors for IgA specific help, polyclonal B cell activation and B cell proliferation. J Exp Med 156:1860.

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

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

Nakanishi K, Howard M, Muraguchi A, Farrar J et al. (1983) Soluble factors involved in B cell differentiation: Identification of two distinct T cell-replacing factors (TRF). J Immunol 128:1296.

Okada M, Sakagushi N, Yoshimura N, Hara H et al. (1983) B cell growth factors and cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J Exp Med 157:583.

Swain SL, Howard M, Kappler J, Marrach P et al. (1983) Evidence for two distinct classes of murine B cell growth factor with activities in different functional assays. J Exp Med 158:822.

Interleukin 3 Dependent Retrovirus Induced Lymphomas: Loss of the Ability to Terminally Differentiate in Response to Differentiation Factors

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INTRODUCTION

The mechanisms by which murine retroviruses induce lymphomas are largely unknown. A number of requirements have been defined which play an essential role. In Moloney leukemia virus (MoLV) induced leukemia, one potential component is an immune response (Lee and Ihle, 1981; Lee and Ihle, 1981). This has been speculated to be due to a requirement to generate an appropriate target cell population, which is accomplished by viral antigen-induced production of several lymphokines by antigen-specific helper T cells. These lymphokines subsequently induce the proliferation and differentiation of a variety of cell types. As a consequence of the acute viremia and the presence of antigen-specific helper T cells, the frequency of cells proliferating to T cell-derived lymphokines is 50- to 200-fold higher than in control mice. It is from this expanded population that the lymphomas ultimately arise (Pepersack et al., 1980).

One of the T cell-derived factors which has been speculated to play an integral role in leukemogenesis is interleukin 3 (IL 3). This lymphokine is a T cell-derived glycoprotein with an apparent molecular weight of 28,000 daltons which has been purified to homogeneity and the N-terminal sequence determined (Ihle et al., 1982; Ihle et al., 1983). IL 3 regulates the differentiation of an early stem cell. In particular, in the presence of IL 3 a stem cell is induced to proliferate after which it is obligated to continue differentiation. Among the progeny are a variety of cell types including prothymocytes, mast-like cells, granulocytes, macrophages, megakaryocytes and possibly others (Ihle, 1984; Ihle et al., 1984). Recently we have observed that a number of murine retrovirus-induced lymphomas express markers characteristic of the IL 3 lineage of differentiation. Moreover, whereas these lymphomas have been extremely difficult to establish in tissue culture under normal conditions, in the presence of IL 3, long-term cell lines were easily established. In order to study the mechanisms involved in the transformation of hematopoietic cells we have compared the properties of several lymphoma cell lines to the properties of normal cells responding to IL 3. The results demonstrate that IL 3-dependent lymphoma cell lines retain an absolute dependence on IL 3 for growth but have lost the ability to be induced to differentiate by the appropriate factors.

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RESULTS AND DISCUSSION

The wild mouse ecotropic virus (Cas-Br-M-MuLV) was isolated from Lake Casitas animals and has been demonstrated to induce a wide spectrum of hematopoietic tumors (Fredrickson et al., 1984). The distribution of tumors seen in our study using NFS mice is shown in Table 1. The predominant leukemias were thymic lymphomas, myelogeneous leukemias and lymphoblastic leukemias based on histological analysis. Less frequently erythroleukemias, mixed follicular center cell lymphomas and immunoblastic leukemias were observed. With each of the primary tumors we attempted to derive cell lines by culturing in either media alone or media supplemented with IL 3. In media alone long-term lines were rarely established and there was no apparent propensity for a particular tumor type to adapt to culture better than the others. In contrast, a number of the lymphomas showed either limited growth in media supplemented with IL 3 or were established as long-term cell lines in IL 3. There was a clear relationship between lymphoma type and the ability to be established as IL 3-dependent cell lines. Rarely did the thymic lymphomas or lymphoblastic leukemias give rise to long-term growth. In contrast the myelogeneous leukemias and erythroleukemias quite consistently grew in vitro in the presence of IL 3 and were readily established as long-term cell lines.

Table 1. Establishment of long-term lymphoma cell lines from Cas-Br MuLV-induced tumors

	No.	No growth	Limited growth[a] (3-5 months)	IL 3 dependent lines	Factor independent
Thymic lymphomas	14	7	5	1	1
Myelogenous leukemias	13	1	2	8	1
Mixed follicular center cell lymphomas	3	0	3	0	0
Erythroleukemias	6	1	0	4	1
Immunoblastic leukemias	4	0	0	3	1
Lymphoblastic leukemias	13	5	3	3	2

[a]Limited growth was observed only in cultures supplemented with IL 3.

Most aspects of hematopoietic stem cell differentiation have not been elucidated however recent studies have demonstrated the sequential roles of IL 3 and CSF-2 in myeloid differentiation. In particular IL 3 induces a stem cell to begin to proliferate and differentiate. The sequence is characterized by the induction of Thy 1 expression. The Thy 1^+ cells have the potential to differentiate into a variety of phenotypes including granulocytes and macrophages. A typical experiment is illustrated in Table 2. In this experiment bone marrow was initially depleted of Thy 1^+ cells and cultured in IL 3. After 7 days the induced Thy 1^+ cells were isolated by a fluorescence activated cell sorter (FACS) and cultured under the conditions described. In the absence of any factors the cells rapidly lost viability without any evidence

of differentiation. In the presence of IL 3 viability was maintained and by 7 days after sorting approximately 40% of the cells were morphologically defineable as granulocytes and macrophages. In addition, however, lymphoblastic cells as well as cells having the morphology of mast cells were present. Continued culture in IL 3 resulted in the loss of the granulocyte/macrophage component and the population became homogeneous for the mast-like cells. In contrast to the results obtained in IL 3, when the Thy 1^+ population was cultured in CSF-2 the populations were 100% granulocytes/macrophages at 7 days after sorting. These populations, as with the cultures in IL 3, did not persist but rather were lost by 14 days after sorting. These results demonstrate that the IL 3-induced Thy 1^+ cells have the potential to differentiate into granulocytes/macrophages and that this differentiation can occur in the presence of IL 3 or CSF-2. In either case differentiation occurs over a relatively short time in vitro and is terminal.

Table 2. The relationship of IL 3 and CSF-2 in the differentiation of granulocytes and macrophages

Culture conditions	Percentage granulocyte/macrophages Day 0	Day 7	Day 14
Media	10%	N.V.[a]	N.V.
IL 3	-	43%	<5%
CSF-2	-	100%	N.V.

[a]In these cultures no viable cells were detectable.

The above results provided a basis with which we could next compare the properties of the myelogenous cell lines. As noted above, all but one of the cell lines established from myelogenous leukemias required the continued presence of IL 3 for growth in vitro. We therefore initially examined the proliferative response of the cell lines to IL 3 or CSF-2. As shown in Table 3, a proliferative response to CSF-2 was detectable. The lymphomas examined included two of the Cas-Br-M-MuLV-induced tumors (NFS-58 and NFS-60). For comparison, a comparable leukemia obtained from a Moloney leukemia virus-induced tumor (DA-3), is also shown. Last, an IL 3-dependent cell line obtained by Harvey sarcoma virus (HaSV) transformation of fetal liver cells is shown. This cell line is phenotypically distinguishable from the myelogenous leukemia cell lines and has properties of the IL 3 induced mast-like cells. All four lines required IL 3 for continued growth and as indicated in Table 3, ^{3}H-thymidine incorporation. The myelogenous leukemias showed varying levels of ^{3}H-thymidine incorporation in the presence of CSF-2 relative to IL 3 whereas no proliferation was detectable with the HaSV transformed line. To eliminate the possibility that the CSF-2 contained contaminating IL 3 the ability of an immune Ig against IL 3 to inhibit each response, was examined. As shown, the immune Ig could completely inhibit the response to IL 3 but did not affect the response to CSF-2 of any of the cell lines. These results suggested that the cell lines from myelogenous leukemias uniquely responded to CSF-2 as well as IL 3.

The possibility existed that the lymphoma cell lines were not homogeneous and there existed two populations with different growth factor requirements. To examine this, cells from the DA-3 line were grown in either IL 3 or CSF-2 and their responses evaluated.

Table 3. Proliferation of myelogeneous leukemia cell lines to IL 3 and CSF-2

Culture Conditions	Percent of maximum ^{3}H-thymidine incorporation[a]			
	NFS-58	NFS-60	DA-3	HaSV-JK
	<1	<1	<1	<1
IL 3	100	100	100	100
CSF-2	44	57	28	<1
IL 3 + 20μg immune Ig	<1	<1	<1	N.D.
IL 3 + 20μg control Ig	100	100	100	N.D.
CSF-2 + 20μg immune Ig	44	53	25	N.D.
CSF-2 + 20μg control Ig	40	48	31	N.D.

[a]The cells were cultured under the indicated conditions for 24 hours and were pulsed with ^{3}H-thymidine for 6 hours. The results are presented as the percentage of the response seen with IL 3 for each cell line. IL 3 and CSF-2 were used at 20-40 units/ml.

As shown in Table 4, in the absence of either IL 3 or CSF-2 no viable cells were recovered after 4 days. In the presence of IL 3 there was a 40-fold increase in cell number and the calculated doubling time was approximately 17 hours. In the presence of CSF-2 there was only a 40% increase in cell numbers and the calculated doubling time was approximately 8 days. The recovered cells were subsequently assessed for their responses to IL 3 or CSF-2. As shown in Table 4, cells maintained in IL 3 proliferated in response to IL 3 and CSF-2 and the response to CSF-2 was approximately 10% of that seen with IL 3. More importantly cells which had been maintained in CSF-2 remained responsive to IL 3 comparable to that observed with cells maintained in IL 3. The response to CSF-2 was also retained and was increased relative to the cells from the IL 3 cultures. Nevertheless, the response to CSF-2 was only approximately 40% of that observed with IL 3. These results demonstrate that CSF-2 can maintain cells that require IL 3 for optimal growth in vitro. Moreover, the results suggest that IL 3 and CSF-2 provide qualitatively different signals to these cells. In spite of the clear difference in the responses, examination of stained cell preparations from the IL 3 or CSF-2 cultures indicated no morphological differences suggesting differentiation.

The above results provide evidence to support the hypothesis that myelogenous leukemias are blocked in their ability to differentiate to factors which normally promote terminal differentiation. This block in differentiation is apparently after the commitment for differentiation along the myeloid lineage. In particular, IL 3 induces differentiation of a relatively early stem cell which can differentiate to a variety of cell types. The earliest events detectable in the sequence are the induction of 20αSDH expression and Thy 1 expression. CSF-2 does not induce either of these markers but can support the differentiation of a 20αSDH$^+$ Thy 1$^+$ cell to granulocytes and macrophages. Not all IL 3 dependent cell lines can respond to CSF-2 demonstrating that the response to CSF-2 is characteristic of a subpopulation and therefore is a convenient marker of commitment. Under normal conditions either IL 3 or CSF-2 can support terminal differentiation once the cells

Table 4. Proliferative responses of DA-3 cells grown in either IL 3 or CSF-2

Culture conditions	Cell No.[a] Day 4	^{3}H-thymidine incorporation in response to[b] IL 3	CSF-2	Media
Media	$<10^2$	N.A.	N.A.	N.A.
IL 3	2×10^7	34,290	3,650	130
CSF-2	7×10^5	30,730	12,130	110

[a]DA-3 cells (5×10^5) were cultured in RPMI 1640 containing 10% FCS and 20-40 units/ml of IL 3 or CSF-2 for four days and the recovery of viable cells determined.

[b]The recovered cells were subsequently used to assess proliferation by culturing in microliter plates with either IL 3 or CSF-2 at 20-40 units/ml for 24 hours and pulsing the cells with ^{3}H-thymidine for 6 hours.

are committed. Neither of these factors induces differentiation of the leukemic cell lines. Experiments are currently in progress to determine whether the expression of known onc genes within the IL 3 lineage can induce this phenotype in vitro.

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REFERENCES

Fredrickson TN, Langdon WY, Hoffman PM, Hartley JW, Morse III HC. (1984) Histologic and cell-surface antigen studies of hematopoietic tumors induced by Cas-Br-M murine leukemia virus. J. Natl. Cancer Inst. 72:447-454.

Ihle JN, Keller J, Greenberger JS, Henderson L, Yetter RA, Morse III HC. (1982) Phenotypic characteristics of cell lines requiring interleukin 3 for growth. J. Immunol. 129:1377-1383.

Ihle JN, Keller J, Oroszlan S, Henderson L, Copeland T, Fitch F, Prystowsky MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B (1983) Biological properties of homogeneous interleukin 3: I. Demonstration of WEHI-3 growth factor activity, P-cell stimulating factor activity, colony stimulating factor activity and histamine producing cell stimulating factor activity. J. Immunol. 131:282-287.

Ihle JN (1984) Biochemical and biological properties of interleukin 3. Clinical Immunology Newsletter. In press.

Ihle JN (1984) Biochemical and biological properties of interleukin 3: A lymphokine mediating the differentiation of a lineage of cells which includes prothymocytes and mast-like cells. Contemporary Topics in Molecular Immunology. FP Inman (ed.) Plenum Press, New York. In press.

Lee JC, Ihle JN (1981) Chronic immune stimulation is required for Moloney leukemia virus-induced lymphomas. Nature 209:407-409.

Lee JC, Ihle JN (1981) Increased responses to lymphokines are diagnostic of preleukemia in Moloney virus inoculated mice. Proc. Natl. Acad. Sci. USA 78:7712-7716.

Pepersack L, Lee JC, McEwan R, Ihle JN (1980) Phenotypic heterogeneity of moloney leukemia virus-induced T-cell lymphomas. J. Immunol. 124:279-285.

Growth Regulation of a B-Cell Lymphoma via the Antigen Receptor

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WEHI-231, a murine cell line established from a B-cell lymphoma that arose in a BALB/c x NZB F1 hybrid mouse, was observed to stop growing in the presence of anti-immunoglobulin reagents (Ralph, 1979; Boyd and Schrader, 1981). This growth arrest is rapid and complete: virtually all of the cells stop growing in approximately 20 hours of culture with stoichiometric amounts of anti-Ig (DeFranco et al., 1982a). In contrast, most other B-cell lines grow fairly normally in the presence of anti-Ig. These results suggested that WEHI-231 would be an exceptional model system for studying genetic and biochemical aspects of antigen receptor-mediated regulation of B-cell growth.

The effects of anti-Ig on normal B cells are well-characterized. Anti-immunoglobulins, including anti-μ, anti-δ, and to a lesser extent anti-κ, stimulate all mature, resting, splenic B cells to leave the resting state, G_O, and to enter G_1 phase of the cell cycle (DeFranco et al., 1982b, 1984). This is believed to reflect the action of antigens that can crosslink membrane IgM or membrane IgD. Larger amounts of anti-μ or anti-δ, either in solution or on beads, can stimulate proliferation of a subset of these B cells (Parker, 1975; Sieckman et al., 1981; DeFranco et al., 1982b). Immature B cells, on the other hand, are very readily tolerized by contact with either anti-μ (Pike et al., 1982) or antigen (Nossal and Pike, 1975; Metcalf and Klinman, 1976), suggesting "clonal abortion" or "clonal anergy" as a B cell mechanism for tolerance to some types of antigens.

WEHI-231 may have resulted from the transformation of an immature B cell. This B-cell line lacks Fc receptors (Warner et al., 1979; DeFranco, unpublished results), as do immature B cells, whereas mature B cells have acquired these membrane receptors (Kearny et al., 1977). Furthermore, the inhibition of WEHI-231 cell growth by anti-μ could be viewed as similar to the inactivation of immature B cells by antigen or anti-μ. An alternative explanation for the growth inhibition of WEHI-231 comes from the observation that WEHI-231 has more mIgM molecules per cell than do many other B-cell lines (Koshland, 1984). Therefore, interaction of anti-μ with these cells may generate more of whatever intracellular signals are regulating the cell's physiology. In either case, WEHI-231 provides a good model system for studying the biochemical events triggered by the antigen receptor upon crosslinking because of its very clear-cut response.

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GENETIC BASIS FOR ANTIGEN RECEPTOR ACTION

One of the most attractive aspects of studying antigen regulation of WEHI-231 is the potential for genetic analysis. Since the cells stop growing in the presence of anti-μ, mutants can be readily selected because they continue to grow. These mutants are presumably altered in either their antigen receptors, in components required for signaling (e.g., ion channels, protein kinases, etc.), or in components of the growth-regulating machinery that receive these signals. Our laboratory has now isolated 34 independent mutants of this type. They have the characteristics of bona fide mutants: their frequency is greatly increased by mutagenesis with standard mutagens, and their phenotype is very stable in the absence of genetic selection. Approximately one-half of these mutants were obtained from cells mutagenized with ethyl methane sulfonate, a point mutagen. The others were obtained from cells mutagenized with ICR191, a frameshift mutagen, or with 1,2,7,8-diepoxyoctane, which creates short deletions We are currently analyzing the phenotypes of these mutants. For example, only 4 of the 34 have significantly less mIgM than do the parental WEHI-231 cells. The other 30 mutants have normal numbers of receptors and therefore must result from either mutations in μ that affect its ability to transmit a signal, or mutations in later components. These mutants will be especially valuable for dissecting the biochemistry of receptor signaling.

BIOCHEMICAL EVENTS STIMULATED BY mIgM CROSSLINKING

Very little is known about the biochemical events that mediate the effects of antigen binding to mIgM in B cells. Braun et al. (1979) observed that normal B cells treated with anti-Ig had increased uptake and increased efflux of calcium. These experiments used ^{45}Ca to follow movements of calcium. The results were consistent with the idea that anti-Ig caused an increase in the cytoplasmic concentration of free calcium. This was later demonstrated to be true by Pozzan et al. (1982) using a fluorescent dye, quin 2, that can be loaded into the cytoplasm of cells and serves as a dynamic measure of the free calcium concentration. We have reproduced those results in WEHI-231. These cells have a dramatic influx of extracellular calcium that raises the internal concentration of calcium about three-fold in less than one minute after the addition of anti-μ. Control antibodies (e.g., affinity-purified anti-ferritin) have no such effect.

Experiments are under way to determine whether this influx of calcium occurs via voltage-dependent calcium channels, as occurs in many nerve cells, or whether it occurs via voltage-independent calcium channels. In the latter case, one would expect the influx of calcium to be a graded response, related to the amount of receptor binding antigen. In the former case, an all-or-none response might be expected: if the membrane is depolarized sufficiently, all of the channels would open. Our preliminary results favor the voltage-independent mechanism. Artificial depolarization of the WEHI-231 cells by adding high concentrations of potassium ions outside the cell does not cause an influx of calcium. Furthermore, direct measurement of membrane potential with the fluorescent probe oxonol (Rink et al., 1980) fails to reveal a rapid depolarization of WEHI-231 upon the addition of anti-μ. We do not see such a depolarization, suggesting that the influx of calcium results from activation of voltage-independent calcium channels.

Monroe and Cambier (1983, 1984) have reported significant depolarization of normal B cells in response to anti-Ig or antigen (for purified antigen-specific B cells). This depolarization can be observed within 5 minutes of stimulation of the B cell. They have argued that this depolarization is required and sufficient to initiate early events in B-cell activation such as entry into G_1 phase and increased expression of Ia antigens. In our experiments, membrane-potential changes are not observed within the first 5-10 minutes after adding anti-Ig. More work needs to be done to resolve this apparent discrepancy.

One clear advantage of the WEHI-231 system is the opportunity to bring genetics to bear on the biochemical problems. We are currently measuring the calcium influx in each of the 34 mutants to see if any are altered in this event. One could argue, for example, that calcium influx is required for capping but not for growth regulation. If, however, any mutants selected for loss of the growth regulation result from loss of the calcium influx, this would be direct evidence for the role of calcium in this regulation. We are also examining several other biochemical reactions that may also be part of antigen receptor signaling and here again, we hope the availability of mutants will indicate the importance of these events.

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REFERENCES

Braun J, Sha'afi R, Unanue ER (1979) Crosslinking by ligands to surface immunoglobulin triggers mobilization of intracellular $^{45}Ca^{+2}$ in B-lymphocytes. J Cell Biol 82: 755-766.

Boyd AW, Schrader JS (1981) The regulation of growth and differentiation of a murine B cell lymphoma II. The inhibition of WEHI-231 by anti-immunoglobulin antibodies. J Immunol 126: 2466-2469.

DeFranco AL, Davis MM, Paul WE (1982a) WEHI-231 as a tumor model for tolerance induction in immature B lymphocytes. In: Vitteta E (ed) Band T cell tumors: Biological and clinical aspects. Academic Press, New York, pp 445-450.

DeFranco AL, Raveche ES, Asofsky R, Paul WE (1982b) Frequency of B lymphocytes responsive to anti-immunoglobulin. J Exp Med 155: 1523-1536.

DeFranco AL, Raveche ES, Paul WE (1984) Separate control of B lymphocyte enlargement and proliferation in response to anti-immunoglobulin. In preparation.

Kearny JF, Cooper MD, Klein J, Abney ER, Parkhouse RME, Lawton AR (1977) Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. J Exp Med 146: 297-301.

Koshland ME (1983) Presidential address: molecular aspects of B cell differentiation. J Immunol 131: i-ix.

Metcalf ES and Klinman NR (1976) In vitro tolerance induction of neonatal murine B cells. J Exp Med 143: 1327-1340.

Monroe JG, Cambier JC (1983) B cell activation II. Receptor cross-linking by thymus-independent and thymus-dependent antigens induces a rapid decrease in the plasma membrane potential of antigen-binding B lymphocytes. J Immunol 131: 2641-2644.

Monroe JG, Cambier JC (1984) B cell activation I. anti-immunoglobulin induced receptor crosslinking results in a decrease in the plasma membrane potential of murine B lymphocytes. J Exp Med, in press.

Nossal GJV, Pike BL (1975) Evidence for the clonal abortion theory of B-lymphocyte tolerance. J Exp Med 141: 904-917.

Parker DC (1975) Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin. Nature (London) 258: 361-363.

Pike BL, Boyd AW, Nossal GJV (1982) Clonal anergy: The universally anergic B lymphocyte. Proc Natl Acad Sci, USA 79: 2013-2017.

Possan T, Arslan P, Tsien RY, Rink TJ (1982) Anti-immunoglobulin, cytoplasmic free calcium, and capping in B lymphocytes. J Cell Biol 94: 335-340.

Ralph P (1979) Functional subsets of murine and human B lymphocyte cell lines. Immunol Rev 48: 107-121.

Rink TJ, Montecucco C, Hesketh TR, Tsien RY (1980) Lymphocyte membrane potential assessed with fluorescent probes. Biochim Biophys Acta 595: 15-30.

Sieckmann DG, Habbersett R, Sher I, Paul WE (1981) Activation of mouse lymphocytes by anti-immunoglobulin III. Analysis of responding B lymphocytes by flow cytometry and cell sorting. J Immunol 127: 205-211.

Warner NL, Daley MJ, Richey J, Spellman C (1979) Flow cytometry analysis of murine B cell lymphoma differentiation. Immunol Rev 48: 197-243.

Interleukin-2 Receptors on Activated Malignant and Normal B-Cells

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INTRODUCTION

A battery of antigen non-specific, genetically unrestricted factors derived from T cells have been shown to play a role in the regulation of B-cell responses (Howard and Paul 1983). One such factor designated B-cell growth factor (BCGF) appears to be required for the proliferation of a subset of B cells following their interaction with antigen or with anti-immunoglobulin molecules. A second set of factors termed B-cell differentiation or T-cell replacing factors (TRF) is involved in the terminal maturation of such proliferating B-cells into immunoglobulin secreting cells. There has been controversy concerning the possible involvement of T-cell growth factor or interleukin-2 (IL-2) in B-cell responses and concerning the ability of this growth factor to act directly on B lymphocytes. The proponents of a direct action of IL-2 on B-cells have shown that depletion of IL-2 from cofactor rich supernatants by absorption on IL-2 dependent T-cell lines also removes a factor required for B-cell differentiation (Parker 1982; Leibson et al. 1981). The view that IL-2 acts directly on B-cells has been challenged since the IL-2 containing supernatants generally used in the previous studies also contained BCGF and one or more TRF's (Howard and Paul 1983). Furthermore, IL-2 was not absorbed by resting B-cells, LPS stimulated splenic lymphoblasts or by either of the two Burkitt's lymphoma B-cell lines examined suggesting that B-cells do not manifest receptors for IL-2 (Robb et al. 1981).

We have re-explored the possibility that certain activated B-cells display receptors for Interleukin-2 using the anti-Tac monoclonal antibody produced in our laboratory (Uchiyama, Broder and Waldmann 1981) that identifies the receptor for Interleukin-2 (Leonard et al. 1982). The observations that led to the conclusion that anti-Tac identifies the IL-2 receptor include: 1) anti-Tac blocks the proliferation of IL-2 dependent T-cell lines but does not affect the proliferation of IL-2 independent T-cell, B-cell or monocyte lines; 2) anti-Tac blocks the binding of radiolabeled IL-2 to its specific receptor on activated T-cells; and 3) IL-2 blocks the binding of radiolabeled anti-Tac to its antigenic target on activated T-cells and T-cell lines. In the present study we demonstrate that certain leukemic B-cell populations and certain activated normal B-cells display the Tac antigen and manifest high affinity receptors for radiolabeled Interleukin-2.

ANTI-TAC INHIBITS HELPER T-CELL DEPENDENT IMMUNOGLOBULIN SYNTHESIS

The ability of anti-Tac to inhibit helper T-cell dependent immunogloblin synthesis by normal B-cells was evaluated using an *in vitro* biosynthesis

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technique described previously (Waldmann, et al. 1974). Extensively washed peripheral blood mononuclear cells from normal individuals were stimulated to synthesize and secrete immunoglobulin molecules by pokeweed mitogen, wheat germ agglutinin, Streptolysin-O and Nocardia water soluble mitogen. The immunoglobulins synthesized and secreted during the 12 day culture period were assayed by quantitating the immunoglobulins in the media at the end of the culture using sensitive double antibody radioimmunoassays for IgM, IgG and IgA. The addition of 20 micrograms of the purified anti-Tac monoclonal antibody to the two million cells cultured in this system inhibited the synthesis of immunoglobulin by 57%, 64%, 47% and 53% for cultures stimulated by pokeweed mitogen, wheat germ agglutinin, streptolysin-O and Nocardia water soluble mitogen respectively. A control monoclonal antibody (RPC5) of the same IgG 2a isotype as anti-Tac did not inhibit this polyclonal immunoglobulin synthetic response. The suppression of immunoglobulin synthesis by 20 micrograms of anti-Tac was reversed when 50 nanograms of purified IL-2 was added to the pokeweed mitogen stimulated cultures supporting the view that anti-Tac and IL-2 directly compete for the same receptor. The maturation of B-cells into immunoglobulin synthesizing cells with each of these polyclonal activators requires the participation of helper T-cells. Thus, the site of action of anti-Tac in this system cannot be defined with both an effect directly on T-cells to inhibit their secretion of B-cell growth and differentiation factors or a direct action at the B-cell level being possible.

INTERLEUKIN-2 RECEPTORS ON B-CELLS

In order for there to be a direct action of anti-Tac on B-cells, the B-cells must manifest the Tac antigen on their surface and thus must bear receptors for IL-2. To determine whether B-cells manifest IL-2 receptors B-cells from normal indivduals and from individuals with leukemias of B-cells were examined by indirect immunofluorescence using the anti-Tac antibody. The anti-Tac monoclonal antibody did not react with purified resting normal B-cells. Furthermore, four of five unselected B-cell lines from normal individuals established using the Epstein-Barr virus were Tac antigen negative. In contrast, the Tac antigen was demonstrable on the surface of Hairy Cell leukemic cells in 8 of 8 cases examined (Korsmeyer et al. 1983), on Burkitt's lymphoma lines of 5 of 10 cases examined, and in 5 of 6 cases of B-cell lines established from patients who had the adult T-cell leukemia which is associated with human T-cell leukemia/lymphoma virus. In each of the cases with positive reactivity in the indirect immunofluorescence assay with anti-Tac antibody the cells were nonreactive when an irrelevant mouse IgG2a myeloma protein was utilized in place of the anti-Tac monoclonal. Similarily, each of the leukemic cell populations and cell lines was nonreactive when the Leu 4 antibody that defines virtually all mature T-cells was utilized confirming that the lines examined were not contaminated by T lymphocytes.

Although as discussed above, the majority of unselected Epstein-Barr virus transformed B cell lines from normal individuals were Tac antigen negative we have shown that the Tac antigen can be induced on normal B-cells and have established cloned normal B-cell lines that bear the Tac antigen. Populations containing 90% purified B-cells from normal individuals and 10% autologous T-cells that had received 2,000R of λ irradiation were stimulated with pokeweed mitogen and cultured for 6 days. At the end of the culture period the T-cells were removed by a sheep red blood cell rosetting procedure. The residual population of B lymphocytes did not react with monoclonal antibodies directed toward T-cells but contained a proportion (21-43%) of Tac positive cells as identified by indirect immunofluorescence with the anti-Tac monoclonal antibody. Thus, normal B-cells could be induced to display the Tac antigen in the

pokeweed mitogen stimulation system. Continuous Tac positive B-cell lines were established from these Tac positive B-cells. Following the procedures discussed above, the stimulated B-cells were incubated with the mouse monoclonal anti-Tac antibody and the Tac positive B cells were isolated by a rosetting procedure with ox erythrocytes whose surfaces were coupled with rabbit anti-mouse immunoglobulin. Lines from these Tac positive B-cells were established by addition of Epstein-Barr virus. Five to 39% of the cells in the 10 Epstein-Barr virus transformed B-cell lines established by this method were Tac positive. Cloned lines were established by limiting dilution from one of these Tac positive lines. Two of the clones, 1C9 that constitutively produced the Tac antigen and 5B4 that could be induced to manifest the Tac antigen were examined more extensively. The addition of 50 nanograms of purified IL-2 as well as IL-2 that was the product of the expression of the IL-2 gene in E. coli blocked the binding of anti-Tac to its receptor on these cloned lines. The binding of ^{3}H-anti-Tac to the line 1C9 was examined and the data analyzed by Scatchard analysis. 1.84ng of anti-Tac were bound per million cells yielding an average of 7.5×10^{3} antibody molecules bound per cell with a K_d of 1×10^{-10} mol/L. In parallel studies using tritiated IL-2 1.7×10^{3} high affinity (10^{-12} mol/l) receptors per cell for IL-2 as well as additional receptors for IL-2 with a lower affinity were defined. These studies support the view that normal B-cells as well as cloned B-cell lines from normal individuals can express the Tac antigen and that such Tac positive B-cell lines display high affinity receptors for purified IL-2.

IL-2 Induction of IL-2 Receptors on B-Cells

The cloned normal B-cell line, 5B4, prepared as described above manifested only small numbers of Tac receptors with about 5% of the cells identifiable as Tac positive by fluorescence activated cell sorter analysis and with only 550 receptors per cell as determined by Scatchard analysis of ^{3}H-anti-Tac binding to this cloned cell line. The ability of this population to be induced to express larger numbers of high affinity Tac receptors was explored after an initial observation that exposure to lectin depleted IL-2 led to an increase in the proportion of Tac positive cells from 5% prior to induction, to over 30% following induction. Affinity purified IL-2 as well as recombinant IL-2 in the range from 1 to 100 nanograms per 10^{6} cells increased the number of anti-Tac molecules bound per million cells from 550 receptors per cell to 4.5×10^{3} receptors per cell following 48 hours of culture. The addition of cyclohexamide or actinomycin D abrogated the increase in IL-2 receptors following exposure to purified IL-2. However, λ irradiation of the cells with doses sufficient to inhibit thymidine uptake (2,000, 4,000 or 8,000R) did not inhibit the induction of IL-2 receptor expression following exposure to IL-2. Thus, there was up regulation of the number of IL-2 receptors by its ligand. The fact that both purified JURKAT IL-2 and recombinant DNA derived IL-2 could cause this induction supports the view that IL-2 itself rather than a potential contaminant was involved in this induction. The requirements for protein syntheis and RNA transcription suggest that induction of the receptor involves de novo synthesis of the receptor rather than unmasking of cryptic receptors. Although in many receptor ligand systems, the addition of the ligand leads to down regulation of the number of receptors, there is considerable precedence for up regulation of receptor numbers by certain ligands. For example, Fc receptors for IgE can be induced on activated T-cells by the addition of IgE (Yodoi and Ishizaka 1980). Similarly, Fc receptors for IgA were induced on T-cells by the addition of IgA to such cells in vitro (Hoover, Dieckgraefe and Lynch 1981). Furthermore, the number of insulin receptors on 3T3 cells and chondrosarcoma cell lines increased following the addition of insulin (King, Lechler and Kahn 1982; Stevens, Austen and Nissley 1983). In the present study we show that IL-2 in the media may lead to the augmentation in the number of IL-2 receptors on B lymphocytes and

thus may play a role in the maintenance of high numbers of cell surface IL-2 receptors required for the action of this lymphokine.

Structure of IL-2 Receptors on B-cells

On the basis of previous studies, the Tac antigen present on normal activated T-cells were shown to have a molecular weight of 53 to 57,000 daltons (Leonard et al. 1982). The size of the antigen identified by the anti-Tac monoclonal antibody on the cloned normal B cell line 1C9 and of the line 5B4 following induction were analyzed on cells that had been surface labeled with radioiodine using the lactoperoxidase technique as well as on cells that were labeled with ^{3}H-Glucosamine for the terminal four hours of culture. The radiolabeled cells were lysed, immunoprecipitated with anti-Tac and the size of the receptor precipitated, analyzed by SDS polyacrylamide electrophoresis. The size of the receptor identified by the anti-Tac monoclonal on these B-cell lines was identical (53-57,000 daltons) to the receptors on simultaneously studied PHA stimulated T-cell lymphoblasts, supporting the view that activated B-cells can bear a receptor that is comparable to the IL-2 receptor on activated T lymphocytes.

Functional Effects of IL-2 and Anti-Tac on the B-Cell Line 5B4

The addition of various concentrations of purified IL-2 or of anti-Tac had no effect on the thymidine uptake of the cells of the 5B4 line either prior to or following the induction of IL-2 receptors. However, the synthesis and secretion of IgM molecules into the media were augmented by a factor of 2 to 4 by exposure of the uninduced line to 50 nanograms of IL-2 per million cells in culture. The augmentation of IgM synthesis was completely abrogated if 20 micrograms of the anti-Tac monoclonal was added along with the purified IL-2. In contrast, the addition of the control monoclonal (RPC5) did not effect this IL-2 stimulated immunoglobulin synthesis. Thus IL-2 did not effect B-cell proliferation although it facilitated the terminal differentiation of cloned normal B cells into immunoglobulin synthesizing cells.

DISCUSSION

In previous reports no receptors for IL-2 could be demonstrated on resting B cells or on the activated B-cells examined. In the present report, we present the following data that support the conclusion that certain activated B cells bear a receptor for IL-2: 1) the monoclonal, anti-Tac that identifies the human receptor for IL-2 binds to certain leukemic B-cell populations and to certain normal B-cells activated by pokeweed mitogen. Furthermore, cloned EBV transformed lines derived from Tac positive activated normal B-cells continued to produce the Tac antigen in long term culture; 2) Purified IL-2 blocked the binding of the anti-Tac monoclonal to Tac positive B cells: 3) radiolabeled purified IL-2 bound to the cloned EBV transformed normal Tac positive B-cell line 1C9 with 1,700 high affinity IL-2 receptors and a higher number of low affinity IL-2 receptors demonstrable per cell and 4) The size of the receptor identified by anti-Tac on B-cells was similar to that on activated T-cells.

Although it appears likely that IL-2 receptors develop on certain B-cells during their activation, the functional relevance of the interaction of IL-2

with this receptor is not clear. It is evident for example, in studies of Tac negative EBV transformed B-cell lines that this interaction is not required for all immunogloublin synthesis nor is the addition of IL-2 alone to B-cells sufficient to lead to B-cell proliferation or differentiation. However, anti-Tac inhibited polyclonal immunoglobulin biosynthesis in response to helper T-cell dependent polyclonal activators such as pokeweed mitogen, Streptolysin-O, wheat germ agglutin, and Nocardia water soluble mitogen. Here the effect can either be on B-cells directly or on the production of B-cell growth and differentiation factors by T-cells. Howard and coworkers (1983) have indeed demonstrated the requirement for IL-2 for the synthesis of B cell growth factor by T-cells. However, IL-2 may also have a direct affect on B-cells. In studies not presented here, we have shown that the immunoglobulin biosynthetic response of purified B-cells to crude T-cell replacing factors (the supernatants of T-cells stimulated by pokeweed mitogen) was abrogated by the addition of anti-Tac to the culture system. Such cells however could theoretically be contaminated by small numbers of T-cells. To avoid the potential effects of anti-Tac on such contaminating T-cells we have studied the effect of the monoclonal antibody anti-Tac and of IL-2 on the immunoglobulin synthesis by a cloned normal B-cell line 5B4. The immunoglobulin synthesis by this line was increased by a factor of 2 to 4 by the addition of purified JURKAT and recombinant DNA derived IL-2. Furthermore, this augmentation of immunoglobulin synthesis was reduced to the baseline levels by the addition of anti-Tac to the IL-2 stimulated B-cells. The observation of this augmentation of immunoglobulin synthesis taken in conjunction with the observation that neither IL-2 nor anti-Tac affected DNA replication by B-cells suggests that IL-2 acts on the terminal differentiation of B-cells but not on their proliferation. Clearly further studies are required to address this issue. However, these observations taken in conjunction with the studies reported by others (Howard and Paul 1983) would support the following model of B-cell activation growth and differentiation; B-cells are activated initially by the binding of antigen to its specific receptor, cell surface immunoglobulin. B-cell activation then continues and the B-cells enter a proliferative cycle following the action of IL-1 and BCGF on receptors for these molecules that become expressed on the B-cell surface. The terminal maturation of these B-cells involves the interaction of a series of antigen non specific B-cell factors including T cell replacing factors and in certain cases, IL-2 with their specific receptors on activated proliferating B-cells. Thus, IL-2 would join other antigen non specific factors produced by T-cells as a potential regulator of the terminal maturation of B-cells into immunoglobulin synthesizing and secreting cells.

REFERENCES

Hoover RG, Dieckgraefe BK, and Lynch RG (1981) T cells with Fc receptors for IgA: induction of T cells in vivo and in vitro by purified IgA. J Immunol 127:1560-1563

Howard M, Matis L, Malek TR, Shevack E, Kell W, Cohen D, Nakanishi K, and Paul WE (1983) Interleukin 2 induces antigen-reactive T cell lines to secrete BCGF-1. J Exp Med 158:2024-2039

Howard M and Paul WE (1983) Regulation of B-cell growth and differentiation by soluble factors. Ann Rev Immunol 1:307-333

King GL, Rechler MM, and Kahn CR (1982) Interaction between the receptors for insulin and the insulin-like growth factors on adipocytes. J Biol Chem 257:10001-10006

Korsmeyer SJ, Greene WC, Cossman J, Hsu S, Jensen JP, Neckers LM, Marshall SL, Bakhshi A, Depper J, Leonard WJ, Jaffe ES, and Waldmann TA (1983) Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in Hairy Cell Leukemia (1983) Proc Natl Acad Sci USA 80:4522-4526

Leibson H, Marrack P, Kappler J (1981) B cell helper factors I. requirement for both IL-2 and another 40,000 Mr factor. J Exp Med 154:1681-1693

Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, and Greene WC (1982) A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor: partial characterization of the receptor. Nature 300:267-269

Parker D.C. (1982) Separable helper factors support B cell proliferation and maturation to Ig secretion. J Immunol 129:469-474

Robb R, Munck A, Smith K (1981) T cell growth factor receptors. Quantitation, specificity and biological relevance. J Exp Med 154:1455-1474

Stevens RL, Austen KF, and Nissley SP (1983) Insulin-induced increase in insulin binding to cultured chondrosarcoma chondrocytes. J Biol Chem 258:2940-2944

Uchiyama T, Broder S, and Waldmann TA (1981) A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. J Immunol 126:1398-1403

Waldmann TA, Broder S, Blaese RM, Durm M, Blackman M, and Strober W (1974) Role of suppressor T cells in the pathogenesis of common variable hypogammaglobulinemia. Lancet II 609-613

Yodoi J, and Ishizaka K (1980) induction of Fc-receptor bearing cells in vivo in human peripheral lymphocytes. J Immunol 124:934-938

Immuno Affinity Purified Interleukin 2 (IL2) Induces B Cell Growth

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INTRODUCTION

With the recent availability of immuno-affinity purified IL2 (Robb 1983) we attempted to investigate whether B cell growth can be directly influenced by this lymphokine. In view of our recent findings that large "activated" B cells are more receptive than small "resting" B cells to the growth stimulating effects of EL4 derived B cell growth factor (Mond, 1984) we undertook to study the effects of IL2 on size separated populations of B cells. B cells were prepared by a modification of the method of Leibson (1981) and then separated by counterflow Centrifugation into five size dependent fractions (Thompson, 1983). The average size of the separated B cells in the smallest sized fraction (fraction 1) was 115 μ^3 and each successive fraction had a progressively larger mean cell volume with the largest sized (fractions) being 168 μ^3. Less than 2% Thy 1.2^+ cells were present in any of the fractions. The immuno-affinity purified IL2 used in these experiments was prepared by passing supernatant coupled with a murine monoclonal antibody reactive with human IL2. Bound IL2 was recovered by elution of the column with 1.5% acetic acid (Robb, et al 1983).

RESULTS AND DISCUSSION

IL2 Induces B Cell Proliferation in Large But Not Small B Cells

B cells were cultured at a density of 2×10^5 per microwell together with concentrations of IL2 varying from 1 to 100 units per ml of culture media. 1.0 μCi of ^{3}H-Tdr was added at 48 hours and cells were collected using a MASH harvested 18 hours later. All concentrations of IL2 tested stimulated proliferation in large but not small B cells which could be detected as early as two days after the onset of culture. To minimize the possibility that T cells were contributing in any way to the IL2 stimulated proliferative response, B cells were cultured at a lower cell density of 3×10^4/microwell (Table 1). IL2 stimulated significant thymidine incorporation by the large B cells as stimulation of these B cells with Concanavalin A never induced proliferative responses above that seen in control cultures. Although it is not possible to unequivocally exclude the possible involvement of T cells in this response we think this is unlikely for a number of reasons: 1) even if one were to assume a 2% contamination of T cells in the B cell population which is an overestimate of the number of T cells actually present in the B cell population, the calculated number of contaminating T cells in culture would be 600. Addition of this number of T cells to cultures of small B cells showed no significant proliferation in response to IL2 suggesting that the proliferation seen in response to IL2 does not reflect direct T cell stimulation; 2) antigen specific T cell clones in numbers under 10^3 do not secrete enough B cell growth factors in response to IL2 to stimulate B cell proliferation. Thus it is most probable that the proliferation induced in B cells even when such cells are cultured with anti-Ig antibodies cultured with IL2 is a result of direct B cell stimulation rather than an indirect effect via the stimulation of T cells to secrete B cell growth factors.

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The ability of IL2 to stimulate B cell proliferation extends the observations of Malek, et al (1983) who demonstrated the presence of receptors for IL2 on LPS stimulated B cells. Recently we have extended these findings (Finkelman, et al) by demonstrating that B cells cultured in vitro with anti-Ig induces the expression of IL2 receptors on B cells. Taken together the above data indicates that B cells cultured in vitro in media containing fetal bovine serum can be induced to express IL2 receptors and to show enhanced growth in the presence of homogenous preparations of IL2. From other preliminary data it appears that the B cell growth promoting effects of IL2 differs from that of EL4 derived BCGF in that the latter does not maintain B cells in a proliferative cycle over as long a period as IL2. It is interesting to speculate that when T cells are in close proximity to B cells as occurs when antigen specific T cells are focused via antigen onto B cells, the IL2 secreted by such activated T cells plays an important role in maintaining B cell clonal expansion.

Table 1. IL2 induced proliferation in size separated B cells

Source of B cell	Cell number ($x10^4$)	Medium	IL2 (100/ml)	ConA
		CPM		
Experiment 1				
fraction #1 ("small")	20	13,452±2,214	14,994±1,276	8,389±175
fraction #2 ("large")	20	23,603±1,686	71,653±2,227	23,133±1,178
Experiment 2				
fraction #5 ("large")	3	925±83	7,588±169	7,588±169

1. B cells were size separated using counterflow centrifugation and cultured in modified Mishell Dutton medium with 10 units/ml of immuno-affinity purified IL2. ^{3}H-Tdr was added at 48 hours and cultures were harvested 18 hours later. Results represent arithmetic mean ± s.e. of triplicate wells.

REFERENCES

Leibson H, Marrack P, Kappler J (1981) B cell helper factors. II. Synergy among three helper factors in the response of T cell and macrophage depleted B cells. J Immunol 139:1398

Malek R, Robb RJ, Schevach EM (1983) Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. Proc Natl Acad Sci USA 80:5694-5698

Mond JJ, Thompson CB, Schaefer ME, Farrar J, Finkelman FD (1984) Responsiveness of size separated Lyb5$^-$ B cells from immune defective (CBA/N x DBA/2) mice to a chromatographically purified B cell growth factor. J Immunol (Submitted for publication)

Robb RJ, Kutny RM, Chowdry, V (1983) Purification and partial sequence analysis of human T-cell growth factor. Proc Natl Acad Sci USA 80:5990-5994

Thompson CB, Ryan JJ, Sieckmann G, Finkelman FD, Mond JJ, Scher I (1983) A method for size separation of murine spleen cells using counterflow centrifugation. J Immunol Methods 63:299-307

Factors Affecting Normal and Malignant B Lymphocyte Precursors

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INTRODUCTION

There is a large scale daily production of B lymphocytes within bone marrow of mammals, however until recently, little has been known about cellular interactions or humoral factors which normally regulate this process. Monoclonal antibodies to B lineage antigens of mice and humans now permit the detection and manipulation of B cell precursors which lack surface immunoglobulin. We have been using these with several culture techniques to identify factors which may be important in this process and have been particularly aided in our studies by materials from genetically defective animals and patients. The properties and possible target cells of two newly discovered cytokines will be summarized in this communication. An established tumor cell line is responsive to at least some of the factors which influence normal B cell precursors and it seems likely that a better understanding of such mechanisms will be instructive with respect to malignant cell proliferation and differentiation.

FORMATION OF MURINE B CELLS IN CULTURE

There have been many studies where spontaneous or induced maturation steps were observed in cultures of murine B lymphocyte precursors (reviewed in Kincade, 1981). Our own experience indicated that their functional maturation was facilitated by cellular interaction in high density cultures and that two types of regulatory cells might be involved (Kincade, et. al. 1981). More recently, we found that sIg^- precursors could become $sIgM^+$ and mitogen responsive even when cultured in semisolid cultures, provided that appropriate humoral factors and/or adherent regulatory cells were present (Jyonouchi and Kincade, 1983; Jyonouchi, et. al. 1983). The well-defined monokine, interleukin 1 (IL-1) was also effective in this respect (Giri, et. al. 1984).

New Zealand Black strain mice provide a unique experimental model for genetically determined dysregulation of B lymphocyte formation. We recently found that B lymphocyte lineage precursors are demonstrable within their hemopoietic tissues during embryonic life 2-3 days earlier than normal and in substantially elevated numbers (Jyonouchi and Kincade, 1984). This hyperactivity continues into the first few weeks of life and bone marrow from 4-week-old NZB mice contains abnormally high numbers of pre-B cells and precursors which can quickly mature in culture (Jyonouchi, et. al. 1983). At this time, but not later when the mice become deficient, a humoral factor(s) was demonstrable in their serum which enhances the functional maturation of normal B cell precursors. We interpreted these findings to mean that this interesting strain of mice has defects in microenvironmental elements which are important for the final steps in B lymphocyte formation.

A partial biochemical characterization has been done of the NZB serum factor(s). Activity elutes over a broad size range on initial gel filtration chromatography and we pooled Sephadex G-100 fractions corresponding to 1,800 - 48,000 MW for further

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studies. A single peak was eluted from Phenyl-Sepharose when the initial column equilibration was done in 0.8M $(NH_4)_2SO_4$, suggesting that the material is relatively hydrophobic. Three active peaks were consistently observed when preparative isoelectric focusing was performed (approximate pIs = 3.2, 7.9 and 8.4) and the two alkaline ones merged when the sample was treated with neuraminidase before focusing. All active fractions were found to be heat labile, pH 2.0 resistant, trypsin sensitive, and lentil lectin adherent. The biochemical and biological properties seem sufficient to distinguish this activity from IL-1, IL-2 (T cell growth factor), B cell growth factor, and granulocyte-macrophage colony stimulating activity. Antiviral titers of our fractionated material are insufficient to explain this in terms of interferons (Jyonouchi, et. al. 1983; Jyonouchi and Kincade, 1984; and unpublished observations).

It remains unclear if the NZB serum derived factor is a single cytokine whose heterogeneity on isoelectric focusing is due to postsynthetic modification or if we have isolated two distinct factors which have similar functions. Addition of combinations of any two of the three isoelectric focusing fractions at optimal doses does not indicate that they act synergistically. Results obtained thus far with preparative and analytical polyacrylamide gel electrophoresis indicate that it may be possible to attribute all of the biological activity to a single band (approximate MW = 18,000) which is stable under reducing conditions (H. Jyonouchi, unpublished observations).

The inducing effect of the NZB serum factor(s) was demonstrable when the cultured cell suspension was depleted of Thy-1 antigen bearing cells or Sephadex G-10 adherent cells as well as sIg^+ B cells. However, this was not true when the suspensions were depleted with our monoclonal 14.8 antibody. This reagent recognizes all B cells, all $c\mu^+$ pre-B cells, and an additional population of lymphocytes which may already be restricted to B lineage differentiation (Kincade, et. al. 1981 ; Landreth, et. al. 1983). Numbers of phenotypically identified B cells emerging in cultures of B cell depleted marrow were increased in the presence of factor but neither pre-B nor B cells were induced when the material was added to 14.8 depleted cell suspensions (H. Jyonouchi, unpublished observations). These findings suggest that the target of the NZB serum factor(s) may be committed B cell precursors which respond by becoming phenotypically and functionally mature.

Studies with an established pre-B leukemia cell line are relevant to the question of target cell specificity as well as to the theme of this meeting. Since its original description by Paige, et. al. in 1978, the 70Z/3 cell line has been thoroughly studied. Both the genes for immunoglobulin light and heavy chains are configured for transcription but only μ chains are constitutively produced (Maki, et. al. 1980). On exposure to LPS at the G1 - S boundary of the cell cycle, nuclease sensitive sites are exposed in the light chain gene and a variety of messengers are produced (Sakaguchi, et. al. 1980; Parslow and Granner, 1982; Perry and Kelly, 1979). This is followed by display of assembled IgM on the cell surface. Of special interest was the finding that κ synthesis and display was inducible with a T lymphocyte derived factor (Paige, et. al. 1982; Sidman, et. al. 1984). In addition the cell line was responsive to IL-1 and an interferon-like material (Giri, et. al. 1984). The latter result has now been confirmed with recombinant immune interferon donated by Genentech. All fractions of young NZB serum that induced normal B cell precursors also caused expression of sIg on 70Z/3 cells (Jyonouchi and Kincade, unpublished observations).

FORMATION OF HUMAN B LINEAGE CELLS IN CULTURE

Regulatory events in human B lymphopoiesis have not been studied, primarily because no *in vitro* assays for early B lineage cells has been developed. Repeated attempts in

this and other laboratories to develop such an assay with normal bone marrow cells have met with failure (Pearl, et. al. 1978; Pearl, 1983 and K.S. Landreth, unpublished observations). Two reports suggested that cytoplasmic μ chains of IgM (cμ) could be induced in a proportion of non-T, non-B acute lymphoblastic leukemia cells which lacked detectable cμ when incubated with the phorbol diester 12-*O* tetradecanolyphorbol 13-acetate (Nadler, et. al. 1982, Cosman, et.al. 1982) and the possibility that some of these cells proceed to sIg expression (Cossman, et. al. 1982). This observation has not been extended to normal cells. One of our goals has been to establish *in vitro* conditions necessary for assay of normal human pre-B cells to allow extension of our observations on murine cells to normal and abnormal human B lymphocyte development.

Human primary immunodeficiency disease, hemopoietic failure and leukemia are often accompanied by defects in the production of B lymphocytes, but the involvement of regulatory events in these disease states is unknown. In a recent study from this laboratory a 16-month-old male patient with documented cyclic neutropenia was found to have cyclic excessive production of pre-B cells in the bone marrow (Engelhard, et. al. 1983). This prompted us to look for soluble factors in his urine during periods of excessive lymphoproliferation which may promote pre-B cell development. Patient and age/sex matched control urines were collected, dialyzed extensively against phosphate buffered saline and concentrated. These urine preparations were added to cultures of normal human bone marrow cells which were first depleted of sIg^+ cells and changes in pre-B and B cell populations followed by immunofluorescence. Addition of this patient's urine collected during the week preceding, but not during, neutropenia resulted in the generation of new pre-B cells within the first 24 hours of human marrow culture (Landreth, et. al. 1984). This effect was not achieved in control cultures or cultures supplemented with any tested concentration of normal urine. Absolute numbers of B cells increased over the first 72 hours in cultures containing the patient's urine, but not in other cultures.

Active urine fractions were tested on murine bone marrow cells and were found to be equally, if not more, effective in promoting pre-B cell generation and/or differentiation *in vitro* (Landreth, et. al. 1984). These observations were extended by depleting murine marrow of pre-B cells as well as B cells with monoclonal 14.8 antibody. Cell suspensions prepared in this way generated pre-B cells *in vitro* only in the presence of active patient urine fractions and the greatest numbers of newly generated pre-B cells were observed 48 hours after the initiation of cultures.

Isoelectric focusing of these urine samples resulted in a single peak of activity with a pI of 5.9 to 6.4 and initial characterization suggests that the molecular weight of the active component(s) is between 10,000 and 30,000. This activity could not be replaced with IL-1 or immune interferon and as such appears to be distinct from other regulatory molecules which have been shown to affect the expression of sIg on normal bone marrow precursors. Sources of colony stimulating factors, IL-2 or IL-3 also appear not to replace this urinary factor activity.

CONCLUDING REMARKS

The observations summarized here indicate that the final steps in the process of normal B lymphocyte formation are subject to multiple kinds of regulation. Sensitivity to interleukin 1 and immune interferon provide a possible explanation for reports of nonspecific environmental antigen effects on bone marrow lymphocytes (Fulop and Osmond, 1983). In addition, we have defined two types of stimuli which may be restricted in their effects on B lineage cells. The human derived factor(s) acts at

a relatively early stage in being able to induce pre-B cell formation in cultures of pre-B cell depleted marrow. Thus far, this material is unique in this respect and in being able to induce differentiation of human B lineage cells in culture. The factor(s) isolated from young NZB serum promotes the phenotypic and functional maturation of murine B cell precursors in culture, but only when 14.8 marked cells are present. There are presumably also inhibitory networks which will be found that can limit the production of B cells within bone marrow.

As yet we have no clear distinctions between effects of these mediators on proliferation and differentiation events, we do not know if cells are responsive at particular stages of the proliferative cycle, if proliferation is obligatory in the process, and if different factors can commonly interact with the same receptors. Use of cloned tumor cell lines like 70Z/3 which are responsive to such factors will be very helpful in defining their modes of action. It is also possible that a survey of other malignant cells will reveal important defects in regulation by soluble mediators.

The human derived factor has made it possible for the first time to observe formation of human pre-B and B cells in culture. This should provide important new approaches to dissection of human immunodeficiency diseases, immunoregulatory dysfunctions, and malignancies.

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REFERENCES

Cossman J, Neckers LM, Arnold A, Korsmeyer SJ (1982) Induction of differentiation in a case of common acute lymphoblastic leukemia. New Engl J Med 307:1251-1254

Engelhard D, Landreth KS, Kapoor N, Kincade PW, DeBault LE, Theodore A, Good RA (1983) Cycling of peripheral blood and marrow lymphocytes in cyclic neutropenia. Proc Natl Acad Sci USA 80:5734-5738

Fulop GM, Osmond DG (1983) Regulation of bone marrow lymphocyte production. IV. Cells mediating the stimulation of marrow lymphocyte production by sheep red blood cells: studies in anti-IgM-suppressed mice, athymic mice, and silica treated mice. Cellular Immunology 75:91-102

Giri JG, Kincade PW, Mizel SB (1984) Interleukin 1-mediated induction of κ light chain synthesis and surface immunoglobulin expression on pre-B cells. J Immunol 132: 223-228

Jyonouchi H, Kincade PW (1983) Changes in B lineage cell populations in liver and spleen of normal neonatal mice. J Immunol 130:1616-1621

Jyonouchi H, Kincade PW, Good RA, Gershwin ME (1983) B lymphocyte lineage cells in newborn and very young NZB mice: Evidence for regulatory disorders affecting B cell formation. J Immunol 131:2219-2225

Jyonouchi H, Kincade PW (1984) Precocious and enhanced functional maturation of B lineage cells in New Zealand Black mice during embryonic development. J Exp Med 159: in press

Kincade PW (1981) Formation of B lymphocytes in fetal and adult life. Adv Immunol 31:177-245

Kincade PW, Lee G, Paige CJ, Scheid MP (1981) Cellular interactions affecting the maturation of murine B lymphocyte precursors in vitro. J Immunol 127:255-260
Kincade PW, Lee G, Watanabe T, Sun L, Scheid MP (1981a) Antigens displayed on murine B-lymphocyte precursors. J Immunol 127:2262-2268
Landreth KS, Kincade PW, Lee G, Medlock ES (1983) Phenotypic and functional characterization of murine B lymphocyte precursors isolated from fetal and adult tissues. J Immunol 131:572-580
Landreth KS, Engelhard D, Beare MH, Kincade PW, Good RA (1984) Identification of a human pre-B cell differentiation activity associated with cyclic neutropenia. Fed Proc in press
Maki R, Kearney J, Paige CJ, Tonegawa S (1980) Immunoglobulin gene rearrangement in immature B cells. Science 209:1366-1369
Nadler LM, Ritz J, Bates MP, Park EK, Anderson KC, Sallan SE, Schlossman SF (1982) Induction of human B cell antigens in non T cell acute lymphoblastic leukemia. J Clin Invest 70:433-442
Paige CJ, Kincade PW, Ralph P (1978) Murine B cell leukemia line with inducible surface immunoglobulin expression. J Immunol 121:641-647
Paige CJ, Schreier MH, Sidman CL (1982) Mediators from cloned T helper cell lines affect immunoglobulin expression by B cells. Proc Natl Acad Sci USA 79:4756-4760
Parslow TG, Granner DK (1982) Activation of a rearranged immunoglobulin kappa gene: Evidence for a control region inside the gene. Immunobiology 163:182-183
Pearl ER, Vogler LB, Okos A, Crist WM, Lawton AR, Cooper MD (1978) B lymphocyte precursors in human bone marrow: An analysis of normal individuals and patients with immunodeficiency states. J Immunol 120:1169
Pearl ER (1983) Pre-B cells in normal human bone marrow and in bone marrow from patients with leukemia in remission: Persistent quantitative differences and possible expression of cell surface IgM in vitro. Blood 61:464
Perry PP, Kelly DE (1979) Immunoglobulin messenger RNAs in murine cell lines that have characteristics of immature B lymphocytes. Cell 18:1333
Sakaguchi N, Kishimoto T, Kikutani H, Watanabe T, Yoshida N, Shimizu A, Yamawaki-Kataoka Y, Honjo T, Yamamura Y. (1980) Induction and regulation of immunoglobulin expression in a murine pre-B cell line, 70Z/3. I. Cell cycle-associated induction of sIgM expression and k-chain synthesis in 70Z/3 cells by LPS stimulation. J Immunol 125:2654-2659
Sidman CL, Paige CJ, Schreier MH (1984) B cell maturation factor (BMF): A lymphokine or family of lymphokines promoting the maturation of B lymphocytes. J Immunol 132:209-222

Immunoglobulin Heavy Chain Switching in Cultured I.29 Murine B Lymphoma Cells: Commitment to an IgA or IgE Switch

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A clone of B cells bearing IgM can be induced to switch to the expression of other isotypes, i.e. to other C_H genes, while maintaining expression of the identical heavy chain variable region (V_H) gene. Studies of the structure of Ig genes in myelomas (Davis et al., 1980), in hybridomas (Hurwitz et al., 1980), and in a B cell lymphoma (Stavnezer et al., 1982) which have undergone isotype switching, and also in normal splenic B cells treated with lipopolysaccharide (LPS) (Hurwitz and Cebra, 1982) indicate that DNA recombinations between tandemly repeated switch (S) sequences located 5' to each C_H gene except δ result in the deletion of the μ gene and the other C_H genes located 5' to the C_H gene to be expressed, and effect the isotype switch. The production of δ is an exception, as cloned cell lines which produce μ and δ simultaneously contain δ genes in the same context as in μ - producing cells and must produce μ and δ by alternative RNA processing. Other investigators have questioned whether alternative RNA processing may be used to express other H chains simultaneously with μ (Yaoita et al., 1982) in order to explain the surprisingly large number of cells in the spleen which express two isotypes simultaneously (~5% of spleen cells). But because H chain switching can occur rapidly and frequently, partly because the target for switch recombination is large, it is entirely possible that the double-producing cells are cells which have recently deleted their μ genes but still have μ mRNA and protein.

Numerous investigators have been attempting to understand what induces H chain switching, and how it is determined which isotypes will be expressed. The results appear contradictory among different systems. For example, in experiments by Kiyono et al. (1982) and Kawanishi et al. (1983) antigen or LPS was found to induce switching by purified normal B cells, but only in the presence of T cells, whereas in experiments using the spleen focus assay in nude mice by Mongini et al. (1982, 1983), normal splenic B cells stimulated with antigen switched in the absence of T cells, although T cells increased the frequency of switching to most isotypes.

As one B cell clone can generate multiple isotypes, it is generally believed that splenic B cells are not committed to one isotype (Gearhart et al., 1975; Teale, 1982) but whether they can become committed at a certain stage (for example, by an interaction with a T cell) to switch to particular isotypes is unknown. The C_H genes which are switched to more frequently in the absence of T cells, i.e. γ_3, ε, and α (Mongini et al., 1982, 1983) have S regions which are more homologous to Sμ than do the other C_H genes (Nikaido et al., 1982). However, the sequence of Sε is more homologous to Sμ than is the Sα sequence, whereas switches to α are more common than switches to ε. The basis for the choice of isotype in the absence or in the presence of T cells is not understood.

To understand the mechanism of isotype switching it would be useful to have a cloned cell line which can be induced to switch. The murine B cell lymphoma, I.29, is such a system. The I.29 lymphoma arose spontaneously as an ascites in

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the I/St strain of mice in 1961. It consists of cells expressing either IgM or IgA with the identical idiotype (Sitia et al., 1981), and with identical V_H genes, as determined by nucleotide sequencing (Klein and Stavnezer, unpublished).

By genomic DNA blotting experiments, by karyotype analyses and by examination of cloned H chain genes, we have found that the IgM cells contain two H chain chromosomes, bearing two rearranged μ genes: an expressed μ gene (which has undergone VDJ_4 recombination) and a non-expressed μ gene which has undergone a DJ_2 recombination (Fig. 1) (Stavnezer et al., 1982; unpublished data).

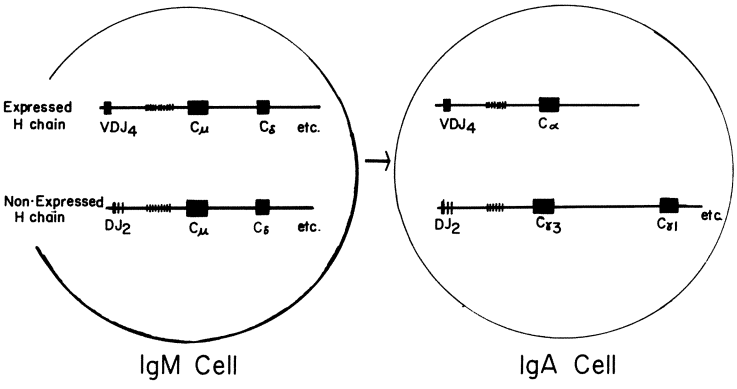

Fig. 1. Diagram of H chain gene chromosomes in the IgM^+ and IgA^+ cells from the I.29 lymphoma.

The C_H genes 3' to the Cμ genes are present in a germline configuration on both chromosomes in the IgM cells. In the IgA cells, both μ genes have been deleted, having undergone switch recombination events between Sμ and Sα sequences on the expressed H chain chromosome, and between Sμ and $S\gamma_3$ sequences on the non-expressed chromosome. These recombinations resulted in the deletion of all the C_H genes except Cα from the expressed chromosome, and deletion of the μ and δ genes from the non-expressed chromosome. The C_H genes 3' to the $C\gamma_3$ genes are in germline configuration on the non-expressed chromosome.

The structure of the μ and α genes established that the IgM and IgA cells in the I.29 lymphoma are related by switch recombination events, but to determine if the IgM cells could actively switch to IgA, it was necessary to be able to culture and to clone the IgM cells. IgM cells were purified on the fluorescence-activated cell sorter by selecting the 30% most brightly staining IgM cells (Hammerling, U., unpublished). These cells have been passaged in vivo for 18 passages, and IgA cells have never been observed, as assayed by light microscopy of fixed cells stained with fluorescent antibodies (a rhodamine-labeled goat anti-IgA antibody, IgG fraction, Cappel, or a rhodamine-labeled rabbit anti-IgA antibody, R. Sitia, and a fluorescein-labeled monoclonal rat anti-mouse IgM antibody, G. Hammerling). At passage 17 no IgA cells among 18,360 IgM cells were detected, i.e. there were less than 0.01% IgA cells present. This pure IgM cell line is termed "I.29μ". These cells were adapted to culture.

Induction of switching

In the first successful attempts to induce the cultured IgM cells to switch, LPS, a B cell mitogen, was added to the cultures for four days, after which time it was removed. IgA cells appeared in the culture about three days

after the addition of LPS and increased to 40% of the cells 15 days after LPS was added. At early times after the addition of LPS, cells whose cytoplasm stained with both anti-IgA and anti-IgM were detected; at later times, the IgA cells did not also stain with anti-IgM. We interpret the double-staining cells to be cells which had recently deleted their μ genes and rearranged their α genes but still retained μmRNA and protein. We have examined Ig H chain genes in cells which had been treated with LPS or other inducing agents described below, and find that the expressed VDJ_{H4} gene segment has recombined with the α gene and that the μ-containing fragments from the expressed chromosome are reduced in quantity relative to that in the IgM cells, especially in cultures containing a high proportion of IgA cells. (Because the J_{H1-3} gene segments are deleted from the expressed H chain chromosome, but only J_{H1} was deleted from the non-expressed chromosome, we can distinguish these chromosomes in genomic DNA blots.)

The presence of double-stained cells proved that the IgA cells were derived by H chain switching from the IgM cells, and not by outgrowth of contaminating IgA cells from the IgM cell preparation. But to obtain further proof of active switching, we produced eight clones of IgM cells by plating one or three IgM cells into 20μl microtiter wells. Two clones were obtained from the 50 wells which had received one cell per well, and six clones from 50 wells which had received three cells per well. The clones were tested for ability to switch to IgA. Six of the eight clones yielded IgA cells. This provided unequivocal proof that the IgM cells could switch to IgA since the level of contamination of the starting population of IgM cells with IgA cells was <0.01%.

To attempt to obtain a greater amount of isotype switching and to learn what may induce isotype switching in vivo, we began a series of experiments to search for other molecules, besides LPS, which would induce switching. The greatest amount of switching was consistently induced by a culture supernatant from a mouse hybridoma producing an anti-I.29 idiotype (Id) antibody (Tada et al., 1981), or by the purified anti-Id antibody itself, when these reagents were added to the cells in the presence of LPS (10 μg/ml) for four days. Eight days after the I.29μ cells were treated with LPS plus purified anti-Id, or with LPS plus a supernatant from the hybridoma producing anti-Id, the cultures contained 14% and 30% IgA cells, respectively. The culture treated with LPS alone contained 8% IgA cells. If the amount of LPS added was increased to 50 μg/ml no increase in the proportion of IgA^+ cells was observed.

These experiments proved that T cells were not required for isotype switching by I.29 cells. It is possible that the cell from which the I.29 lymphoma arose had already been directed by a T cell to become committed to switch to IgA. Hamano and Asofky (1983) have described a B cell hybridoma which could be induced by anti-IgM to switch to IgG_{2a} in the absence of T cells. We have not yet determined whether T cells can increase the rate at which the IgA cells accumulate, perhaps by selecting cells which have switched.

I.29μ cells sometimes switch to IgE rather than to IgA. We do not know what determines which isotype will be expressed. The IgE cells were often detected at 4-11 days after the inducing agent was added; by 15 days they have usually disappeared, and either IgM or IgA cells eventually dominate the cultures. Only in a very few cultures did IgE cells eventually dominate. The IgE bearing cells could be stained with anti-mouse IgE and also with anti-I.29 idiotype antibodies in immunofluorescence assays on fixed cells. Further proof that these cells expressed IgE was obtained by RNA blotting experiments. A nick-translated DNA fragment containing the ε gene detected a 1.9 kb poly(A)+ RNA molecule (the expected size for ε mRNA, Zajdel-Blair et al., 1981) and also larger RNAs, some of which may code for the ε chain present in membrane-bound IgE, and same of which may be nuclear RNA precursors for ε mRNA (Fig. 2). Southern blots have also been performed on genomic DNA from IgE clones. They

contain one germline ε gene and one rearranged ε gene, which migrated at the same size as the expressed V_HDJ gene segment when the DNA was digested with BglII (data not shown).

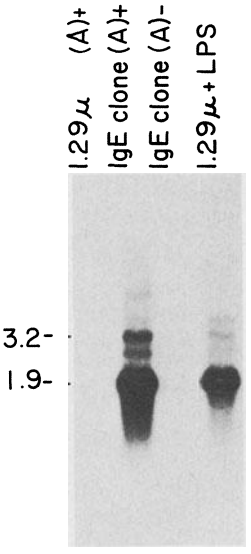

Fig. 2. Blot of I.29 cell RNAs hybridized with the ε gene probe. Lanes from left to right contained I.29μ(A)+: 6 μg poly(A)+ RNA from the in vivo line of I.29μ; IgE clone(A)+: 6 μg poly(A)+ RNA from the IgE^+ cloned cell lines (pooled); IgE clone(A)-: 44 μg of poly(A)- RNA from the pooled IgE clones; I.29μ + LPS: 20μg of total cell RNA from one LPS-treated culture of I.29μ cells which became predominantly IgE^+.

The sizes of the predominant ε RNAs are indicated in kb on the left side, as determined by reference to HindIII fragments of λ phage DNA.

The ε probe was a nick-translated 2.9 kb Bam/HindIII fragment which encoded the germline Cε gene (Nishida et al., 1981).

The finding that I.29μ cells can switch to either IgE or IgA is consistent with the observations that B cell clones are multipotential as to isotype (Gearhart et al., 1975; Teale, 1983). Moreover, IgA and IgE have frequently been found to be expressed in the same B cell clone, and both IgA and IgE precursors arise in the Peyer's patch (Durkin et al., 1981; Clough and Cebra, 1983).

DNA rearrangements accompany switching

To determine if rearrangements of H chain genes accompany the isotype switch at early times after addition of inducers, and to determine what kinds of rearrangements occur, and to search for intermediate DNA rearrangements in the process of switching, we have performed numerous blots of DNA from I.29 cells which had been induced to switch with LPS and/or anti-Ig reagents, at various times after induction. At the earliest time we examined after addition of amm. SO_4-precipitated anti-Id to the IgM cells (three days), changes in the μ and α genes relative to their configuration in cultured IgM cells were observed. The Sμ region on the non-expressed H chain chromosome underwent a series of deletions. The Sα region underwent a 0.9 kb deletion. Three days after the addition of inducers only 3% of the cells expressed IgA; this may explain why a rearranged expressed α gene was not detected. At later times after addition of LPS or anti-Ig reagents, rearranged expressed α genes were readily detected. The sizes of the restriction fragments bearing the rearranged expressed α genes varied among different cultures, indicating that different sites within the Sμ and/or Sα regions were recombined in different cells. However, there also were a number of cultures which appeared to have fragments of the same size, suggesting that there were preferential sites of recombination (data not shown).

The sites of recombination between the μ and α genes and between the μ and ε genes were mapped to be within or near the Sμ, Sα and Sε regions 5' to the C_H genes. As numerous bands were detected which corresponded to the non-expressed allele, due to the variation in the extent of the deletions of the Sμ region and of additional regions of the intervening sequence 5' to the Cμ gene (up to a

deletion of 5 kb), we have not yet mapped the various forms of the non-expressed DJ_H-Cμ gene.

Molecular evidence for the predetermination of I.29μ cells to switch to IgA or IgE

I.29μ cells switch to IgA and less frequently to IgE, but switches to IgG have never been detected. To attempt to understand the molecular basis of this commitment, we examined the level of activity of the C_H genes in the IgM cells as manifested by their state of methylation and by their transcriptional activity. The levels of methylation of the Cα and $C\gamma_{2b}$ genes and of the $S\gamma_{2b}$ region in I.29 cells were compared with their level of methylation in liver cells by comparing the fragments produced by digestion of these DNAs with the restriction enzyme, MspI, which is insensitive to methylation of the CG dinucleotide in its recognition sequence (CCGG), with the fragments produced by the methyl-sensitive isoschizomer, HpaII (Fig. 3). The enzymes and the probes used are indicated in the figure.

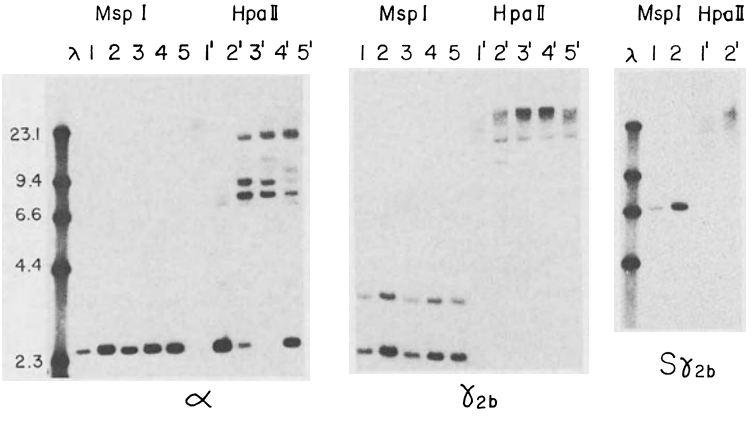

Fig. 3. DNA blots of I.29 cell DNAs digested with the methyl sensitive enzyme, HpaII, and its methyl-insensitive isoschizomer, MspI. The source of the DNAs are explained in the text. The probes are indicated below each blot. γ2b is an 840-bp Pst-Kpn fragment from a cDNA clone (Schibler et al., 1978). Sγ2b is a 1.4-kb fragment containing Sγ2b sequences (Lang et al., 1982). α is a 1.1-kb MspI fragment from pα(J558)[13] (Marcu et al., 1980).

Lanes 1, 1' contain liver DNA; lanes 2, 2' contain DNA from IgM^+ cells passaged in vivo; lanes 3, 3'contain DNA from I.29μ cells cultured for 2 ½ months in vitro; lanes 4, 4'contain DNA from I.29μ cells which were treated with 3 μM 5-azacytidine for the first 18 hr they were in culture, and then cultured for 2 ½ months without azacytidine. These cells were greatly inhibited in their ability to switch to IgA (by more than 10-fold). Lanes 5, 5' contain DNA from the I.29 tumor (IgM.5P), which contained 77% IgA cells, 17% IgM cells. The HpaII sites detected by hybridization with the α probe were hypomethylated, relative to those in liver DNA, in the in vivo passaged IgM cells, probably on both chromosomes and to a lesser extent in the IgM cells cultured in vitro. The IgM cells treated with azacytidine showed more methylation of these HpaII sites than the untreated cells; this finding was suprising since azacytidine has been shown to inhibit methylation, but was in agreement with the fact that these cells would not switch to IgA. Consistent with the fact that I.29 cells have

not been found to switch to γ_{2b}, the γ_{2b} genes and $S\gamma_{2b}$ regions were as highly methylated in I.29 cells as in liver cells.

As genes which are transcriptionally active have generally been found to be hypomethylated, we questioned whether the α genes were being transcribed in the IgM cells. RNA blots containing poly(A)+ RNA from either the IgM cells passaged in vivo, or from cloned IgA cells cultured in vitro were hybridized with the various H chain gene probes indicated (Fig. 4).

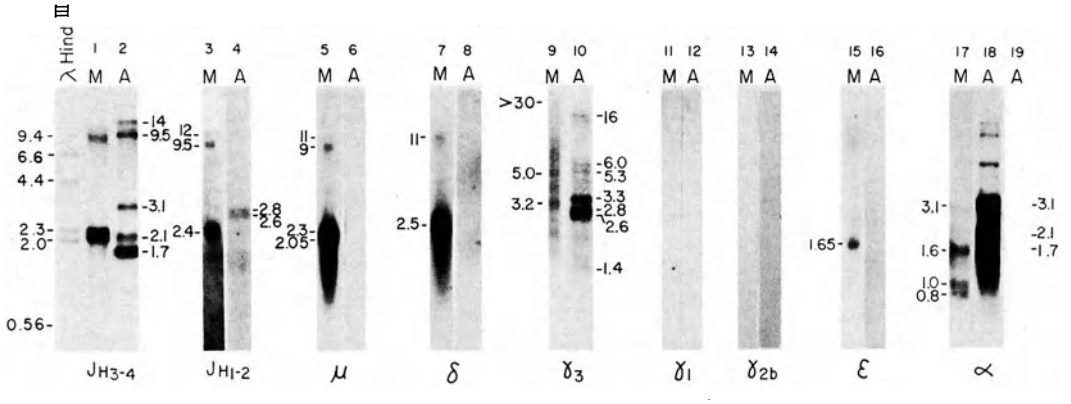

Fig. 4. Blots of poly(A)$^+$ RNA from IgM$^+$ cells (M) and from cloned IgA$^+$ cells (A). 3 μg of RNA were loaded in each lane. The blots were hybridized with the indicated probes. Exposure times for each pair of lanes was identical except lane 19 was exposed a very short time. The sizes of the RNAs were determined by reference to HindIII fragments of λ phage DNA which were labeled at their 5' ends with ^{32}P and electrophoresed in parallel lanes in the same gels. Although the sizes of the μ mRNAs differ from the sizes usually reported. (2.4 and 2.7 kb), the sizes we have determined agree with the sizes predicted from the size of the μ gene (Nelson et al., 1983).

The probes J_{H3-4} and J_{H1-2} were from the plasmid clones Pj_{11} and Pj_0, respectively (Marcu et al., 1980). Cμ was a BglII/HindIII fragment from the plasmid pμ(3741)[9] which contains Cμ cDNA (Marcu et al., 1980). δ was a 0.6 kb PvuII/HindIII fragment from pδ54J (Tucker et al., 1980). γ_3 was a 6.6 kb HindIII fragment containing the BALB/c germline $C\gamma_3$ gene and 5' and 3' flanking sequences (Stanton and Marcu, 1982). γ_1 is a 430 bp Hinf1/HincII fragment from the plasmid clone pH21-1 (Rogers et al., 1979). The other probes were described in the legends for Figs 2, 3.

Both the ε and α probes hybridized to poly(A)+ RNAs in the IgM cells (lanes labeled M) which were not the size of true ε and α mRNAs. α mRNAs are 1.7, 2.1 and 3.1 kb in length; ε mRNA is 1.9 kb long (See lane 19 and Fig. 2). The amounts of these presumably sterile RNAs were much lower than the amount of the functional mRNAs in the IgA and IgE cells. For example, lanes 17 & 18 were both from the same autoradiogram of the same gel (overnight exposure). There was about 100 times more α RNA in the IgA cells than in the IgM cells. There was little or no γ_1 or γ_{2b} RNA detected in the IgM cells, but significant amounts of γ_3 RNA was detected (lanes 9, 11, 13). The non-expressed DJ_2 segment in the IgA cells from the in vivo passaged tumor has undergone a switch recombination with the γ_3 gene; perhaps this was because the γ_3 gene was in an active state on the non-expressed H chain chromosome in the IgM cells. We have not detected, however, any switching of the non-expressed H chain allele to γ_3 in the IgA cells which have switched in vitro. It appears that the recombined DJ_2 segment from the non-expressed chromosome is transcribed in the IgM and IgA cell lines as the J_{H1-2} probe detected RNA species in both the IgM and IgA cells (lanes 3, 4). As the γ_3 gene is the most proximal γ gene to the μ and δ genes, lying 55

kb 3' to the δ gene, the γ_3 gene may be transcribed because it resides within the active μ chromatin domain present in the IgM cells. We do not know, however, if the γ_3 transcription in the IgM cells is from the non-expressed allele. This is a caveat to our results because if transcription from the γ_3 gene is occurring on the expressed H chain chromosome in the IgM cells, the commitment of I.29 cells to switch to IgA or IgE could not solely be determined by the active state of the α or ε genes. To examine the specificity of transcription of the α RNAs, we have hybridized poly(A)+ RNA from the in vitro line of BCL_1, a B cell lymphoma which expresses IgM (Gronowicz et al., 1980) and has not been found to switch to IgA, with α and γ_3 probes. No α RNAs were detected, although γ_3 RNA, the same size as in the I.29μ cells, was detected.

In conclusion, the IgM cells in the I.29 tumor appear to be committed to switch to IgA or to IgE by the state of activation of the α and ε genes. These data suggest that the availability of the α and ε genes for transcription may be essential to, or a result of, the mechanism for predetermination of I.29 cells to switch to IgA or to IgE, and that genes which are available to RNA polymerase may also be genes which are available to switch recombination enzymes.

REFERENCES

Clough ER, Cebra JJ (1983) Interrelationship of primed B cells with the potential for IgE and/or IgA expression. Mol Immunol 20: 903-915

Davis MM, Calame K, Early PW, Livant DL, Joho R, Weissman IL, Hood L (1980) An immunoglobulin heavy-chain gene is formed by at least two recombinational events. Nature 283: 733-739

Durkin HG, Bazin H, Waksman BH (1981) Origin and fate of IgE-bearing lymphocytes. I. Peyer's patches as differentiation site of cells simultaneously bearing IgA and IgE. J Exp Med 154: 640-648

Gearhart PJ, Sigal NH, Klinman NR (1975) Production of antibodies of identical idiotype but diverse immunoglobulin classes by cells derived from a single stimulated B cell. Proc Nat Acad Sci USA 72: 1707-1711

Gronowicz ES, Doss CA, Howard FD, Morrison DC and Strober S (1980) An in vitro line of the B cell tumor BCL can be activated by LPS to secrete IgM. J Immunol 125: 976-980

Hamano I, Asofsky R (1983) Functional studies on B cell hybridomas with B cell surface antigens I. Effects of anti-immunoglobulin antibodies on proliferation and differentiation. J Immunol 30: 2027-2032

Hurwitz JL, Cebra JJ (1982) Rearrangements between the immunoglobulin heavy chain gene J_H and Cμ regions accompany normal B lymphocyte differentiation in vitro. Nature 299: 742-744

Hurwitz JL, Coleclough C, Cebra JJ (1980) C_H gene rearrangements in IgM-bearing B cells and in the normal splenic DNA component of hybridomas making different isotypes of antibody. Cell 22: 349-359

Kawanishi H, Satzman LE, Strober W (1983) Mechanisms regulating IgA class-specific immunoglobulin production in murine gut-associated lymphoid tissues. I. T cells derived from Peyer's patches that switch sIgM B cells to IgA B cells in vitro. J Exp Med 157: 433-450

Kiyono H, McGhee JR, Mostellar LM, Eldridge JH, Koopman WJ, Kearney JF, Michalek SM (1982) Murine Peyer's patch T cell clones. Characterization of antigen-specific helper T cells for IgA responses. J Exp Med 156: 1115-1130

Lang RB, Stanton LW, Marcu KB (1982) On immunoglobulin heavy chain gene switching: two γ2b genes are rearranged via switch sequences in MPC-11 cells but only one is expressed. Nucl Acids Res 10: 611-630

Marcu KB, Banerji J, Penncavage NA, Lang RB, Arnheim N (1980) 5' flanking region of immunoglobulin heavy chain constant region genes displays length heterogeneity in germlines of inbred mouse strains. Cell 22: 187-196

Mongini P, Paul WE, Metcalf ES (1982) T cell regulation of immunoglobulin class expression in the antibody response to trinitrophenol-Ficoll: evidence for T cell enhancement of the immunoglobulin class switch. J Exp Med 155: 884-902

Mongini P, Paul WE, Metcalf ES (1983) IgG subclass, IgE, and IgA anti-trinitrophenyl antibody production within trinitrophenyl-Ficoll-responsive B cell clones. J Exp Med 157: 69-85

Nelson KJ, Haimovich J, Perry RP (1983) Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of μm and μs mRNA. Mol Cell Biol 3: 1317-1332

Nikaido T, Yamawaki-Kataoka Y, and Honjo T (1982) Nucleotide sequences of switch regions of immunoglubulin Cε and Cγ genes and their comparison. J Biol Chem 257: 7322-7329

Nishida Y, Kataoka T, Ishida N, Nakai S, Kishimoto T, Bottcher I, Honjo T (1981) Cloning of mouse immunoglobulin ε gene and its location within the heavy chain gene cluster. Proc Natl Acad Sci USA 78: 1581-1585

Rogers J, Clarke P, Salser W (1979) Sequence analysis of cloned cDNA encoding part of an immunoglobulin heavy chain. Nucl Acids Res 6: 3305-3321

Schibler U, Marcu KB, Perry RP (1978) The synthesis and processing of the messenger RNAs specifying heavy and light chain immunoglobulins in MPC-11 cells. Cell 15: 1495-1509

Sitia R, Rubartelli A, Hammerling U (1981) Expression of 2 immunoglobulin isotypes, IgM and IgA, with identical idiotype in the B cell lymphoma I.29. J Immunol 127: 1388-1394

Stanton LW, Marcu KB (1982) Nucleotide sequence and properties of the murine γ_3 immunoglobulin heavy chain gene switch region: implications for successive Cγ gene switching. Nucl Acids Res 10: 5993-6006

Stavnezer J, Marcu KB, Sirlin S, Alhadeff B, Hammerling U (1982) Rearrangements and deletions of immunoglobulin heavy chain genes in the double-producing B cell lymphoma I.29. Mol Cell Biol 2: 1002-1013

Tada N, Kimura S, Binari R, Liu Y, Hammerling U (1981) New mouse immunoglobulin A heavy chain allotype specificities detected using the hybridoma-derived IgA of I/St mice. Immunogen 13: 475-481

Teale JM (1982) The potential of B lymphocytes for isotype expression. Fed Proc 41: 2497-2501

Teale JM (1983) The use of specific helper T cell clones to study the regulation of the isotype expression by antigen-stimulated clones. J Immunol 131: 2170-2177

Tucker PW, Liu C-P, Mushinski JF, Blattner FR (1980) Mouse immunoglobulin D: messenger RNA and genomic DNA sequences. Science 209: 1353-1360

Yaoita Y, Kumagai Y, Okumura K, Honjo T (1982) Expression of lymphocyte surface IgE does not require switch recombination. Nature 297: 697-699

Zajdel-Blair ME, Blair GE, Bennich H (1981) Purification and characterization of the messenger RNA for the heavy chain of rat immunoglobulin E. Nucl Acids Res 9: 4547-4555

The Regulation of c-myc by Growth Signals

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ABSTRACT

c-myc mRNA is a cell-cycle associated transcript whose induction is regulated by agents that initiate the first phase of a proliferative response. Specifically, c-myc mRNA levels are increased between 10 and 40 fold within one to three hours after incubation of lymphocytes with lipopolysaccharide or Concanavalin A or fibroblasts with platelet-derived growth factor (PDGF). This induction of c-myc does not require the synthesis of new protein species and therefore is regulated directly by the biochemical events that immediately follow PDGF binding in fibroblasts and mitogen binding in lymphocytes. In fibroblasts that have been synchronized with respect to the cell-cycle by a short incubation in PDGF, c-myc mRNA levels are maximal in early GO/G1 and diminish to a quiescent level by the time the cells enter the S phase. Thus, c-myc RNA is a labile transcript with a maximal half-life of three hours. Finally, our experiments provide data that places the action of two oncogenes in a hierarchy: c-sis, the putative structural gene for PDGF, regulates the expression of c-myc.

INTRODUCTION

Viral oncogenes act as dominant growth transforming agents, and in some cases, somatically-altered cellular proto-oncogenes also transform certain cells to tumorigenicity (Bishop 1983; Cooper, 1982). It seems likely that some oncogenes may deregulate normal proliferation by acting at control points in the cell cycle. Growth control is governed by growth factors that exert their effect in the G1 phase of the cell cycle (Pardee, 1978). In fibroblasts, two control points have been defined that are regulated by either platelet derived growth factor (PDGF), epidermal growth factor (EGF) and the somatomedins. PDGF has been shown to act in the initial competence phase of G1, while EGF and somatomedins are required for the progression of cells through a later stage in G1 (Stiles 1979; Leof 1982). Thus, the identification of two oncogenes, sis and erb B, as the structural gene for PDGF II (Doolittle 1983; Waterfield 1983) and a truncated EGF receptor-like gene (Downward, 1984), respectively, lends strong support to the idea that at least some oncogenes mediate their effects by deregulating control of the cell cycle.

In considering the large number of oncogenes that have been identified, it seems likely that some of these are positioned in successive steps of a single metabolic pathway. The metabolic pathway(s) regulated by growth signals are of obvious significance in considering the physiological action of oncogenes. In this paper we consider the c-myc gene, identified originally as the cellular homolog of the transforming determinant carried by avian myeloblastosis virus. We asked whether normal c-myc expression is regulated in relation to the cell cycle as a function of growth signals. In order to test such a possibility, we analyzed the temporal expression of c-myc mRNA in three types of quiescent cells that can be stimulated to growth by mitogens.

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The three systems that we have utilized are lipopolysaccharide (LPS) activated B cells, Concanavalin A (Con A) activated T cells and PDGF stimulated fibroblasts. By analogy to the previously discussed growth requirements of fibroblasts, lymphocytes also appear to require two temporally separated signals in order to transit from a quiescent state to DNA synthesis. The first signal is mediated in T cells via a lectin-receptor interaction (Larsson 1979; Hall 1981), and the second signal can be replaced by the T cell-specific growth factor, interleukin 2 (Maizel 1981). Although LPS activation of B cells does not require any additional comitogens, indirect evidence suggests that LPS stimulates proliferation by the delivery of two separate signals to B cells (Bretscher 1975; DeFranco 1982).

Our results show that c-myc mRNA is strongly induced in several different cells by agents that initiate the first phase of a proliferative response, termed competence (Pledger 1977). Enhanced c-myc expression occurs very soon following the activation of T lymphocytes with Con A, B lymphocytes with LPS and 3T3 cells with PDGF. By contrast, c-myc mRNA is not induced following EGF or insulin/somatomedin treatment of quiescent 3T3 cells. Thus, c-myc is an inducible gene that is shown to be modulated by a specific type of growth signal and expressed in a cell cycle dependent manner.

RESULTS

Transient Induction of c-myc mRNA in B Lymphocytes

In order to investigate the role of c-myc in normal lymphocyte proliferation, resting spleen cells were stimulated with the B cell specific mitogen, LPS, or the T cell specific mitogen, Con A. To look at the possible cell cycle assoiated expression of c-myc, aliquots of splenic lymphocytes were harvested at various times following the addition of polyclonal mitogens. A constant amount of total RNA, extracted from the various cell aliquots, was analyzed for c-myc RNA by an S1 nuclease assay.

A representative experiment showing the temporal expression of c-myc mRNA following LPS stimulation of B cells is seen in Figure 1A. c-myc mRNA is induced approximately 20-fold between one and two hours after addition of LPS to lymphocyte cultures. Induced c-myc mRNA initiates at the two previously-described promoters (Battey 1983) in approximately the same relative abundance as compared to uninduced c-myc mRNA. As shown by the incorporation of tritiated uridine and tritiated thymidine, the increase in c-myc expression precedes by 6 hours general increases in RNA synthesis associated with cell enlargement and precedes by 13 hours the onset of replicative DNA synthesis (Kelly 1983). c-myc mRNA levels peak between 2 and 9 hours, remain at an induced level for approximately 48 hours, and subsequently drop to near background levels. Qualitatively and quantitatively similar results concerning c-myc induction have been found following the stimulation of spleen cells by the T cell-specific mitogen, Con A (Kelly 1983). The asynchrony of mitogen-stimulated lymphocytes in the late G1 phase of the cell cycle (Cantrell 1983) precludes any conclusions concerning the subsequent relative levels of c-myc mRNA following early G1 induction.

To control for the relative amount of mRNA in each sample, aliquots of total RNA were analyzed for beta-2 microglobulin mRNA by the S1 nuclease assay. As shown in Figure 1B, beta-2 microglobulin mRNA remains nearly constant relative to total RNA throughout the course of LPS-induced proliferation. We interpret small variations in the amount of beta-2 microglobulin mRNA as resulting from experimental error in the S1 assay.

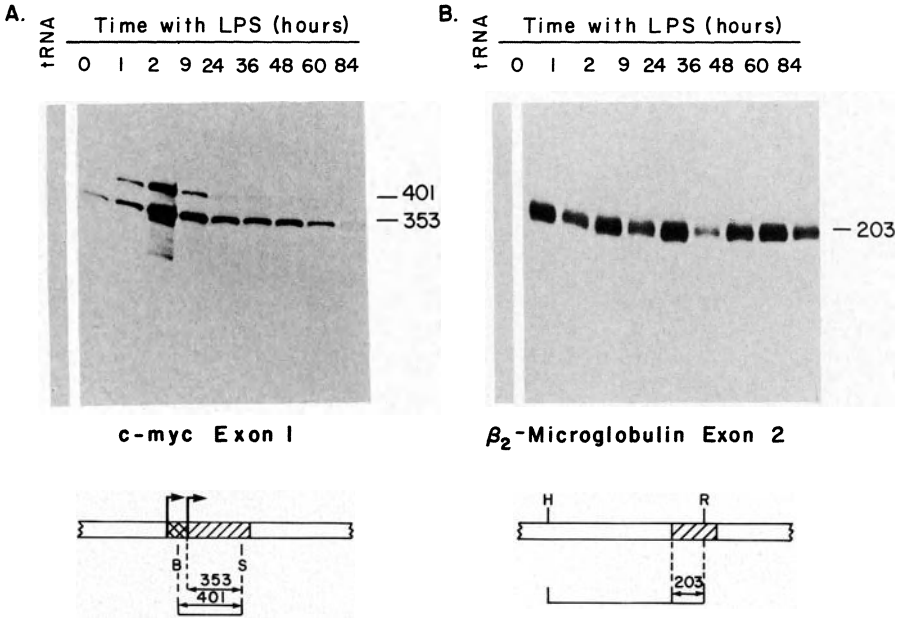

Fig. 1. c-myc mRNA expression is enhanced in LPS-treated B cells. A. S1 nuclease analyses were performed on RNA samples extracted from spleen cell cultures stimulated for the indicated times with LPS. A uniformly-labeled, single-stranded c-myc probe was utilized. Ten micrograms of total RNA from each sample were analyzed. The expected S-nuclease resistant DNA products are shown in the diagram below the figure. The map position of the 5' boundary of the c-myc clone, relative to the two promoter regions, is indicated. The S1-resistant products are displayed on a 5% denaturing polyacrylamide gel. Size determinations were made relative to radioactive phosphate-end labeled molecular weight markers (HinfI-digested pBR327). The control tRNA lane show the result when the appropriate amount of probe is incubated with 10 micrograms of tRNA and digested with S1 nuclease. Unstimulated spleen cells cultured for various lengths of time (up to 84 hours) showed no variation in the amount of c-myc mRNA that was expressed. B. The amounts of beta-2 microglobulin mRNA contained in parallel identical samples relative to those shown in panel A were determined by S1 nuclease protection of a uniformly-labeled beta-2 microglobulin probe. The differing GC contents of the c-myc and beta-2 microglobulin probes preclude their hybridization in a single sample. The expected S1-nuclease resistant DNA product is shown in the diagram below the figure. All other experimental details are identical to those described in part A.

PDGF-Mediated Induction of c-myc mRNA in 3T3 cells

The observation that c-myc mRNA levels dramatically increase promptly after the delivery of a proliferation signal to resting lymphocytes suggests that c-myc expression may be an important component in controlling the transit of cells through the cell cycle. The generality of such a control mechanism is of obvious interest. Since there are parallels between the proliferative response of resting lymphocytes to mitogens and quiescent fibroblasts to growth factors, we have analyzed c-myc expression in quiescent and growth factor-treated 3T3 cells.

Density arrested monolayers of BALB/c-3T3 cells were incubated overnight in medium supplemented with platelet-poor plasma which lacks PDGF. The cul-

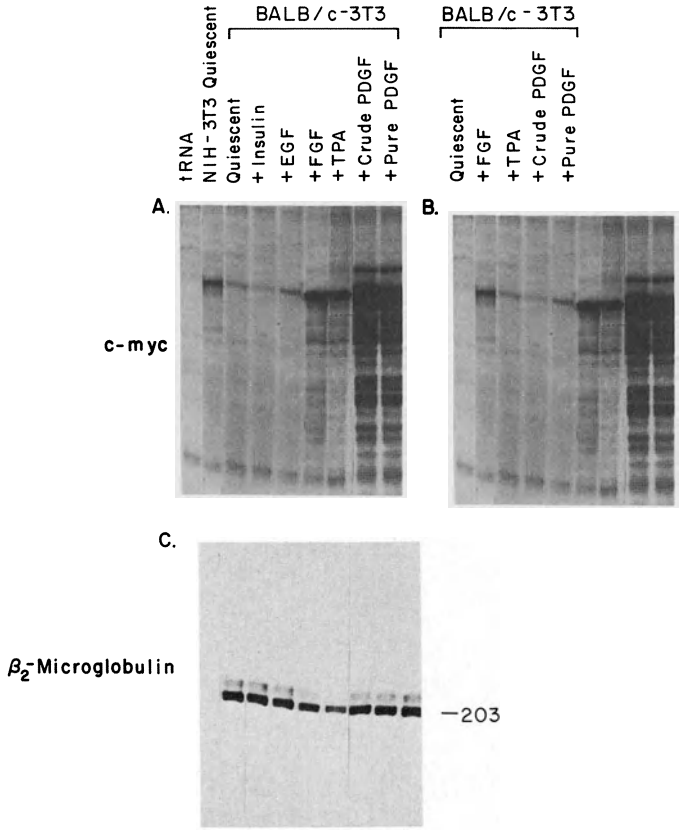

Fig. 2. c-myc mRNA is induced in quiescent 3T3 cells by PDGF, FGF, and TPA. A. S1 nuclease analyses were performed on RNA samples extracted from quiescent BALB/c-3T3 cells, quiescent NIH-3T3 cells, and quiescent BALB/c-3T3 cells treated for 3 hours with partially-purified PDGF, 38 ng/ml electrophoretically pure PDGF, 100 ng/ml EGF, 10 microgram/ml insulin, 100 ng/ml FGF, 250 ng/ml TPA and 5% platelet poor plasma. The c-myc probe utilized is as described in the legend to Fig. 1A. No induction of c-myc mRNA was observed in the legend to Fig. 1A. No induction of c-myc mRNA was observed with 5% platelet poor plasma (not shown). The control tRNA lane displays the nonspecific background following S1 nuclease digestion of the labeled c-myc probe which is observed in the absence of homologous c-myc mRNA. B. Panel B is an approximately ten-fold shorter exposure of the indicated lanes from panel A. C. Parallel samples to those shown in Fig. 2A were analyzed for beta-2 microglobulin mRNA as previously described.

tures were then exposed to PDGF, EGF, insulin or platelet poor plasma. High concentrations of insulin will substitute for somatomedins in promoting 3T3 cell growth, and platelet poor plasma contains both EGF-like and somatomedin-like growth factors (Stiles 1979). Total RNA was extracted from cell monolayers harvested after 3 hours in the presence of growth factors. The amount of c-myc mRNA detected by S1 nuclease analyses of a constant amount of RNA from the various samples is shown in Figure 2A. Quiescent fibroblasts have almost undetectable levels of c-myc mRNA. c-myc mRNA is induced approximately 40-fold after 3 hours in fibroblasts treated with either crude or electrophoretically homogeneous PDGF. We have performed a quantitative analysis of the number of uniformly-labeled DNA mole-

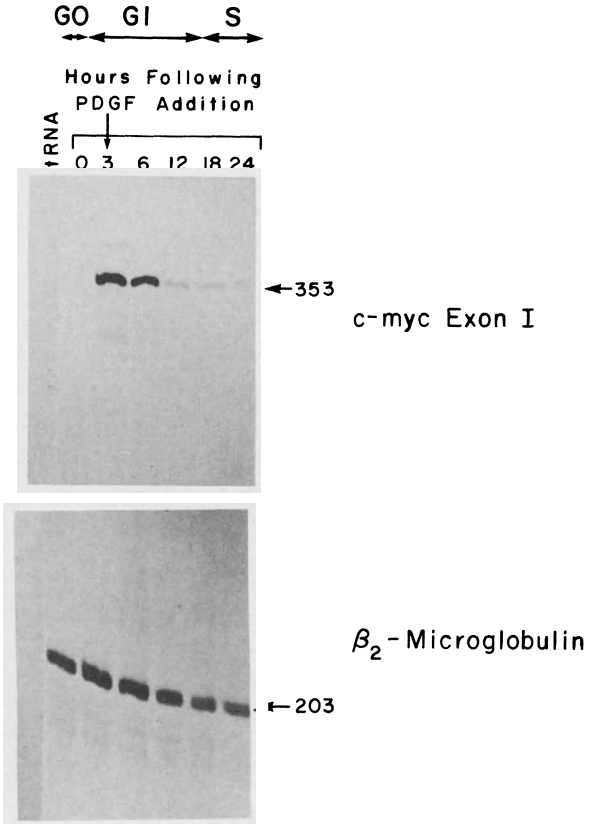

Fig. 3. The expression of c-myc with relation to progression of fibroblasts through the cell cycle. S1 nuclease analyses were performed on RNA samples extracted from BALB/c-3T3 cells at various points in the cell cycle as described in Results. The c-myc and beta-2 microglobulin probes which were used are as described in the legend to Fig. 1.

cules which are protected from S1 nuclease digestion by 10 μg of RNA extracted from PDGF-treated fibroblasts. We estimate that there are approximately 10 molecules of c-myc mRNA per cell after 3 hours of PDGF treatment. Consistent with the role of growth factors required late rather than early in a mitogenic response, EGF, and insulin alone have virtually no effect on the induction of c-myc mRNA (Fig. 2A). Agents such as fibroblast growth factor (FGF) and TPA that mimic PDGF action (Stiles 1979; Frantz 1979) also stimulate c-myc mRNA expression (Figs. 2A and 2B). In contrast to the variability of c-myc mRNA detected in the samples from growth factor treated cells, the amount of beta-2 microglobulin mRNA in parallel samples remains relatively constant (Fig. 2C).

As discussed earlier, PDGF II is the putative translation product of the sis gene (Doolittle 1983; Waterfield 1983). Therefore, we have assayed in two ways the effect of the v-sis gene product on c-myc expression of fibroblasts. Firstly, supernatants from v-sis transfected NRK cells (Devare 1983) were substituted for PDGF in the protocol described for Fig. 2. Secondly, v-sis transfected BALB/c 3T3 cells were grown to confluence, incubated overnight in 5% platelet poor plasma and subsequently assayed for

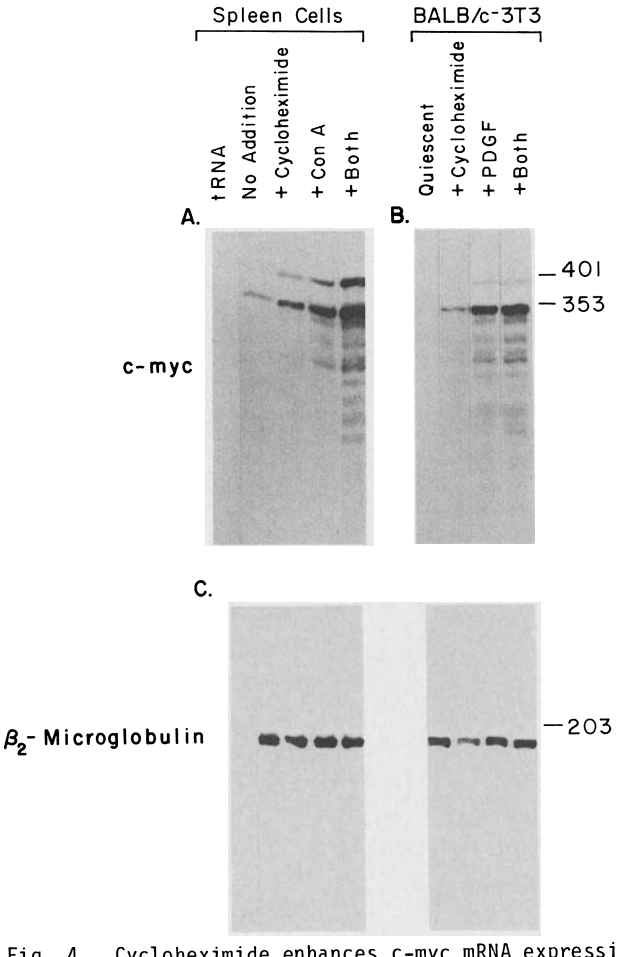

Fig. 4. Cycloheximide enhances c-myc mRNA expression in resting cells and superinduces c-myc mRNA in mitogen-treated cells. A. S1 nuclease analyses were performed on RNA samples extracted from resting spleen cells or from spleen cells treated for 3 hours with cycloheximide (10 μg/ml), ConA (2 μg/ml), or both cycloheximide and ConA as indicated. Similar analyses were performed on RNA samples extracted from quiescent BALB/c-3T3 cells and from BALB/c-3T3 cells treated for 3 hours with cycloheximide (10 μg/ml), partially purified PDGF (200 U/ml), or both cycloheximide and PDGF as indicated. The c-myc probe which was used is as described in the legend to Fig. 1A. B. Duplicate samples to those displayed in panel A were analyzed for beta-2 microglobulin mRNA by S1 nuclease analyses as presented in the legend to Fig. 1B.

c-myc expression. In both experiments, c-myc mRNA levels were found to be comparable to those found after a 3 hour incubation of quiescent BALB/c 3T3 cells with optimum concentrations of PDGF (data not shown). Therefore, we have directly shown that the product of one oncogene, v-sis, regulates the expression of a cellular proto-oncogene, c-myc.

In order to better define the kinetics of c-myc induction with relationship to the progression of fibroblasts through the cell cycle, we analyzed c-myc mRNA levels at various times after treatment with PDGF. Quiescent BALB/c

3T3 cells were incubated with an optimum concentration of PDGF for 3 hours, the cells were washed and placed in 5% platelet poor plasma. RNA was extracted from the monolayers at the times indicated in Fig. 3. As has been shown by Pledger (1978), competent 3T3 cells synchronously enter S phase approximately 12 hours after incubation in platelet poor plasma. Seventy percent of the cell nuclei in the above experiment were labeled after a 24 hour incubation in platelet-poor plasma containing 5 uCi/ml tritiated thymidine. As shown in Fig. 3, the expected induced levels of c-myc mRNA are seen after a 3 hour incubation of 3T3 cells with PDGF. c-myc mRNA levels subsequently decline as the cells progress through G1 in the presence of platelet poor plasma. At the G1/S border (18 hours, Fig. 3), c-myc mRNA has been reduced to nearly background levels. Therefore, c-myc mRNA expression in fibroblasts is confined to the early G1 phase of the cell cycle. Thus, c-myc mRNA must turnover with a half-life of less than 3 hours, a finding similar to the early G1-specific expression of other PDGF-inducible genes (Cochran 1983).

Superinduction of c-myc mRNA by Mitogens in the Presence of Cycloheximide

The prompt induction of c-myc mRNA within 2 hours after mitogen activation of lymphocytes or fibroblasts suggests that the c-myc gene is regulated directly by the biochemical events that immediately follow mitogen binding. To test whether the induction of c-myc mRNA requires the translation of new protein species, the effect of cycloheximide preincubation on c-myc mRNA induction by ConA or PDGF was analyzed.

Spleen cells were activated with Con A for 3 hours in the presence or absence of 10 microgram/ml of cycloheximide. The amount of c-myc mRNA detected in these cells by S1 nuclease analyses is shown in Fig. 4A. Cycloheximide treatment of activated spleen cells enhances the induction of c-myc mRNA 3- to 5-fold over that seen with Con A treatment alone. Cycloheximide alone is seen to increase the expression of c-myc mRNA in resting cells to a level slightly lower than that seen with Con A alone. The effect of cycloheximide on quiescent and PDGF-treated 3T3 cells is comparable to that in resting and Con A-treated spleen cells (Fig. 4A). Cycloheximide has virtually no effect on the amount of beta-2 microglobulin mRNA detected in either quiescent or activated lymphocytes and 3T3 cells (see Fig. 4B), indicating that cycloheximide-mediated superinduction does not reflect merely a generalized stabilization of cellular mRNA. As expected, induction of c-myc mRNA does not occur when PDGF is added together with actinomycin D (data not shown).

DISCUSSION

The induction of c-myc mRNA is regulated in fibroblasts by agents (PDGF, TPA, FGF) which induce competence, the initial priming step for progression through G1. Thus c-myc mRNA increases very early in GO/G1 after incubation of quiescent fibroblasts with PDGF. By analogy, c-myc mRNA is induced within 2 hours after the treatment of lymphocytes with Con A or LPS. Polyclonal mitogens are known to either directly or indirectly mediate more than one growth signal (see Introduction). However, the kinetics of c-myc induction in lymphocytes implies that the myc gene is regulated by the first signal of mitogen binding, a signal which is likely to be comparable to antigen binding to its matching receptor. In addition, c-myc mRNA is induced in the presence of protein synthesis inhibitors. Thus, the c-myc gene appears to be regulated directly by those biochemical events which immediately follow PDGF binding in fibroblasts and mitogen binding in lymphocytes.

How can we interpret various tumor-associated quantitative and qualitative changes in c-myc mRNA in light of the results suggesting that c-myc is an early G1 associated transcript? Quantitative changes in c-myc expression seen in ALV-induced bursal lymphomas (Hayward 1981), in cell lines carrying c-myc gene amplifications (Collins 1982; Alitalo 1983), in plasmacytomas, and in certain Burkitt lymphomas (reviewed in Leder 1983) may confer a selective growth advantage on cells by affecting their efficiency of transit through the G1 phase of the cell cycle. In addition, a deregulation of c-myc transcription relative to the cell cycle may disrupt the normal control of proliferation by effecting the transit of cells from GO to G1. We have shown that noncycling cells have reduced levels of c-myc mRNA relative to cells transiting through early G1. Thus, inappropriate temporal expression of c-myc may preclude the entry of cells from mitosis into a quiescent state. Indeed, variable increases of c-myc mRNA relative to nonmalignant B cells have been observed in various Burkitt lymphomas (Taub 1984). Perhaps, the temporal deregulation of c-myc expression may be far more important than the absolute levels.

In addition, it has recently been shown (Campisi 1984) that two chemically transformed BALB/c 3T3 cell derivatives, BPA31 and DA31, express high levels of c-myc mRNA when the cells are growth arrested in G1 by serum starvation. Transformed fibroblasts have been shown to produce growth factor-like molecules (Todaro 1979). Therefore, a likely explanation for the elevation of c-myc mRNA in BPA31 and DA31 would be the constitutive induction of the c-myc gene by an endogenously-produced competence factor (e.g. PDGF). Consistent with this interpretation is the reduced requirement for serum displayed by BPA31 and DA31 and the more stringent conditions necessary to growth arrest these cells (Dubrow 1979; Campisi 1984). An important question concerns whether BPA31 and DA31 cells are competent and thus simply arrest in G1 as a result of deficiencies in progression factors (e.g. EGF). Alternatively, relaxed regulation of c-myc may result from somatic changes in the regulatory regions of the c-myc gene (see Siebenlist, this volume) or putative trans-acting factor(s) which may control c-myc expression (Leder 1983).

The fundamental role of growth factors in regulating the control of normal proliferation is well established. Although the physiological consequences of growth factor-receptor interactions are largely unknown, the identification of the sis and erbB genes as a growth factor and an altered growth factor receptor, respectively, suggests a mechanism of action associated with normal cell cycle restriction or control points. This idea is further strengthened by the identification of the c-myc gene as a cell cycle associated transcript whose expression is regulated by competence factors. Importantly, this work then links the action of two oncogenes, sis and myc. It is tempting to speculate that yet another class of oncogenes may be added to this hierarchy that disrupts normal proliferation control by acting upon those physiological pathways which mediate the signals between cell surface growth factor receptors and the expression of genes required for cell cycle progression.

REFERENCES

Alitalo K, Schwab M, LIN CC, Varmus HE (1983) Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80:1707-1711

Battey J, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G, Leder P (1983) The human c-myc oncogene: structural consequences of translocation into the IgH in Burkitt lymphoma. Cell 34:779-789.

Bishop JM (1983) Cellular oncogenes and retroviruses. Ann Rev Biochem 52:301-354
Bretscher PA (1975) The two signal-model for B-cell induction. Transpl Rev 23:37-48
Campisi J, Gray HE, Pardee AB, Dean M, Sonnenshein GE (1984) Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell 36:241-247
Cantrell DA, Smith KA (1983) Transient expression of interleukin 2 receptors: consequences for T cell growth. J Exp Med 158:1895-1911
Cochran BH, Reffel AC, Stiles CD (1983) Molecular cloning of gene sequences regulated by platelet-derived growth factor. Cell 33:939-947
Collins S, Groudine M (1982) Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemia cell line. Nature 298:679-681.
Cooper GM (1982) Cellular transforming genes. Science 217:801-806
DeFranco AL, Kung JT, Paul WE (1982) Regulation of growth and proliferation in B cell populations. Immunological Rev 64:161-182
Devare SG, Reddy EP, Law KD, Robbins KC, Aaronson SA (1983) Nucleotide sequence of the simian sarcoma virus genome: demonstration that its acquired cellular sequences encode the transforming gene product $p28^{sis}$. Proc Natl Acad Sci USA 80:731-735
Doolitte RF, Hunkapiller MW, Hood, LE, Devare SG, Robbins KC, Aaronson SA, Antoniades HN (1983) Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221:275-277
Downward J, Yarden Y, Mayes E, Scrace G, Fotty N, Stockwell G, Ullrich A, Schlessinger J, Waterfield MD (1984) Close similarity of epidermal growth factor receptor and v-erbB oncogenic protein sequences. Nature 307:521-527
Dubrow R, Riddle VGH, Pardee AB (1979) Different responses to drugs and serum of cells transformed by various means. Cancer Res 39: 2718-2726
Frantz CN, Stiles CD, Scher CD (1979) The tumor promoter 12-O-tetradecanoylphorbol-13-acetate enhances the proliferative response of BALB/c-3T3 cells to hormonal growth factors. J Cell Physiol 100:413-424
Hall DJ, O'Leary JJ, Sand TT, Rosenberg A (1981) Commitment and proliferation kinetics of human lymphocytes stimulated in vitro: effects of alpha-MM addition and suboptimal dose on concanavalin A response. J Cell Physiol 108:25-34
Hayward WS, Neel BG, Astrin SM (1981) Activation of cellular onc genes by promoter insertion in ALV-induced lymphoid leukosis. Nature 296: 474-479
Kelly K, Cochran BH, Stiles CD, Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603-610
Larsson EL, Coutinho A (1979) The role of mitogenic lectins in T cell triggering. Nature 304:596-602
Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T, Taub R (1983) Translocations among antibody genes in human cancer. Science 222:765-771
Leof EB, Wharton W, Van Wyk JJ, Pledger WJ (1982) Epidermal growth factor and somatomedin C regulate G1 progression in competent BALB/c-3T3 cells. Exp Cell Res 141:107-115
Maizel AL, Mehta SR, Hauft S, Franzini D, Lachman L, Ford RJ (1981) Human T lymphocyte/monocytic interaction in response to lectin: kinetics of entry into the S-phase. J Immunol 127:1058-1064
Pardee AB, Dubrow R, Hamlin JL, Kletzien RF (1978) Animal cell cycle. Ann Rev Biochem 47:715-750

Pledger WJ, Stiles CD, Antoniades HN, Scher CD (1977) Induction of DNA synthesis in BALB/c-3T3 cells by serum components: re-evaluation of the commitment process. Proc Natl Acad Sci USA 74:4481-4485

Pledger WJ, Stiles CD, Antoniades HN, Scher CD (1978) An ordered sequence of events is required before BALB/c-3T3 cells become committed to DNA synthesis. Proc Natl Acad Sci USA 75:2839-2843

Stiles CD, Capone GT, Scher CD, Antoniades HN, Van Wyk JJ, Pledger WJ (1979) Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc Natl Acad Sci USA 76:1279-1283

Taub R, Moulding C, Battey J, Murphy W, Vasicek T, Lenoir G, Leder P (1984) Activation and somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. Cell 36:339-348

Todaro GJ, DeLarco JE, Marquardt H, Bryant BR, Sherwin SA, Slistin AH (1979) Polypeptide growth factors produced by tumor cells: a possible growth advantage for the producer cells. In: Cold Spring Harbor Conferences on Cell Proliferation, Vol. 6, Hormones and Cell Culture. Ross R, Sato GH (eds), Cold Spring Harbor Laboratory, NY, p 113

Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnson A, Wasteson A, Westermark B, Heldin CH, Huang JS, Deuel TF (1983) Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304: 35-39.

Immune Regulation of the c-myc Oncogene in a Murine B Lymphoma

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The c-myc oncogene has been implicated in a wide spectrum of B cell neoplasias (1-4). Reports of c-myc gene amplification (5), of insertion of viral elements near the c-myc promoter (1), and of point mutations within the c-myc gene (6) have appeared. However, the most frequently observed alteration is a chromosomal translocation, and usually this is to an immunoglobulin gene element (3-5). The observed amplification of the c-myc gene in some tumors led to the proposal of activation via enhanced levels of expression of c-myc RNA; however, increased transcription does not appear to be a general mechanism of activation (5). Since the first exon of the c-myc gene is often lost during translocation, it was postulated that the product of the rearranged myc gene would be different from that of the germ-line gene. However, DNA sequencing demonstrated that initiation of protein synthesis would occur within the second exon, and thus both protein products would be the same (7). Since the large majority of these modifications affect the promoter region, subtle alterations in the regulation of c-myc expression might result. Therefore we decided to examine the regulation of expression of the normal c-myc gene within a B cell system.

WEHI 231 is a murine B cell lymphoma originally isolated in an F1 progeny of a Balb/c and NZB mouse cross (8). This cell line, adapted to tissue culture, expresses surface IgM (sIgM), but not IgD, and has been classified as an early B cell lymphoma. The c-myc genes within WEHI 231 have not been rearranged (4). It has been shown that the growth of WEHI 231 can be inhibited by incubation with anti-mouse Ig antisera (8,9). We have confirmed that upon incubation with a goat anti-mouse Ig antiserum, (GaMIg) (1/500 dilution), WEHI 231 cells undergo only one further round of division (10). We report here on the specific effect of this treatment on c-myc gene expression in this B lymphoma cell line.

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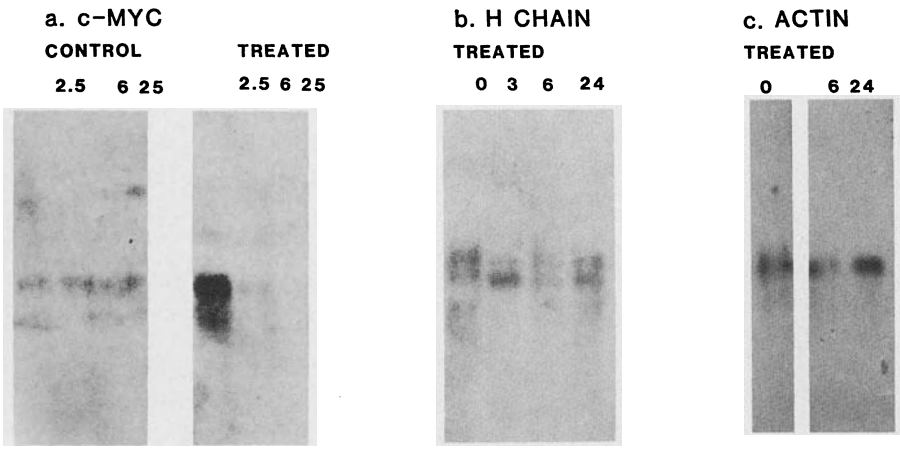

Figure 1. Specific Fluctuations in c-myc RNA Levels.

Results

Antibody Treatment Specifically Affects Levels of c-myc RNA in the Cytoplasm

Cytoplasmic RNA, isolated from mock treated control and GaMIg treated cultures after 2.5, 6 and 25 hours, was enriched for poly(A)-containing RNA by oligo(dT)-cellulose chromatography (11). No change in total RNA ($O.D._{260}$) or poly(A)-containing RNA [as evaluated by hybridization to ^{3}H-poly(U) (12)] occurs on a per cell basis as a result of this treatment. Hybridization of a ^{32}P-labelled c-myc probe to an RNA blot of these samples (11) indicates that c-myc RNA levels in control cells do not fluctuate significantly (Figure 1a). In contrast, in treated cultures, c-myc RNA levels undergo a rapid increase within 2 1/2 hours, followed by an even more dramatic decrease during the next 3 1/2 hours (Figure 1a).

As a control for the specificity of these changes, we monitored the effect on mRNA species for other proteins. No significant changes are observed in the mRNAs for either the μ heavy chain (Figure 1b) or for actin (Figure 1c).

Translation in a rabbit reticulocyte cell-free system was employed to evaluate the effect of GaMIg on the levels of other major mRNAs. Analysis of the radiolabelled products reveals no significant changes in products of RNA from 2 and 6 hour treated cells compared to control samples. (Levels of c-myc RNA are too low to be able to evaluate by translation without a specific

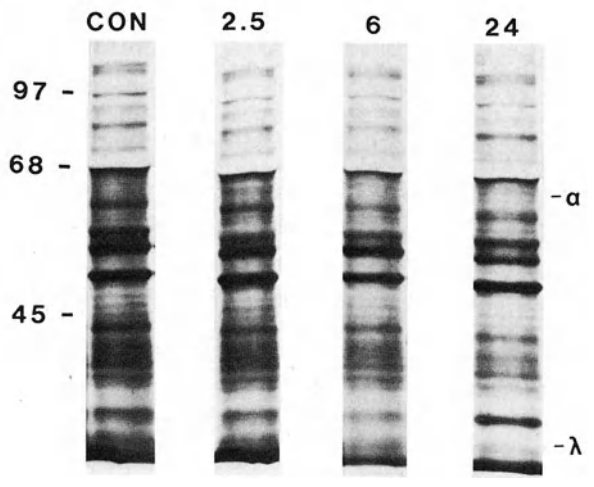

Figure 2. Levels of mRNAs for Most Polypeptide Chains are Unaffected.

antiserum.) The only significant change observed in the RNA population occurs after 24 hours of treatment; there is an increase in a band of approximately 28 kd (Figure 2). A slightly larger polypeptide is seen in intracellular labelling at this time (10). (It is interesting to note that Rahmsdorf et al. (13) have reported the specific synthesis in resting B cells of a 35 kd nuclear glycoprotein which is processed from a 28 kd primary translation product.) We conclude from the analysis of the products of the cell-free system that the relative amounts of most major mRNAs are not changed as a result of the GaMIg treatment, and the effect on c-myc RNA is rather specific.

Cytoplasmic c-myc mRNA is in Polysomes

In order to evaluate the translational activity of c-myc mRNA, in the absence of a specific antiserum recognizing mouse c-myc protein, we assayed for the presence of c-myc message in polyribosome structures. The polyribosome profiles observed for lysates of control and 2.5 hour treated cell cultures, prepared as described previously (11), were essentially identical (Figure 3). The fractions from the regions containing large polysomes, small polysomes, 80S ribosomes and 40-60S messenger ribonucleoprotein particles (mRNP) were pooled as indicated, and poly(A)-containing RNA analyzed for c-myc sequences (11). In the control (data not shown) and after 2.5 hours of treatment (Figure 3), most of the c-

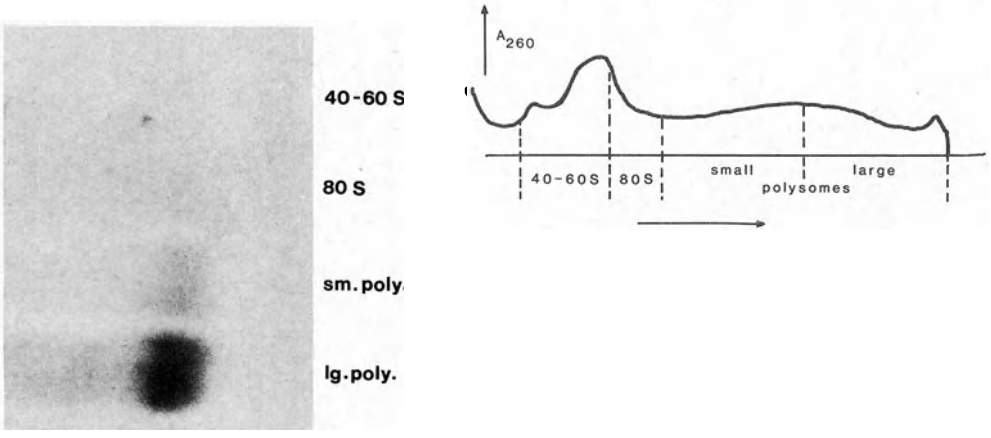

Figure 3. c-myc RNA is in Polysomes.

myc RNA is in large polysomes and a minor amount in small polysomes. (The profile of hybridization is the same with RNA from control cells; however, the intensity is much lower as expected, data not shown) Therefore in control and Ig-treated cells, the transcript from the unrearranged myc gene appears functionally active.

c-myc mRNA has a short half-life

In order to examine in more detail the early events following GaMIg treatment, c-myc RNA levels were assessed after 0, 2, 4 and 6 hours of treatment. A rapid increase is observed in c-myc RNA levels by 2 hours followed by a dramatic decrease within the next 2 hours of treatment (Figure 4a). In contrast, little change is observed in levels of ras^{Ki} mRNA species (Figure 4b). Although difficult to achieve accurate quantitation with such large variations, the level of c-myc RNA decreases at least 50-fold between the 2 and 4 hour time points. Thus during this period c-myc RNA has a half-life of less than 25 minutes.

Discussion

Upon incubation with the appropriate concentration of GaMIg, a culture of growing WEHI 231 cells will only divide once. We have employed this sytem as a model to evaluate the molecular events occurring during anti-Ig inhibition of B cell growth. A

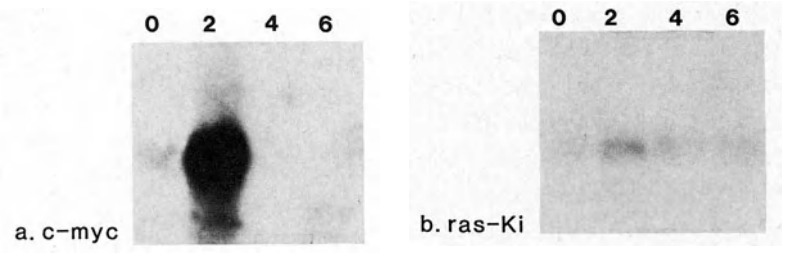

Figure 4. Levels of c-myc RNA Increase and Decrease Rapidly.

rapid increase of cytoplasmic c-myc mRNA levels occurs within the first two hours of incubation. Similar effects have been observed following mitogen stimulation of resting B and T cells (14), and when resting Balb/c 3T3 fibroblasts proliferate (12). In contrast, however, the WEHI 231 cells were actively growing before treatment. Localization of c-myc RNA in predominantly large polyribosome structures implies that this RNA is active translationally. Thus there would be a large increase in c-myc protein levels shortly following the initiation of the GaMIg treatment.

The decrease in c-myc RNA levels between 2 and 4 hours of incubation is much more rapid than the decline observed following LPS stimulation of B cells (14). The half-life of 25 minutes for c-myc RNA is similar to those reported for histone messages (15). In analogy, it seems likely that c-myc mRNA is subject to a specific turnover mechanism. Saito et al. (16) have proposed, on the basis of sequencing studies, that the 5' end of the transcript of the unrearranged c-myc gene can form a "loop-and-stem" secondary structure. While they suggested involvement of such a structure in controlling translational ability, our polysome localization results suggest that the normal c-myc RNA is translated efficiently. An alternative role for the "loop-and-stem" structure may be in RNA degradation. An endonucleolytic clip within this structure would eliminate the capped 5' end of the message. This would result in both loss of translational capability for the mRNA as well as enhanced sensitivity to 5' exonuclease degradation. Products of a translocated gene which have lost the first exon would be unable to form this structure and thus would become resistant to degradation via this pathway.

The results of our experiments are consistent with the c-myc

gene product performing an essential role during growth. In view of c-myc RNA appearance in the early G0/G1 phase of the cell cycle (12,14), we would expect that the decrease of c-myc RNA following GaMIg treatment would block WEHI 231 cells at this point. Further studies of WEHI 231 cells should clarify the mechanism whereby GaMIg treatment mediates effects on gene expression.

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1. Hayward, W.S., B.G. Neel and S.M. Astrin (1981) Nature 290:475.
2. Taub, R., I. Kirsch, C. Morton, G. Lenoir, D. Swan, S. Tronick, S. Aaronson, and P. Leder (1982) Proc. Nat'l. Acad. Sci. U.S.A. 79:7837.
3. Shen-Ong, G., E. Keath, S. Piccoli, and M. Cole (1982) Cell 31:443.
4. Adams, J., S. Gerondakis, E. Webb, J. Mitchell, O. Bernard and S. Cory (1982) Proc. Nat'l. Acad. Sci. U.S.A. 79:6966.
5. Taub, R., C. Moulding, J. Battey, W. Murphy, T. Vasicek, G. Lenoir, and P. Leder (1984) Cell 36:339.
6. Rabbitts, T., P. Hamlyn, and R. Baer (1983) Nature 306:760.
7. Stanton, L.W., R. Watt and K.B. Marcu (1983) Nature 303:401.
8. Ralph, P. (1979) Immunol. Rev. 48:107.
9. Boyd, A.W. and J.W. Schrader (1981) J. Immunol. 126:2466.
10. McCormack, J.E., V.H. Pepe, R.B. Kent, M. Dean, A. Marshak-Rothstein, and G.E. Sonenshein (manuscript submitted).
11. Dean, M., R.B. Kent, and G.E. Sonenshein (1983) Nature 305:443.
12. Campisi, J., H. Gray, A.B. Pardee, M. Dean, and G.E. Sonenshein (1984) Cell 36:241.
13. Rahmsdorf, H., U. Mallick, H. Ponta, and P. Herrlich (1982) Cell 29:459.
14. Kelly, K., Cochran, B., Stiles, C. and P. Leder (1983) Cell 35:603.
15. Alterman, R.-B., S. Ganguly, D. Schulze, W. Marzluff, C. Schildkraut, and A. Skoultchi (1984) Mol. Cell. Biol. 4:123.
16. Saito, H., A. Hayday, K. Wiman, W. Hayward and S. Tonegawa (1983) Proc. Nat'l. Acad. Sci. U.S.A. 80:7476.

The Translocated c-myc Oncogene of Burkitt Lymphoma is Differentially Regulated in Lymphoblastoid vs Plasma Cells

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INTRODUCTION

We have shown that the c-myc oncogene translocates from its normal position on band q24 of chromosome 8 to the heavy chain locus on chromosome 14 in Burkitt lymphomas with the t(8;14) chromosome translocation, while the c-myc oncogene remains on chromosome 8 and either the lambda or the kappa chain locus translocates to a region distal to the c-myc oncogene in Burkitt lymphoma with the t(8;22) and t(2;8) translocations, respectively (Dalla Favera 1982, 1983; Erikson 1983, 1983a; Croce 1983). Independently of whether it is structurally rearranged, the c-myc oncogene involved in the translocation is transcriptionally active, while the c-myc oncogene on the normal chromosome 8 is transcriptionally silent (Erikson 1983, 1983a; Croce 1983; Nishikura 1983; ar-Rushdi 1983). We have also shown that while the c-myc oncogene involved in the translocation escapes normal transcriptional control, the untranslocated c-myc oncogene on normal chromosome 8 is repressed in plasma cells (Erikson 1983; 1983a; Croce 1983; Nishikura 1983; ar-Rushdi 1983). To determine whether the activation of the translocated c-myc oncogene depends on the differentiated state of the B cells harboring the c-myc oncogene involved in the translocation, we have hybridized different human Burkitt lymphoma cells with human lymphoblastoid cells and with mouse plasmacytoma cells and examined the hybrids for the expression of the normal and of the involved c-myc oncogene.

In addition since we have found that the first exon of the c-myc oncogene, which is separated from the c-myc coding exons by the chromosomal break occurring in the first c-myc intron, is located 5' of the heavy chain enhancer and is transcriptionally very active in ST486 Burkitt lymphoma cells (ar-Rushdi 1983), we have also examined the hybrids between ST486 and either lymphoblastoid or plasmacytoma cells for the expression of the separated first exon to determine whether its transcriptional activity depends on the differentiated state of the hybrids.

MATERIALS AND METHODS

CELLS Human GM1500-6TG-OUB lymphoblastoid cells derived from the Epstein-Barr virus (EBV) transformed cell line GM1500-6TG (Croce 1980; Kozbor 1982) and are deficient in hypoxanthine phosphoribosyltransferase and resistant to 10^{-4} M oubain. The GM1500-6TG cell line is a 6-thioguanine resistant mutant of the EBV transformed GM1500 cell line (Croce 1980; Kozbor 1982). We have tested these three lymphoblastoid cell lines for tumorigenicity by injecting 10^7 cells per mouse into Balb/c nude mice. No tumors were obtained in three of three mice injected with GM1500 cells which were observed for 91 days. Only one of five

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mice injected with GM1500-6TG cells developed a tumor (0.8 cm in diameter) which appeared 64 days after injection. The five mice were observed for 103 days. All three mice injected with GM1500-6TG-OUB cells developed tumors within 23 days after injection which were larger than 1.5 cm in diameter 70 days after injection. All three lymphoblastoid cell lines carry a germ line c-myc oncogene.

Three Burkitt lymphoma cell lines with the t(8;14) chromosome translocations were studied: Daudi, CA46 and ST486. Daudi cells contain a translocated but unrearranged c-myc oncogene on the $14q^+$ chromosome (Dalla Favera 1982, 1983; Erikson 1983). CA46 and ST486 cells carry a translocated and altered c-myc oncogene which is rearranged head-to-head with one of the immunoglobulin heavy chain constant region genes on the $14q^+$ chromosome (Dalla Favera 1983; ar-Rushdi 1983). In both cell lines the rearrangement occurred within the first c-myc intron (Dalla Favera 1983; ar-Rushdi 1983). We have previously shown that ST486 cells in addition to transcribing the translocated coding exons of the c-myc oncogene also express high levels of transcripts of the first exon that has been separated from the coding exons by the chromosomal breakpoint. Each of the three Burkitt lymphoma cell lines was fused with the GM1500-6TG-OUB lymphoblastoid cell line in the presence of polyethylene glycol 1000 (PEG) according to standard procedures (Croce 1979) and the hybrids were selected in HAT medium (Littlefield 1964) containing 10^{-5}M ouabain (Croce 1977). ST486 cells were also hybridized with NP3 mouse plasmacytoma cells (Erikson 1983 1983a; Croce 1983; Nishikura 1983) and the hybrids selected in HAT medium containing 10^{-5} ouabain (Croce 1977).

DNA GEL ELECTROPHORESIS AND SOUTHERN TRANSFER

Agarose gel (0.7% or 1%) electrophoresis was carried out in 40 mM Tris·HCl/5 mM NaOAc/2.0 mM EDTA, pH 8.0. HindIII-digested λ phage DNA molecular weight markers (0.75 μg per lane) (Bethesda Research Laboratories) were included on every gel. Cellular DNA samples were digested with restriction enzymes and then subjected to electrophoresis in a horizontal agarose (Sigma) slab gel (10 μg of DNA per lane). Transfer of DNA from gel to nitrocellulose sheet (Millipore) was performed essentially as described by Southern (1975).

RNA TRANSFER

Cytoplasmic RNA was extracted by the cesium chloride method as described (ar-Rushdi 1982). RNA was denatured in 1 M glyoxal in 10 mM $NaPO_4$, pH 6.5, at 50°C for 1 hr, electrophoresed in 1% agarose gel, transferred to nitrocellulose, prehybridized and hybridized to 0.2 μg of the myc probe (about 4×10^7 cpm) according to the method of Thomas (1980). Twenty micrograms of RNA were loaded in each lane. Prehybridizations and hybridizations were performed as reported (Erikson 1983; ar-Rushdi 1983). Molecular weight markers were electrophoresed in each gel.

PROBES

The first exon probe is an XhoI-PvuII leader fragment of 449 base pairs (bp) within exon 1 (ar-Rushdi 1983). The probe for the coding exons of the c-myc gene is a 1029-bp fragment obtained from the PstI digestion of a cDNA clone (pRyc 7.4) which includes 221 bp of the 3' end of the exon II and all of exon III (Nishikura 1983; ar-Rushdi 1983). The probe used for S_1 mapping analysis to detect novel initiation sites or cryptic splicing sites within the intervening sequences between the first and second exons was a double-stranded 1.4 kb XbaI-BstEII fragment, 5' ^{32}P-labeled at the BstEII site within the second exon (ar-Rushdi 1983). The immunoglobulin Cu gene probe was a 1.2-kb EcoR1 genomic probe described elsewhere (Erikson 1983).

S_1 NUCLEASE ANALYSIS OF HUMAN C-MYC TRANSCRIPTS

Nuclease S_1 analysis was carried out according to Sharp et al (1980) with modifications (Weaver 1979) by using 5'-end-labeled human c-myc DNA clones (Nishikura 1983). The 5'-^{32}P end-labeled DNA probes were heat-denatured, hybridized in 80% deionized formamide to 20 µg cytoplasmic RNA at 55°C for 10 hr, digested with 80 units of S_1 nuclease (P.L. Biochemicals), and analyzed by electrophoresis on a 7 M urea 4% polyacrylamide gel (Maniatis 1975). The DNA probe was 5'-end-labeled by the Method of Maxam and Gilbert.

RESULTS

The hybrids between GM1500-6TG-OUB cells and the three different Burkitt lymphoma cells, Daudi, CA46 and ST486, were morphologically identical to the lymphoblastoid parental cells and grew in large clumps like lymphoblastoid cells (data not shown). These hybrids expressed immunoglobulin chains of both parental cells (data not shown).

Since Daudi cells contain a translocated c-myc gene in its germ-line configuration which cannot be distinguished from the normal c-myc gene on the normal chromosomes 8 of Daudi cells and of GM1500-6TG-OUB cells, we examined the Daudi x GM1500-6TG-OUB hybrids (223-A5, 223-C4, 223-B3 and 223-B4) for the presence of the rearranged µ chain gene that is located on the $14q^+$ chromosome of Daudi cells (Erikson 1982). This gene, which is contained within a 18 kb BamH1 restriction fragment band, was present in all four of the hybrid clones examined (data not shown), indicating that the $14q^+$ chromosome carrying the translocated c-myc oncogene (Erikson 1982) was present in all four hybrid clones. As shown in Fig. 1A, two hybrids between CA46 and GM1500-6TG-OUB cells (267-B1 and AC4) lost the translocated and rearranged c-myc oncogene (lane 2 and 4), while hybrid 267-BC4 (lane 3) and hybrid 267-AD5 (data not shown) retained the translocated and rearranged c-myc oncogene in addition to the germ-line gene.

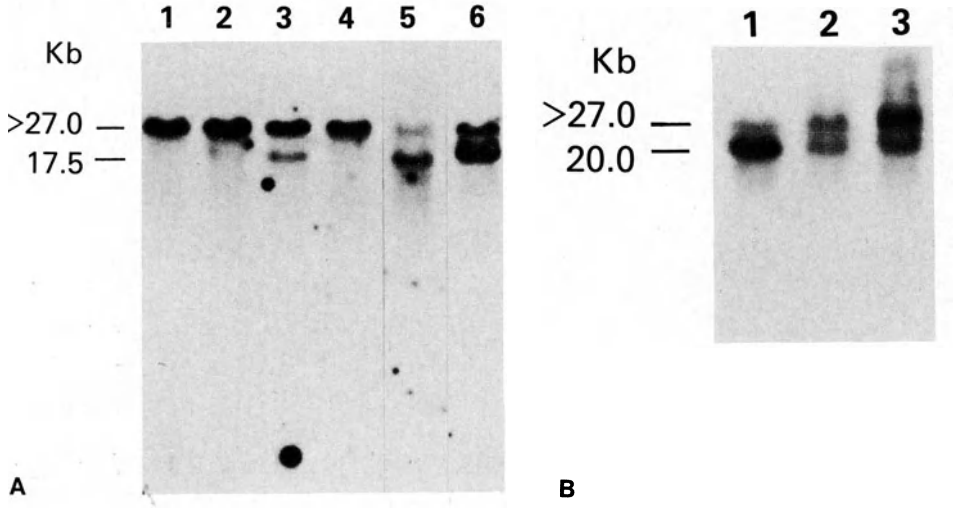

Fig. 1A,B. Southern blotting analysis of BamH1 digested parental and hybrid cell DNAs using the myc (Rcy 7.4) cDNA probe. A. Lane 1, GM1500-6TG-OUB; lane 5, CA46 DNA; lane 6, ST486 DNA; lanes 2-4, CA46 x GM1500-6TG-OUB hybrids 267-AC4, 267-BC4 and 267-B1 DNAs respectively. B. Lane 1, ST486 DNA; lane 2, hybrid 286-2D5 DNA; lane 3, hybrid 286-1C1 DNA.

As shown in Fig. 1B, both hybrids (286-1C1 and 286 3D3) between ST486 and GM1500-6TG cells retained the germ-line and the rearranged and translocated c-myc oncogene (lanes 2 and 3, respectively).

The GM1500-6TG-OUB lymphoblastoid cells express high levels of transcripts of the c-myc oncogene (Fig. 2, lanes 3 and 4). The 6-thioguanine resistant mutant GM1500-6TG line from which the ouabain resistant mutant GM1500-6TG-OUB was derived express lower levels of myc transcripts (Fig. 2, lane 2). On the contrary, the parental GM1500 human lymphoblastoid cell line expresses very low levels of myc transcripts (Fig. 2, lane 1). Interestingly the levels of c-myc transcripts in the three lymphoblastoid cell lines correlate with their tumorigenicity in nude mice (see Materials and Methods).

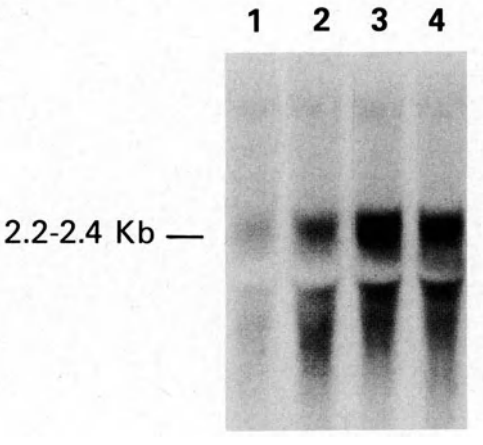

Fig. 2. Northern blotting analysis of the RNA of the GM1500 cell lines using the c-myc cDNA probe (Ryc 7.4). Lane 1, GM1500 cell RNA; lane 2, GM1500-6TG cell RNA; lane 3, GM1500-6TG-OUB RNA; lane 4, RNA derived from a tumor induced in nude mice by injection of GM1500-6TG-OUB cells (Table 1).

As shown in Fig. 3A, all the hybrids between Daudi cells and GM1500-6TG-OUB cells expressed levels of myc transcripts lower than in the Daudi parental cells.

Table 1. Transcripts of the human c-myc gene in B cell hybrids

	Human c-myc gene*			Transcripts of the		
Cells	germ-line	rearranged and translocated	separated first exon*	human germ-line c-myc gene	rearranged and translocated human c-myc gene	separated first exon of the human c-myc gene
ST486	+	+	+	-	+	+
GM1500-6TG-OUB	+	-	-	+	-	-
286-2D5	+	+	+	+	-	+
286-1C1	+	+	+	+	-	+
NP3	-	-	-	-	-	-
287-2D3-BF5	+	+	+	-	+	-

*Cellular DNAs were cut with HindIII and, following agarose gel electrophoresis and Southern blotting the nitrocellulose filters were hybridized with a probe specific for either the second and third exon or the first exon of the c-myc oncogene. The separated first exon appears as a 4.7 kb band following hybridization with the first exon probe.

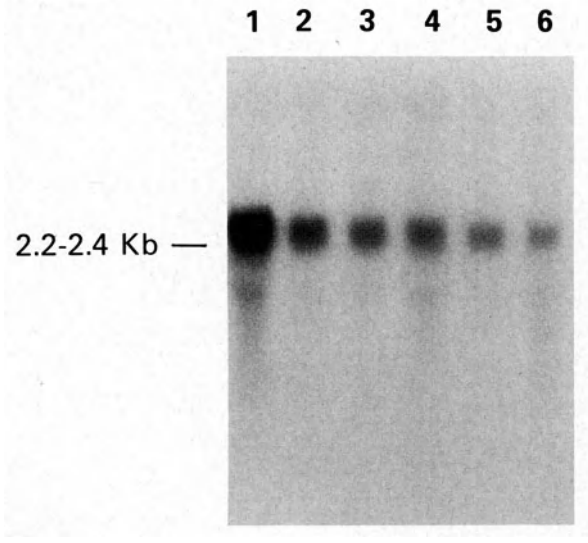

Fig. 3A. Northern blotting analysis of RNA of hybrids between Burkitt lymphoma and GM1500-6TG-OUB lymphoblastoid cells. A. The blot was hybridized with the c-myc cDNA (Ryc 7.4) specific for the second and third exon. Lane 1, Daudi RNA; lanes 2-5, hybrid 223-A5, 223-C4, 223-B3 and 223-B4 DNAs respectively; lane 6, GM1500 RNA.

The hybrids between CA46 and GM1500-6TG cells also expressed levels of myc transcripts much lower than in the CA46 parent (Fig. 3B).

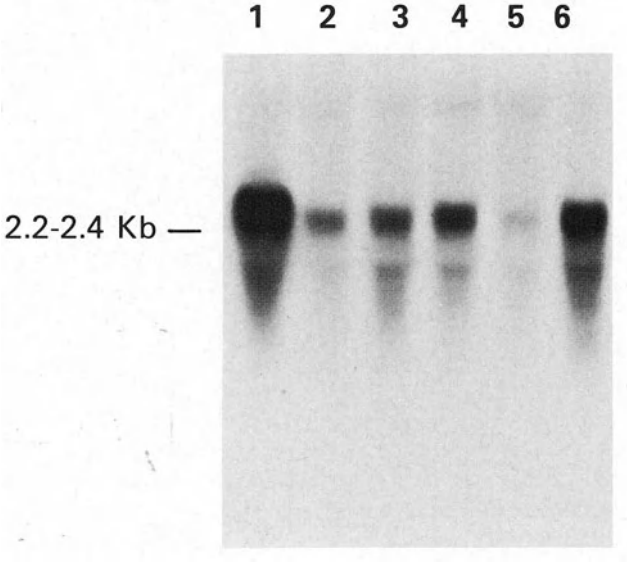

Fig. 3B. Northern blotting analysis of RNA of hybrids between Burkitt lymphoma and GM1500-6TG-OUB lymphoblastoid cells. B. The blot was hybridized with the Ryc 7.4 probe. Lane 1, CA46 RNA, lanes 2-5, hybrid 267-BC4, 267-AC4, 267-B1, and 267-AD5 RNAs respectively; lane 6, GM1500-6TG-OUB RNA.

In these hybrids the levels of myc transcripts were even lower than in the GM1500-6TG-OUB parent. The hybrids between ST486 and GM1500-6TG-OUB cells also expressed levels of c-myc transcripts much lower than both parental cells (Fig. 3C, left).

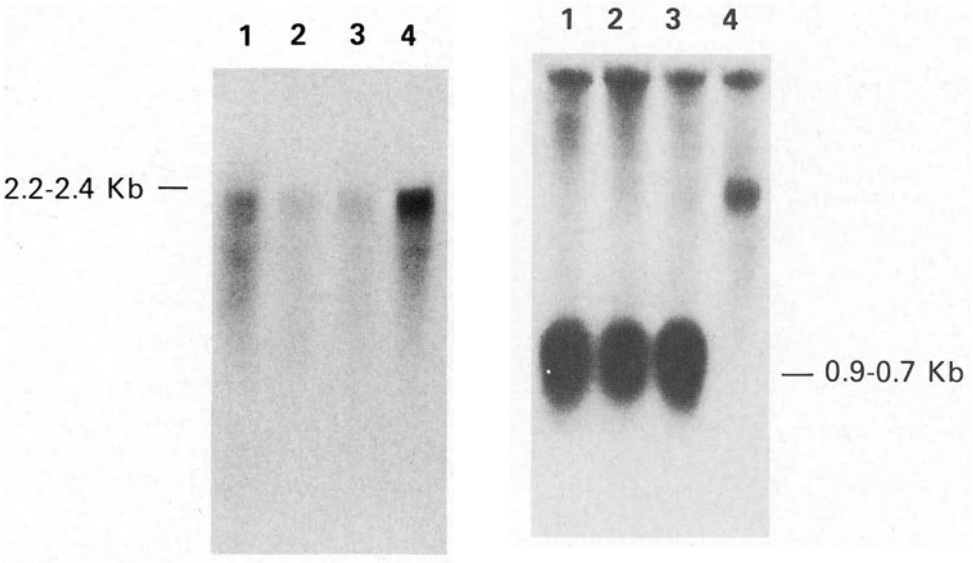

Fig. 3C. Northern blotting analysis of RNA of hybrids between Burkitt lymphoma and GM1500-6TG-OUB lymphoblastoid cells. C. Left panel, the blot was hybridized with the Ryc 7.4 probe; lane 1, ST486; lanes 2-3, hybrid 286-2D3 and 286-1C1 RNAs respectively; lane 4, GM1500-6TG-OUB RNA. Right panel, the blot was hybridized with the c-myc first exon probe; lane 1, ST486 RNA; lanes 2-3 hybrid 286-2D3 and 286-1C1 RNAs respectively; lane 4, GM1500-6TG-OUB RNA.

Since it is possible to distinguish between the transcripts of the normal and of the translocated and rearranged c-myc gene by using the first intron probe (ar-Rushdi 1983), we examined the CA46 x GM1500-6TG-OUB and the ST486 x GM1500-6TG-OUB hybrids for the expression of either the germ-line or the rearranged and translocated c-myc gene. As shown in Fig. 4 all the lymphoblastoid Burkitt hybrids that retained both the germ-line and the rearranged c-myc oncogene expressed only the normal 322 nucleotides band and not the translocated 930, 810 and 770 nucleotides bands c-myc oncogene transcripts (Table 1).

Thus, we conclude that while the normal c-myc oncogene is expressed, the translocated c-myc oncogene is repressed in lymphoblastoid cells (Fig. 4 and Table 1) and that the juxtaposition of the translocated c-myc oncogene to the heavy chain locus is insufficient to activate c-myc transcriptions.

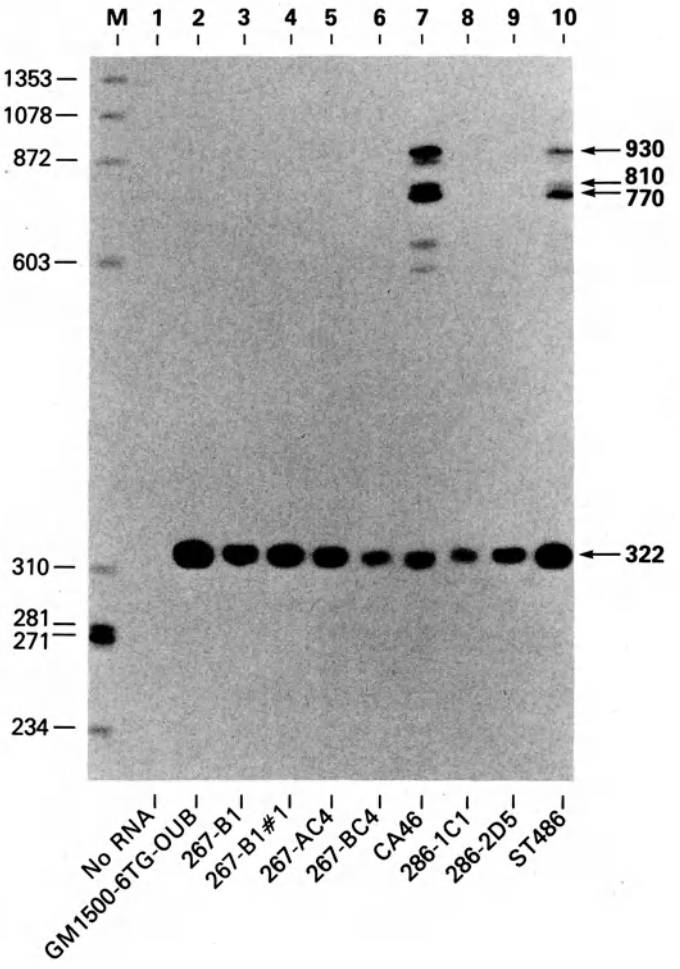

Fig. 4. S_1 nuclease mapping analysis for aberrant c-myc transcripts in the hybrid cells between GM1500-OUB and Burkitt lymphoma cell lines. S_1 probe was prepared from the human genomic c-myc clone pc-myc 41·HE (ar-Rushdi 1983). A DNA fragment XbaI-BstEII, encompassing a part of the first intron and the second exon, 5'-^{32}P-labeled at the 5'-end of the BstEII site, was used as an S_1 probe. Conditions for S_1 nuclease analysis were as described (Nishikura 1983). Hybrid 267-B1#1 derived from a tumor obtained from injecting hybrid 267-B1 into a nude mouse. The results with hybrid 267-AD5 were essentially identical to those obtained with hybrid 267-BC4 (data not shown).

In order to be activated, the translocated c-myc oncogene must be present in Burkitt cells or in plasma cells, since we have previously shown that the translocated c-myc oncogene is expressed in a plasmacytoma background (Croce 1983; Erikson 1983a; Nishikura 1983). To confirm this observation, we hybridized ST486 Burkitt lymphoma cells with NP3 mouse myeloma cells and examined hybrids for the expression of the translocated and of the normal c-myc oncogene. As shown in Fig. 5A and 5B, hybrids containing the translocated and the normal c-myc oncogene expressed only the translocated c-myc oncogene and not the normal c-myc gene transcripts. Thus, the normal and the translocated c-myc oncogene are regulated differently in lymphoblastoid cells and in plasma cells (Table 1).

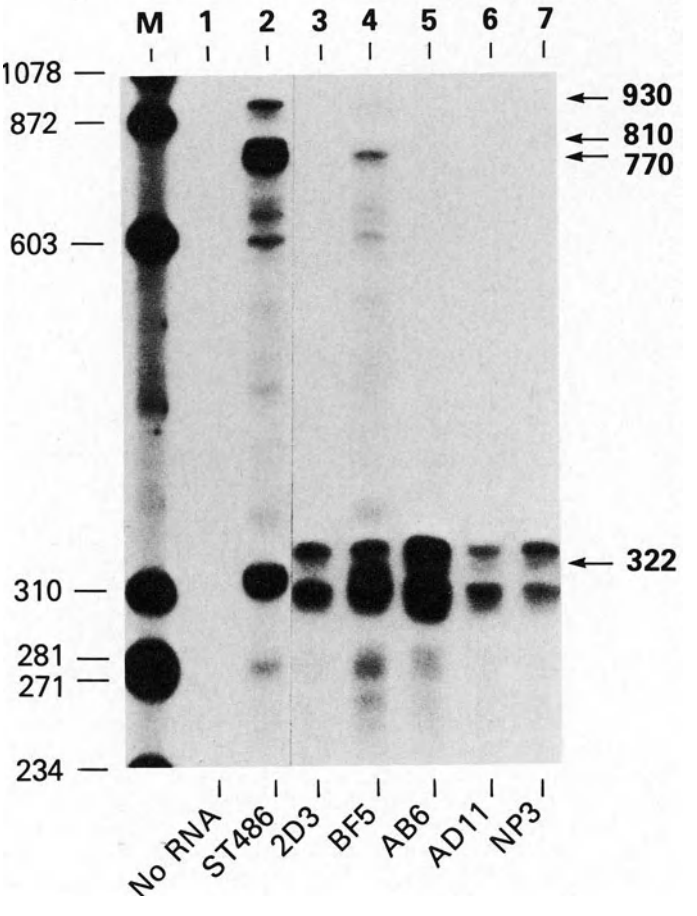

Fig. 5. (A). S_1 nuclease mapping analysis for aberrant c-myc transcripts in the hybrids between NP3 and Burkitt lymphoma cell lines. The double-stranded XbaI-BstEII DNA fragment prepared from pc-Myc 41·HE clone, ^{32}P-labeled at the 5'-end of the BstEII site was used as an S_1 nuclease analysis were as described (Nishikura 1983).

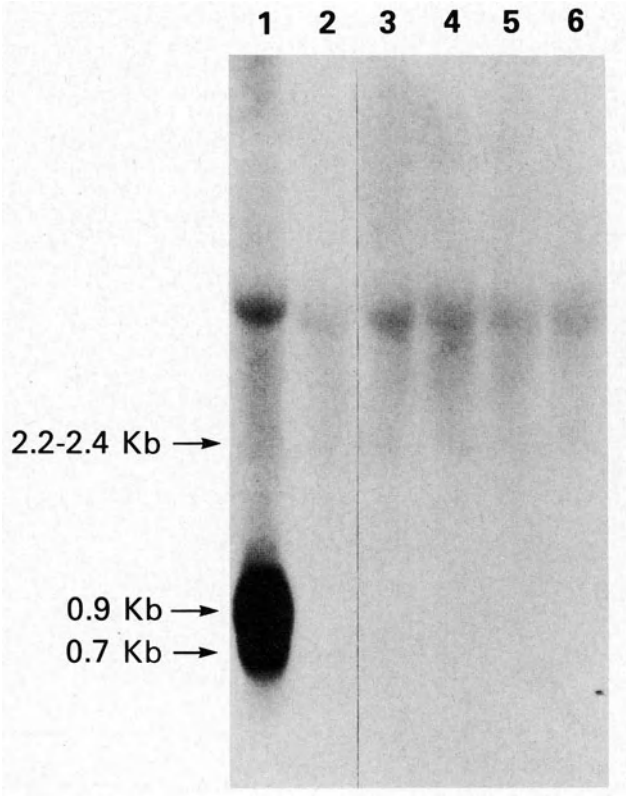

Fig. 5. (B). Northern blotting analysis of hybrids between ST486 and NP3 using a probe specific for the first exon of the c-myc oncogene. Lane 1, ST486 RNA; lane 6, NP3 RNA; lane 2-5 hybrids between ST486 and NP3 cells which contain the separated first exon of the c-myc oncogene. Hybrid 287-2D3 BF5 RNA is in lane 3. Since we do not observe the 2.2-2.4 kb myc RNA we can conclude that the hybrids do not express the normal myc gene.

Because we observed previously that the c-myc first exon which is separated by the chromosomal break, is transcribed at high levels in ST486 Burkitt lymphoma cells (ar-Rushdi 1983), we also examined the two ST486 x GM1500-6TG-OUB hybrids for the expression of the two short transcripts derived from the separated first myc exon (0.9-0.7 kb). As shown in Fig. 3C (right panel), the hybrids expressed very high levels of transcripts of the first exon.

We have also examined the transcription of the separated first c-myc exon in hybrids between NP3 and ST486 cells. As shown in Fig. 5B, the plasma cell hybrids containing the separated first exon of the human c-myc gene failed to transcribe it (Table 1).

DISCUSSION

The results of the analysis of somatic cell hybrids between Burkitt lymphoma and human lymphoblastoid cells indicate that these hybrids, which are morphologically identical to the lymphoblastoid parental cells, transcribe the normal c-myc oncogene but not the translocated c-myc oncogene. On the contrary, hybrids

between Burkitt lymphoma and plasma-cells, which are morphologically identical to the plasmacytoma parental cells, express the translocated c-myc oncogene but not the untranslocated gene on the normal chromosome 8. Thus we postulate the existence of cell type-specific transacting factors, which are active in plasma cells and Burkitt lymphoma cells but are inactive or absent in lymphoblastoid cells and which interact with the rearranged immunoglobulin loci and the c-myc oncogene located in their proximity resulting in the activation of the c-myc oncogene involved in the translocation.

Thus in addition to the immunoglobulin heavy chain gene enhancer (Mercola 1983), which is separated from the c-myc oncogene in some Burkitt lymphomas with a head-to-head rearrangement between the c-myc oncogene and one of the heavy chain genes (ar-Rushdi 1983), a different class of transcriptional enhancing elements must exist. Such enhancing elements must be present within all three immunoglobulin loci since we observe enhanced transcription of the c-myc oncogene involved in the translocation with both the heavy chain and the kappa and lambda light chain loci in plasma cell hybrids. Interestingly, these enhancing elements seem to have the ability to activate gene transcription even if they are located more than 30-50 kb away from the activated gene (Emanuel 1984). This property distinguishes them from immunoglobulin enhancers that have been described, which can activate transcription within 5-20 kb (Khoury 1983; Picard 1984). At present it is not clear why the hybrids between either Burkitt lymphoma and GM1500-6TG-OUB cells express levels of transcripts of the normal c-myc gene which are lower than in GM1500-6TG-OUB parental cells. It is possible that the high levels of myc expression in the GM1500-6TG-OUB lymphoblastoid cells are due to mutation and consequent inactivation of a gene that regulates c-myc transcription. If this is the case, this functional regulatory element could be provided by the Burkitt lymphoma cells, resulting in a decrease of the levels of c-myc transcripts.

Of considerable interest is the fact that the separated first exon of the c-myc oncogene of ST486 cells is transcribed at high levels in ST486 cells and in hybrids with lymphoblastoid cells, but is shut off in hybrids with plasma cells. Since the enhancer between the J and the switch region of the u gene is located 3' of this exon in ST486 cells (Dalla Favera 1983; ar-Rushdi 1983), it is likely that this enhancer is responsible for the high levels of transcripts of the first exon. However, transcription of the ST486 myc first exon does not occur in plasma cells. Thus it seems likely that plasma-cells produce factors capable of inactivating the myc oncogene on normal chromosome 8. It is not clear, however, why in ST486 Burkitt lymphoma cells the normal c-myc gene is repressed and the separated first exon is expressed (ar-Rushdi 1983). It is possible that the close proximity of the heavy chain enhancer to the separated first exon can overcome the transcriptional block to which the normal c-myc oncogene is subjected, in Burkitt lymphoma cells but not in plasma cells.

Recently two reports by Leder et al (1983) and by Saito et al (1983) have proposed two different models to explain the involvement of the c-myc oncogene in Burkitt lymphomas. Leder et al (1983) have suggested that rearrangements involving the first exon of the c-myc oncogene or its separation from the two coding exons results in its failure to respond to a repressor capable of shutting off the expression of the gene. This model is inconsistent with our findings that the rearranged c-myc gene of Burkitt lymphoma is repressed in lymphoblastoid cells. If the deregulation of the c-myc oncogene were due to the structural alteration of the gene, the c-myc gene should have been expressed in the lymphoblastoid cells. In additionally this model and the translational control model proposed by Saito et al (1983) fail to consider that in many Burkitt lymphomas with the t(8;14) translocation the involved c-myc gene is not rearranged (Dalla Favera 1982, 1983, Erikson 1983, 1983a; Croce 1983) and that in the variant lymphomas with the t(2;8) and t(8;22) translocation the rearrangement does not involve sequences 5' of the coding exons, but occurs distally (3') to the c-myc oncogene (Croce 1983; Erikson 1983a).

REFERENCES

ar-Rushdi, A, Tan, KB, Croce, CM (1982) Transcriptional control of the expression of mouse globin genes in myeloma x erythroleukemia cell hybrids. Somatic Cell Genetics 8: 151-161.

ar-Rushdi, A, Nishikura, K, Erikson, J., Watt, R., Rovera, G, Croce, CM (1983) Differential expression of the translocated and of the untranslocated c-myc oncogene in Burkitt lymphoma. Science 222: 390-393.

Croce, CM (1977) Assignment of the integration site for Simian virus 40 to chromosome 17 GM54VA, a human cell line transformed by Simian virus 40. Proc. Natl. Acad. Sci. USA 74: 315-319.

Croce, CM, Shander, M, Martinis, J, Cicurel, L, D'Ancona, GG, Dolby, TW, Koprowski, H (1979) Chromosomal location of the human immunoglobulin heavy chain genes. Proc. Natl. Acad. Sci. USA 76: 3416-3419.

Croce, CM, Linnenbach, A, Hall, W, Steplewski, Z, Koprowski, H (1980) Production of human hybridomas secreting antibodies to measles virus. Nature 288: 488-489.

Croce, CM, Thierfelder, W, Erikson, J, Nishikura, K, Finan, J, Lenoir, G, Nowell, PC (1983) Transcriptional activation of an unrearranged and untranslocated c-myc oncogene by translocation of a Cλ locus in Burkitt lymphoma. Proc. Natl. Acad. Sci. USA 80: 6922-6926.

Dalla Favera, R, Bregni, M, Erikson, J, Patterson, D, Gallo, RC, Croce, CM (1982) Assignment of the human c-myc onc-gene to the region of chromosome 8 which is translocated in Burkitt lymphoma cells. Proc. Natl. Acad. Sci. USA 79: 7824-7827.

Dalla Favera, R, Martinotti, S, Gallo, RC, Nowell, PC, Croce, CM (1983) Translocation and rearrangements of the c-myc onc-gene in human undifferentiated B-cell lymphomas. Science 219: 963-967.

Erikson, J, Croce, CM (1982) Secretion of human immunoglobulins by mouse myeloma x Daudi cells. Eur. J. Immunol. 12: 697-701.

Erikson, J, Finan, J, Nowell, PC, Croce, CM (1982) Translocation of immunoglobulin V_H genes in Burkitt lymphoma. Proc. Natl. Acad. Sci. USA 79: 5611-5615.

Erikson, J, ar-Rushdi, A, Drwinga, HL, Nowell, PC, Croce, CM (1983) Transcriptional activation of the translocated c-myc oncogene in Burkitt lymphoma. Proc. Natl. Acad. Sci. USA 80: 820-824.

Erikson, J, Nishikura, K, ar-Rushdi, A, Finan, J, Emanuel, B, Lenoir, G, Nowell, PC, Croce, CM (1983a) Translocation of an immunoglobulin kappa locus to a region 3' of an unrearranged c-myc oncogene enhances c-myc transcription. Proc. Natl. Acad. Sci. USA 80: 6922-6926.

Emanuel, B, Selden, JR, Chaganti, RSK, Jhanwar, S, Nowell, PC, Croce, CM (1984) The 2p breakpoint of a 2;8 translocation in Burkitt's lymphoma interrupts the V kappa locus. Proc. Natl. Acad. Sci. USA (In Press).

Khoury, G, Gruss, P (1983) Enhancer Elements. Cell 33: 313-314.

Kozbor, D, Lagarde, AE, Roder, JC (1982) Human hybridomas constructed with antigen-specific Epstein-Barr virus transformed cell lines. Proc. Natl. Acad. Sci. USA 79: 6651-6655.

Leder, P, Battey, J, Lenoir, G, Maulding, G, Murphy, W, Potter, H, Stewart, T, Taub, R (1983) Translocations among antibody genes in human cancer. Science 222: 765-771.

Littlefield, JW (1964) Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145: 709-710.

Maniatis, T, Jeffrey, A, Van Sande,H (1975) Chain length determination of small double- and single-stranded DNA molecules by polyacrylamide gel electrophoresis. Biochemistry 14: 3783-3794.

Maxam, AM, Gilbert, W (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymology 65: 499-560.

Mercola, M, Wang, X, Olsen, J, Calame, K (1983) Transcriptional enhancer elements in the mouse immunoglobulin heavy chain locus. Science 221: 663-665.

Nishikura, K, ar-Rushdi, A, Erikson, J, Watt, R, Rovera, G, Croce, CM (1983) Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc. Natl. Acad. Sci. USA 80: 4822-4826.

Picard, D, Schaffner, W (1984) A lymphocyte-specific enhancer in the mouse immunoglobulin gene. Nature (London) 307: 80-82.

Saito, H, Hayday, AC, Wimar, K, Hayward, WS, Tonegawa, S (1983) Activation of the c-myc gene by translocation: A model for translational control. Proc. Natl. Acad. Sci. USA 80: 7476-7480.

Sharp, RA, Berk, AJ, Berget, SM (1980) Transcriptional maps of adenovirus. Methods Enzymology 65: 750-768.

Southern, EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Thomas, PS (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77: 5201-5205.

Weaver, RF, Weissman, C (1979) Mapping of RNA by a modification of the Berk-Sharp procedure: the 5' termini of 15 S β-globin mRNA precursor and mature 10 S β-globin mRNa have identical map coordinates. Nucleic Acids Res. 7: 1175-1193.

Modes of c-myc Oncogene Activation in Murine Plasmacytomas

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INTRODUCTION

The murine c-myc proto-oncogene is activated and inappropriately expressed in murine plasmacytomas (PCs') by virtue of a reciprocal 12;15 chromosomal translocation (Harris et al.,1982a; Shen-Ong et al.,1982; Crews et al.,1982; Marcu et al.,1983; Adams et al.,1983). In most PCs', the c-myc gene is broken by the rcpt(12;15) thereby generating a complex family of aberrant myc transcripts which initiate from normally silent promoters within the first myc intron (Marcu et al.,1983; Adams et al.,1983; Stanton et al.,1983). The disruption of the c-myc locus in the PCs' generally results in elevated levels of such truncated transcripts (Marcu et al.,1983; Mushinski et al.,1983). However, it would seem that the more general consequence of the myc associated rcpt(12;15) is the inappropriate, constitutive expression of c-myc since the normal myc allele is transcriptionally silent in these tumors (Adams et al., 1983; Stanton et al.,1983; Bernard et al.,1983).

The c-myc gene consists of three exons but only the 3' two are coding (Stanton et al.,1983; Bernard et al.,1983). The first exon, which is removed or disrupted by the translocation is a 569 bp non-coding sequence with translation termination codons in all three reading frames and more significantly no ATG initiation codon (see Figure 1) (Stanton et al.,1983; Bernard et al.,1983; Stanton et al.,1984). Therefore, the breakage of the c-myc gene in the PCs' does not alter its coding potential (Stanton et al.,1983). Interestingly, the first exon of the intact c-myc gene has two functional transcription promoters (Bernard et al.,1983; Kelly et al.,1983; Stanton et al.,1984; Marcu et al., 1984) which are also conserved in the human c-myc gene (Battey et al.,1983; Ar-Rushdi et al.,1983; Taub et al.,1984).

In this report, we will discuss other modes of c-myc activation which are also a consequence of myc associated chromosome translocations and/or aberrations in the PCs'. We will also attempt to assimulate these phenomena into a common mechanism of c-myc activation in these neoplasias.

C-MYC TRANSLOCATION IN MURINE PLASMACYTOMAS DOES NOT GENERALLY INVOLVE ANY QUALITATIVE ALTERATIONS IN THE STRUCTURE OF THE C-MYC GENE

We were interested to assess whether mutations in the murine c-myc gene might also be responsible for c-myc activation in the PCs'. To this end, we have completed the nucleotide sequence of a c-myc cDNA clone prepared from normal BALB/c spleen RNA and compared this sequence to those of two rearranged c-myc genes which have retained different portions of the first exon, J558 (Stanton et al.,1983; Bernard et al.,1983) and MPC-11 (Stanton et al.,1984). The cDNA clone was virtually full length only lacking 19 bp at its 5' end. All three c-myc exons were sequenced in the J558 rearranged myc gene. The J558 myc gene suffered a breakage 175 bp 3' of c-myc promoter 1 thereby leaving 384 bp of the non-coding exon attached to the expressed 3' gene segment (see Figure 1). The J558 rearranged myc gene has recombined with the switch region of the C_α

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immunoglobulin heavy chain gene (Harris et al.,1982a). The MPC-11 myc gene was also broken within exon 1 (see Figure 1) (Harris et al.,1983). In MPC-11, we have cloned and sequenced the exon 1 regions of the 5' and 3' reciprocal translocation products (Stanton et al.,1984). The MPC-11 rearranged myc gene has directly recombined with the $C_{\gamma 2a}$ gene's switch region ($S_{\gamma 2a}$) which resulted in the loss of only 11 bp of c-myc exon 1 sequence (Stanton et al.,1984). There are no mutations in either of these rearranged c-myc genes including the 5' reciprocal product of MPC-11 in comparison to the BALB/c spleen c-myc cDNA clone sequence. We conclude that such qualitative changes in the c-myc gene are not a generally important mechanism for either c-myc activation or deregulation in the PCs'.

Two recent structural studies on myc genes in several Burkitt lymphomas (BL) in humans have shown that numerous mutations can accumulate whether the myc locus is broken or not by the translocation (Rabbitts et al.,1983; Taub et al.,1984). In attempting to reconcile these observations on the human myc locus with our results in the mouse, it may be important to consider that the PCs' represent a more terminal stage in lymphocyte differentation than the BLs'. Indeed, the c-myc translocation in PCs' may occur later in B cell differentiation than the analogous translocation in BL. This argument is also borne out by the most frequent targets for c-myc translocation in these neoplasias. The rearranged myc genes in the PCs' are generally found with C_H switch regions downstream from S_μ (Harris et al.,1982b; Adams et al.,1982; Calame et al.,1982; Shen-Ong et al.,1982 Harris et al.,1983) while the myc rearrangements in BL almost exclusively involve the S_μ region (Dalla-Favera et al.,1983; Adams et al.,1983; Leder et al.,1983; Bernard et al.,1983). Somatic mutations may be more common in BL and possibly result from the close association of the myc locus with an immunoglobulin gene. It remains important to demonstrate whether somatic mutations are necessary or essential for either c-myc activation or deregulation in BL.

EXPRESSION OF AN INTACT C-MYC GENE IN MURINE LYMPHOID TUMORS

A number of murine plasmacytomas do not possess a broken c-myc gene but nevertheless contain some type of chromosomal aberration involving the c-myc locus on chromosome 15. These chromosomal aberrations consist of the more common rcpt(12;15), the variant rcpt(6;15) (Ono et al.,1979) and rare examples of PCs' without detectable translocations which contain a chromosome 15 band deletion (del 15) (Weiner, F personal communication). Interestingly, the latter del 15s' occur at the precise chromosome 15 breakpoint (Band D_2/D_3) of the more commonly observed translocations (Ono et al.,1979; Weiner,F personal communication). A number of these tumors were induced by a combination of Pristane and Abelson murine leukemia virus (AbLV) and are termed ABPCs' (Mushinski 1983). The level of myc RNA expressed in the ABPC series is higher than in normal splenic B cells but comparable to the level of myc expression in pre-B lymphomas and lymphosarcomas which were also induced with AbLV (Mushinski et al.,1983; Mushinski 1983).

We decided to compare the modes of c-myc expression by S_1 nuclease analysis in the ABPCs' and other lymphoid tumors which harbor an intact myc gene. For this purpose, an M13 phage derived 1.05 kb DNA fragment containing 535 bp 5' of c-myc and the 5' 515 bp of the 569 bp exon 1 sequence was uniformly labeled and used in an S_1 nuclease protection assay with poly(A)$^+$ and total RNAs obtained from a variety of lymphoid tumor samples. Several representative examples of this analysis are shown in Figure 2 and the results of a survey are presented in Table I. Two S_1 nuclease protected bands corresponding to transcripts originating from one of the two normal myc gene promoters in exon 1 (P_1 and P_2) were detected in all samples tested with one exception (only P_2 is active in ABPC 48) (Table I). Densitometry tracings of the autoradiograms were prepared and the relative outputs of P_1 vs P_2 were assessed (see Table I). ABPCs' or PCs' (NZB strain in origin) with a defined 5' c-myc rearrangement (i.e. an

intact c-myc gene with a rearrangement within 10 kb 5' of exon 1) (ABPC 17, ABPC 33, TEPC 1194, TEPC 1165 and TEPC 1033) utilize P_1 in favor of P_2. Plasmacytomas which display no rearrangement within 10 kb 5' of c-myc, generally favor P_2 over P_1 with several exceptions where P_1=P_2 or P_2 is only somewhat or slightly preferred over P_1 (ABPC 22, CBPC 112, ABPC 60 and PC 3741). All the pre B lymphomas and lymphosarcomas tested favor P_2 over P_1 essentially to the same degree as the plasmacytomas in this category (ABPC 4, ABPC 20, ABPC 103, ABPC 105, ABPC 26, ABPC 47, ABPC 52, ABPC 89 and ABPC 65). One particular pre B lymphoma, 54C12, expresses P_2 at a 50x higher level than P_1 (see Figure 2 and Table I). However, 54C12 is atypical in that it contains an amplified myc locus with 25-30 intact c-myc genes (unpublished results). It is also interesting to mention that the level of myc transcripts in 54C12 is only 4 fold greater than in two other AbLV pre B lymphomas examined. The amount of myc RNA in 54C12 cells is therefore not proportional to myc gene dosage. The simplest explanation for this observation is that myc levels are down regulated in 54C12, and this is mainly controlled by P_1.

Several significant conclusions can be derived from this survey concerning the basis for inappropriate c-myc expression in these neoplasias. A shift in normal myc promoter ratios in favor of P_1 would appear to result from translocations which occur in close proximity to the 5' end of the c-myc gene. A more active P_1 promoter could conceiveably result from the removal of 5' c-myc inhibitory sequences or from the translocation of a transcriptional enhancer element in close proximity 5' of c-myc. This conclusion is also supported by the low P_1/P_2 ratio in MOPC 104E (see Table I). MOPC 104E contains a broken c-myc gene and its normal myc allele is transcriptionally silent (Cole, M personal communication). Therefore, the low P_1/P_2 ratio in MOPC 104E derives from the 5' reciprocal product of the broken c-myc gene which probably lacks any 5' c-myc rearrangement. We would predict that rearrangement negative plasmacytomas, with P_1=P_2 or P_2 only somewhat favored to P_1 (ABPC 22, CBPC 112, ABPC 60 and PC 3741), are likely to have a chromosome breakage within 20 kb 5' of c-myc. The shift in promoter usage in two of the del 15 plasmacytomas demonstrates that the immunoglobulin loci in chromosomes 6 and 12 are not essential in this phenomenon. It is also important to note that a shift in promoter usage is not a hallmark of the myc associated chromosome translocation. Therefore, a high or inappropriate P_1/P_2 ratio can probably be used as a molecular indicator of a nearby translocation but is not likely to be responsible for the inappropriate, deregulated expression of this oncogene. In addition, since the exon 1 probe was completely protected by essentially all of the RNAs tested, there is no obvious evidence of any significant qualitative changes (i.e. mutations) in the myc exon 1 sequences in any these plasmacytomas except for one (see TEPC 1194 in Table I). This would imply that qualitative myc alterations akin to those in the Burkitt lymphomas (Rabbitts et al.,1983; Taub et al.,1984) are not common in the PCs'. Therefore, we conclude that subtle alterations in c-myc gene structure are not likely to be responsible for the inappropriate, constitutive level of c-myc RNAs in these tumors. We suggest that a higher order change in chromatin structure is caused by either translocation or chromosomal aberration and that this is the primary lesion transmitted to the c-myc locus. This notion would be compatible with the observation that the normal c-myc allele is transcriptionally inactive in these tumors (Adams et al.,1983; Stanton et al.,1983; Bernard et al.,1983). The latter phenomenon may either be a characteristic of c-myc expression in these differentiated cells or result from a repressive mechanisim mediated directly or indirectly by the c-myc gene product as originally suggested in Burkitt lymphomas (Leder et al.,1983; Nishikura et al.,1983).

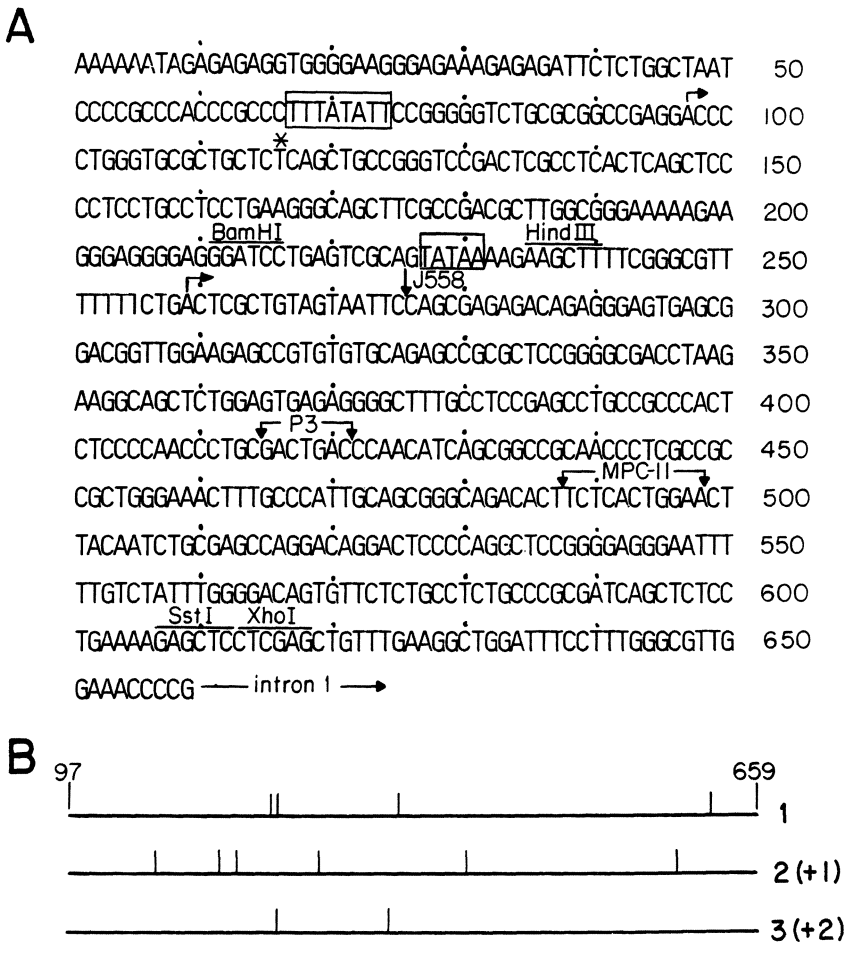

Fig. 1. A. Nucleotide sequence of murine c-myc exon 1. Promoter sequences are boxed and transcription intiation sites are indicated by horizontal arrows. An asterik indicates the 5' end of a BALB/c spleen derived c-myc cDNA clone. The translocation breakpoints in several plasmacytomas are indicated by vertical arrows: J558 (Stanton et al.,1983; Bernard et al.,1983), MPC-11 (Stanton et al.,1984) and P3 (Neuberger and Calabi 1983; Dunnick et al., 1983).

B. Location of stop codons in the three translation reading frames of exon 1.

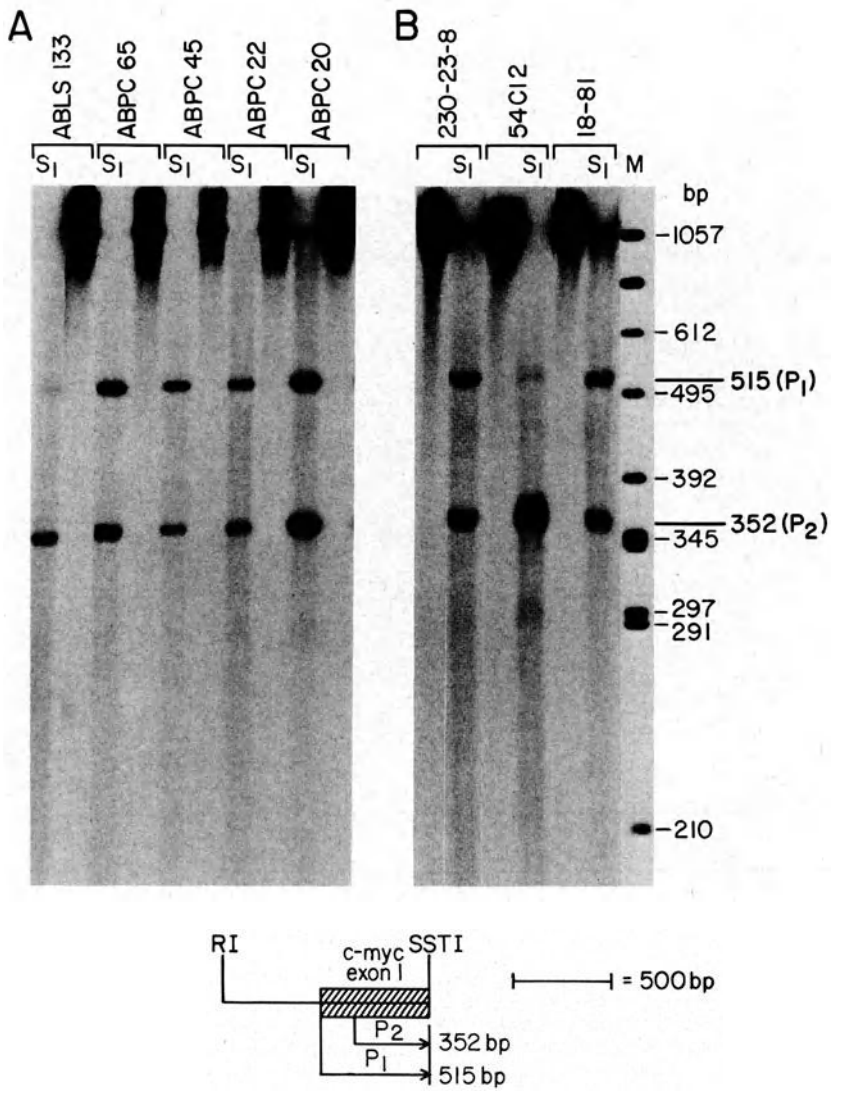

Fig. 2. A and B: A uniformly labeled probe derived from c-myc exon 1 and 5' flanking sequences was hybridized at 58°C overnight to comparable amounts of either poly(A)$^+$ (ABLS 133, ABPC 65, ABPC 45, ABPC 22 and ABPC 20) or total cytoplasmic RNA (230-23-8, 54C12 and 18-81). The RNA:DNA hybrids were treated with 100 units of S_1 nuclease for 1 hr. at 37°C. Samples were analyzed before and after S_1 treatment on a 7M urea polyacrylamide gel. The location of S_1 protected bands corresponding to normal c-myc gene promoters 1 and 2 are indicated.

Table 1. Relative Activity of Two c-myc Promoters in Murine Lymphoid Tumors

		Rearranged c-myc Gene	P1/P2[a]
	Balb/c spleen		0.43
	Balb/c thymus		0.432
rcpt (12;15)	ABPC 47	-	0.42
	ABPC 52	-	0.49
	ABPC 89	-	0.39
	ABPC 60	-	0.85
	ABPC 65	ND	0.54
	ABPC 48	-	P_2 only
	ABPC 33	+ (5'c-myc)[f]	1.71[b]
	TEPC 1194	+ (5'c-myc)[f]	2.62[b]
	TEPC 1165	+ (5'c-myc)[f]	1.79
	TEPC 1033	+ (5'c-myc)[f]	1.49
	MOPC 104E	+ (c-myc intron 1)	0.35[c]
rcpt (6;15)	ABPC 4	-	0.48
	ABPC 20	-	0.38
	ABPC 22	-	0.71
	ABPC 103	-	0.18
	ABPC 105	-	0.50
	ABPC 17	+ (5'c-myc)[f]	2.23
del 15	CBPC 112	-	1.05
	ABPC 45	+ (5'c-myc)[f]	0.75
	ABPC 26	-	0.42
	PC 3741[e]	-	1.07
	PC 7183[e]	-	0.44
AbLV Lymphosarcomas B lymphoma	ABLS 133	-	0.09
	ABLS 5	-	0.45
	WEHI 231	-	0.11
AbLV pre B lymphomas	1881.4	-	0.34
	1881	-	0.35
	230-23-8	-	0.32[d]
	54C12	(25-30 c-myc genes)	0.02[d]

(a) Ratios of densitometric tracings of autoradiograms of 515 and 352 bp S_1 nuclease protected fragments corresponding to transcripts initiating from promoters 1 and 2 (P_1 & P_2) of the c-myc gene. Since the S_1 probe was uniformly labeled, corrections have been made due to the larger size of P_1 protected bands.
(b) The sizes of the S_1 nuclease protected fragments were 467 and 331 bp.
(c) P_1 and P_2 expression arising from the 5'reciprocal fragment of a broken myc gene.
(d) Relative level of P_1+P_2 in 54C12 is ~4 fold higher than observed in 18-81 or 230-23-8.
(e) NZB plasmacytomas of unknown karyotype.
(f) Site of DNA Rearrangement is within 10 kb 5' of c-myc exon 1.

REFERENCES

Adams JM, Gerondakis S, Webb E, Mitchell J, Bernard O, and Cory S (1982) Transcriptionally active DNA region that rearranges frequently in murine lymphoid tumors. Proc. Natl. Acad. Sci. USA 79: 6966-6970.

Adams JM, Gerondakis S, Webb E, Corcoran LM and Cory S (1983) Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc. Natl. Acad. Sci. USA 80: 1982-1986.

Ar-Rushdi A, Nishikura K, Erikson J, Watt R, Rovera G and Croce, C.M. (1983) Differential expression of the translocated and the untranslocated c-myc oncogenes in burkitt lymphoma. Science 222: 390-393.

Battey J, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G and Leder P (1983). The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell 34: 799-787.

Bernard O, Cory S, Gerondakis S, Webb E, and Adams JM (1983) Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumors. EMBO J. 2:2375-2383.

Calame K, Kim S, Lalley P, Hill R, Davis M and Hood L (1982) Molecular cloning of translocations involving chromosome 15 and the immunoglobulin C-alpha gene from chromosome 12 in two murine plasmacytomas. Proc. Natl. Acad. Sci. USA 79: 6994-6998.

Crews S, Barth R, Hood L, Prehn J, and Calame K (1982) Mouse c-myc oncogene is located on chromosome 15 and translocated to chromosome 12 in plasmacytomas Science 218: 1319-1321.

Dalla-Favera R, Martinitti S, Gallo RC, Erikson J and Croce CM (1983) Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219: 963-967.

Dunnick W, Shell BE and Dery C (1983) DNA sequences near the site of reciprocal recombination between a c-myc oncogene and an immunoglobulin switch region. Proc. Natl. Acad. Sci. USA 80: 7269-7273.

Harris LJ, Lang RB and Marcu KB (1982a) Non-Immunoglobulin Associated DNA rearrangements in mouse plasmacytomas. Proc. Natl. Acad. Sci. USA 79: 4175-4179.

Harris LJ, D'Eustachio P, Ruddle FH and Marcu KB (1982b) DNA sequence associated with chromosome translocations in mouse plasmacytomas. Proc. Natl. Acad. Sci. USA 79: 6622-6626.

Harris LJ, Remmers EF, Bordeur P, Riblet R, D'Eustachio P and Marcu KB (1983) C-myc gene rearrangements involving gamma immunoglobulin heavy chain gene switch regions in murine plasmacytomas. Nuc. Acids Res. 11: 8303-8315

Kelly K, Cochran BH, Stiles CD and Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35: 603-610.

Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T and Taub R (1983) Translocations among antibody genes in human cancer. Science 222: 765-771.

Marcu KB, Harris LJ, Stanton LW, Erikson J, Watt R and Croce, C.M. (1983) Transcriptionally active c-myc oncogene is contained within NIARD, a DNA sequence associated with chromosome translocation in B cell neoplasia. Proc. Natl. Acad. Sci. USA 80: 519-523.

Marcu KB, Stanton LW, Harris LJ, Watt R, Yang JQ, Eckhardt LA, Birshtein B, Remmers EF, Greenberg R and Fahrlander P. (1984) Rearrangement and activation of c-myc oncogene by chromosome translocation in B cell neoplasias. Setlow, JK and Hollaender, A.(eds) Genetic Engineering Vol VI, Plenum Press, New York, (in press).

Mushinski JF, Bauer SR, Potter M and Reddy EP (1983) Increased expression of myc-related oncogene in RNA characterizes most BALB/c plasmacytomas induced by pristane or Abelson murine leukemia virus. Proc. Natl. Acad. Sci. USA 80: 1073-1077.

Mushinski JF, (1983) Mechanisms of B Cell Neoplasia. Editones Roches, Basel, pp. 199-209.
Neuberger MS, and Calabi F (1983) Reciprocal chromosome translocation between c-myc and immunoglobulin 2b genes. Nature 305: 240-243.
Nishikura K, Abbas R, Erickson J, Watt R, Rovera G and Croce CM (1983) Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc. Natl. Acad. Sci. USA 80: 4822-4826.
Ohno S, Babonits M, Weiner F, Spira J, Klein G and Potter M (1979) Non-random chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 18: 1001-1007.
Rabbitts TH, Hamlyn PH, Baer R (1983) Altered nucleotide sequences of a translocated c-myc gene in Burkitt lymphoma. Nature 306: 760-765.
Shen-Ong GLC, Keath E, Picolli SP and Cole MD (1982) Novel myc oncogene RNA from abortive immunoglobulin gene recombination in mouse plasmacytomas. Cell 31: 443-452.
Stanton LW, Watt R and Marcu KB (1983) Translocation, breakage and truncated transcripts of c-myc oncogenes in murine plasmacytomas. Nature (London) 303: 401-406.
Stanton LW, Yang JQ, Eckhardt LA, Harris LJ, Birshtein BK and Marcu KB (1984) Products of a reciprocal chromosome translocation involving the c-myc gene in a murine plasmacytoma. Proc. Natl. Acad. Sci. USA, (in press).
Taub R, Moulding C, Battey J, Murphy W, Vasicek T, Lenoir GM and Leder P (1984) Activation and somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. Cell 36: 339-348.

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DNA Sequences Involved in the Rearrangement and Expression of the Murine c-myc Gene

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INTRODUCTION

The characteristic chromosomal translocation found in many B cell tumors results in the joining of a c-myc oncogene (from murine chromosome 15) to an immunoglobulin heavy chain switch region (chromosome 12--Perry, 1983; Robertson, 1983). This translocation has several consequences. One is that the c-myc gene is frequently truncated, losing part or all of its 5'-most exon (Stanton et al., 1983; ar-Rushdi, et al., 1983; Gelman, et al., 1983; Saito, et al., 1983). A second is increased transcription of the truncated c-myc gene; this transcription often originates from adventitious promotors in the first intron (Stanton, et al., 1983; Hamlyn and Rabbitts, 1983; Mushinski et al., 1983; Adams et al., 1983; Erikson, et al., 1983a). Many have hypothesized that a third consequence of this rearrangement is oncogenesis. In virtually all chicken lymphomas induced by avian leukosis virus, the c-myc gene has been activated by proviral insertion (Hayward, et al., 1981). On the other hand, in erythroblastoid tumors induced by the same virus, c-myc is never activated (however, c-erb is activated--Fung, et al., 1983). The conclusion from chickens, that activation of c-myc is necessary for lymphomagenesis, would also seem to apply to activation of murine c-myc by translocation.

We have studied two questions concerning the c-myc translocation. First, do recombinases important to the normal heavy chain switch catalyze the translocation? Second, what is the mechanism of c-myc activation following translocation? Our approach has been to determine the DNA sequence in and near the region of c-myc known to be involved in translocations. We have identified in this region a set of sequences related to immunoglobulin heavy chain switch segments, DNA regions that are important to the DNA rearrangements that mediate the class switch (Davis, et al., 1980). Another unusual feature of this region is the high frequency of CG dinucleotides. Thus, we have considered the possibility that regulation of c-myc expression is either mediated by, or reflected by, the methylation state of the c-myc gene. We have examined the methylation state of c-myc by HpaII digestion both of kidney and liver DNA and of plasmacytoma DNA, following by hybridization to c-myc probes in the Southern blotting technique (1975).

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RESULTS AND DISCUSSION

Switch Recombinases and c-myc Translocations

Previously, we examined the DNA sequences surrounding the translocation sites for five BALB/c c-myc rearrangements (Dunnick et al., 1983). We found a 32 base pair region of partial homology which included the sequence CAGGTTG. Marcu and his colleagues (1982) have found the same sequence upstream of normal heavy chain switch recombination sites. In general, YAGGTTG-like sequences (Y being C or T) are found 7 to 14 base pairs upstream (in the immunoglobulin transcriptional sense) from switch sites. The CAGGTTG sequences lie 8 to 15 base pairs upstream (in the immunoglobulin transcriptional sense) from the 5' translocation site in c-myc (Figure 1). Because this same sequence is found at the same position, in the same orientation, in both normal switches and c-myc translocations, we concluded that switch recombinases recognize YAGGTTG-like sequences in normal switches, and in c-myc translocations (albeit in an abberant way). We have completed the sequence of 1400 base pairs 5' of the murine c-myc gene. We combined our data with that of Stanton and his colleagues (1983), and found many 5 of 7 matches to YAGGTTG in and near the c-myc translocation region. YAGGTTG-like sequences are found in about two-fold excess over what would be expected by random chance, with CAGGTTG sequences predominating. However, these YAGGTTG sequences are not concentrated in the translocation region.

Moreover, if a YAGGTTG-like sequence were the only prerequisite for switch recombinase recognition, then CAGGTTG should have been the only homologous sequences near the c-myc translocation sites. We found modest homology extending about 15 base pairs in either direction from the translocation sites. A modified version of that consensus is shown in Figure 1a. In Figure 1b are shown sequences from the murine c-myc gene around the translocation sites for five different plasmacytomas. These sequences are presented for the antisense strand of c-myc DNA, to put them in the immunoglobulin orientation. If one allows for an insertion of variable length in the middle of the consensus, all five sites share significant homology, including a YAGGTTG-like sequence. Two of the translocation sites (MPC11 and McPC603) have two YAGGTTG-like sequences, one directly following the other. We searched a 3700 base pair region surrounding the 5' most c-myc exon for this homology unit, and found five additional sites (Figure 1c). All of these sites, with the exception of the site at residue 2073, lie within the translocation region of c-myc (Cory et al., 1983). We examined the possibility that this consensus might have homology to some murine heavy chain switch region. It has extensive homology to the 2a switch sequence consensus (Figure 1d). The γ2a switch region is made up of tandemly repeated units of 52 base pairs (Nikaido et al., 1982). Each unit seems to be a duplicated 26 base pair repeat. The c-myc translocation consensus has extensive homology to this 26 base pair subunit; some of the consistent differences are required to generate a YAGGTTG-like sequence. Thus, we conclude that the abberant recognition and translocation of the c-myc gene by switch enzymes requires both YAGGTTG-like sequences and some minimal homology to switch region sequences. This conclusion predicts that most translocation sites will lie in the ten sequences listed in Figure 1b and 1c. In addition, if switch recombinases do recognize c-myc via YAGGTTG-like sequences and γ2a switch region

homology, then the sites of translocation may be restricted by the availability of these sequences (Cory et al., 1983). Alternatively, selection for oncogenesis may restrict the sites of translocation that one can observe (Dunnick et al., 1983).

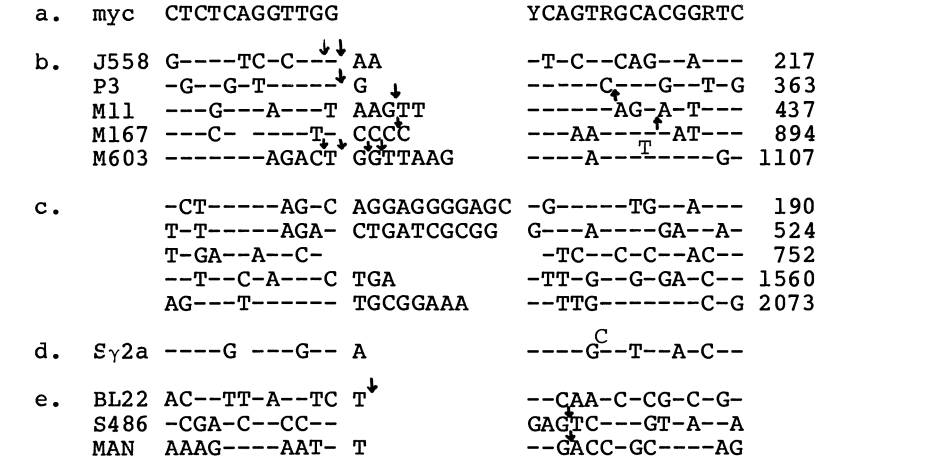

Figure 1: Homology relationships between the c-myc oncogene and the γ2a switch region. a. A consensus sequence generated from the known translocation sites in the murine c-myc is shown. The sequence CAGGTTG is noted by overlining. Throughout the Figure, homology to the c-myc consensus is noted by dashes, deletions by spaces, and insertions by letters above the line. Translocation sites are noted by upward pointing arrows (5') or downward pointing arrows (3'). b. c-myc sequences (anti-sense strand) around the translocation sites in the plasmacytomas J558 (Adams et al., 1983; Stanton et al., 1983), P3 (Neuberger and Calabi, 1983; Dunnick, et al.,1983), MPC11 (Stanton, et al., 1984), MOPC167, and McPC603 (Calame et al., 1982) are shown. c. Five additional regions in the murine c-myc gene with partial homology to the c-myc translocation consensus are shown. Numbering is according to Stanton et al. (1983). The sequence noted by residue 190 was determined by ourselves, and is not found in the sequence published by Stanton et al. (1983). d. Part of the repetitive element (Nikaido et al., 1982) for the Sγ2a segment is shown. This sequence has been interrupted twice, and a nucleotide deleted, to allow for better homology with c-myc sequences. e. Sequences around the translocation site in three Burkitt lymphomas (Battey et al., 1983; E.P. Gelmann et al., 1983; Saito et al., 1983) are shown.

We also examined three sequences surrounding the translocation sites in human Burkitt lymphoma (Figure 1e). These three sequences also demonstrate some YAGGTTG-like sequences, although in each case the match is only 4 of 7. The homology to murine γ2a switch sequences is also much poorer than the murine c-myc sequences, perhaps reflecting the evolutionary divergence of mouse and human switch sequences.

The proposal that switch recombinases recognize weak γ2a switch region homologies in c-myc does not explain two other results.

The first is that c-myc tends to join to μ or α switch sequences, and not to γ2a switch sequences. This is especially perplexing in light of the fact that μ and α switch sequences are not very homologous to γ 2a switch sequences. It is not clear whether this anomoly reflects our lack of understanding of c-myc translocations or of heavy chain switches. Secondly, some c-myc translocations bring this gene near a light chain immunoglobulin gene (Croce, et al., 1983; Erikson, 1983b). It does not seem likely that switch recombinases mediate these translocations. Since there are a wide range of c-myc translocations, with very different resulting structures, perhaps no single proposal can account for them all.

Activation of c-myc expression by translocation

Transcription of the c-myc gene is increased following translocation (Stanton et al., 1983; Erikson et al., 1983; Mushinski, et al., 1983; ar-Rushdi et al., 1983; Rabbitts and Hamlyn, 1983; Adams et al., 1983). Some have suggested that this transcriptional activation is the result of c-myc coming under the influence of a new enhancer element (Erikson et al., 1983b). We (Dunnick et al., 1983) and others (Leder et al., 1983) have suggested that the deregulation of c-myc gene might be the result of escape from a negative regulatory protein, perhaps even c-myc protein itself. This negatively acting protein has been hypothesized to act at the 5' end of the c-myc gene, the region of the gene lost in most translocations. One of the most attractive features of negative control is that it explains the lack of c-myc transcripts from the normal (non-translocated) allele in B cell tumors (Stanton et al., 1983; Nishikura et al., 1983). We have sequenced an extensive portion of the 5' environment of the murine c-myc gene, and Battey and his colleagues (1983) have sequenced a similar portion of the human gene. In Figure 2 these two sequences are compared. The human and mouse genes retain extensive homology to at least 700 base pairs (the extent of the human sequence) 5' of the transcription start site. This remarkable homology suggests a function for these sequences, perhaps in negative regulation.

In addition, we found that this region has an unusually high number of CG dinucleotides. CG dinucleotides are found about ten times more frequently in this region than in other eucaryotic genes (Subak-Sharpe, 1967). This high frequency is reflected in the number of HpaII (recognition sequence CCGG) restriction enzyme cleavage sites, the largest HpaII fragment being only 300 base pairs in length. CG dinucleotides are one substrate for DNA methylation, and there is a good correlation between the transcriptional activation of eucaryotic genes and undermethylation of CG dinucleotides (Razin and Riggs, 1980). Because HpaII does not cleave the methylated sequence, it can be used as a probe for methylated DNA. Using HpaII cleavage, and the Southern (1975) hybridization technique, we examined the methylation state of c-myc in a mixture of kidney and liver DNA, and in P3 plasmacytoma DNA (Table 1). Of course, neither allele is rearranged in kidney and liver DNA, so both c-myc genes are found in a 9.3 kb KpnI fragment. With the NIARD probe (for the second and third exons and introns of c-myc), we detect both the germline and translocated alleles (9.3 and 6.5 kb fragments) in P3 DNA. HpaII digestion of kidney and liver DNA reveals that a single HpaII site, or group of sites, is undermethylated in these DNA samples, the bulk of the c-myc gene remaining in two large

HpaII fragments. In P3 DNA, at least one allele is extensively undermethylated, yielding small HpaII fragments (many of which are probably too small to be detected). Since the translocated allele is actively transcribed, it seems likely that this allele would be undermethylated. One c-myc gene in P3 DNA is more extensively methylated that the corresponding gene in kidney and liver DNA, yielding a 5.8 kb HpaII fragment. Our preliminary results indicate that this fragment, and the 4.0 kb fragment, hybridize to both the NIARD probe and the 5' flanking region probe. Thus, these fragments must originate from the normal allele. It would appear that the more extensive methylation at the 5' end of the normal allele in plasmacytoma DNA correlates well with its low rate of transcription. It should also be noted that 5' end of the translocated allele, separated from the bulk of the c-myc gene, does not appear to be methylated. No fragments that hybridize to the 5' probe, but not to the NIARD probe, are detected.

Table 1. Methylation patterns of c-myc DNA in kidney and liver DNA and in plasmacytoma DNA.

Probe:	NIARD (2nd and 3rd c-myc exons and introns)		5' flanking sequences of c-myc	
DNA sample:	kidney/liver	P3	kidney/liver	P3
Digest				
KpnI strong	9.3	9.3,6.5	9.3	9.3
KpnI/ strong	4.0,3.4	5.8,2.4,1.4,1.2	4.0	5.8
HpaII weak	2.4,1.4,1.2	3.4		4.0

High molecular weight DNA from pooled kidneys and livers or from plasmacytoma P3 was digested with KpnI alone or with KpnI and HpaII, fractioned on a 0.8% agarose gel, and blotted onto nitrocellulose paper (Southern, 1975). Hybridization to nick-translated probes (Rigby et al., 1977) was in 6xSSC at 65°. Sizes of DNA fragments (in kb) that hybridize well (strong) or poorly (weak) to the indicated probes are shown.

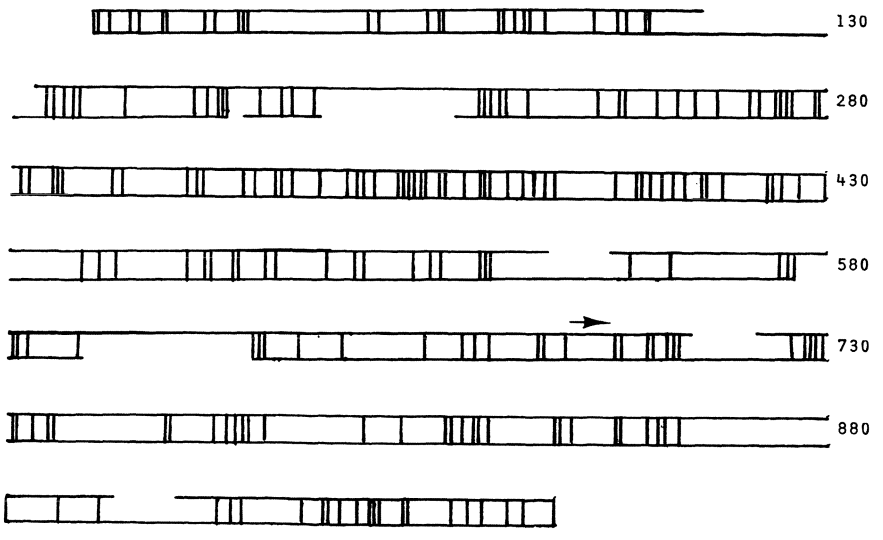

Figure 2: Homology between the 5' flanking sequences of the murine and human c-myc genes. The sequences of the murine (upper line) and the human (lower line) c-myc genes are presently schematically. Differences in the sequences of the two genes are denoted by a vertical bar, and deletions in either gene relative to the other as spaces in the horizonal line. Each line represents 150 base pairs. The arrow near residue 680 notes the transcription initiation site.

REFERENCES

Adams, J.M., Gerondakis, S., Webb, E., Corcoran, L.M., and Cory, S. 1983. Proc. Natl. Acad. Sci. USA 80:1982.

ar-Rushdi, A., Nishikura, K., Erikson, J., Watt, R., Rovera, G., and Croce, C.M. 1983. Science 222:390.

Battey, J., Moulding, C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G., and Leder, P. 1983. Cell 34:779.

Calame, K., Kim, S., Lalley, D., Hill, R., Davis, M., and Hood, L. 1982. Proc. Natl. Acad. Sci. USA 79:6994.

Cory, S., Gerondakis, S., and Adams, J. M. 1983. EMBO Journal 2:697.

Croce, C.M., Thierfelder, W., Erikson, J., Nishikura, K., Finan, J., Lenoir, G.M., and Nowell, P.C. 1983. Proc. Natl. Acad. Sci. USA 80:6922.

Davis, M.M., Kim, S.K., and Hood, L. 1980. Cell 22:1.

Dunnick, W., Shell, B.E., and Dery, C. 1983. Proc. Natl. Acad. Sci. USA 80:7269.

Erikson, J., Ar-Rushdi, A., Drivinga, H.L., Nowell, P.C., and Croce, C.M. 1983a. Proc. Natl. Acad. Sci. USA 80:820.

Erikson, J., Nishikura, K., ar-Rushdi, A., Finan, J., Emanuel, B., Lenoir, G., Nowell, P.C., and Croce, C.M. 1983b. Proc. Natl. Acad. Sci. USA 80:7581.

Fung, Y.-K.T., Lewis, W.G., Crittenden, L.B., and Kung, H.-J. 1983. Cell 33:357.

Gelmann, E.P., Psallidopoulos, M.C., Papas, T.S., and Favera, R.D. 1983. Nature 306:799.

Hamlyn, P.H. and Rabbitts, T.H. 1983. Nature 304:135.

Hayward, W.S., Neel, B.G., and Astrin, S.M. 1981. Nature 290:475.

Leder, P., Battey, J., Lenoir, G., Moulding, C., Murphy, W., Potter, H., Stewart, T., and Taub, R. 1983. Science 222:765.

Marcu, K.B., Lang, R.B., Stanton, L.W., and Harris, L.J. 1982. Nature 298:87.

Mushinski, J. F., Bauer, S. R., Potter, M., and Reddy, E. P. 1983. Proc. Natl. Acad. Sci. USA 80:1073.

Neuberger, M.S., and Calabi, F. 1983. Nature 305:240.

Nikaido, T., Yamawaki-Kataoka, Y., and Honjo, T. 1982. J. Biol. Chem. 257:7322.

Nishikura, K., ar-Rushdi, A., Erikson, J., Watt, R., Rovera, G., and Croce, C.M. 1983. Proc. Natl. Acad. Sci. USA 80:4822.

Perry, R.P. 1983. Cell 33:647.

Razin, A., and Riggs, A.D. 1980. Science 210:604.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. 1977. J. Molec. Biol. 113:237.

Robertson, M. 1983. Nature 306:733.

Saito, H., Hayday, A.C., Wiman, K., Hayward, W.S., and Tonegawa, S. 1983. Proc. Natl. Acad. Sci. USA 80:7476.

Southern, E.M. 1975. J. Molec. Biol. 98:503.

Stanton, L.W., Watt, R., and Marcu, K.B. 1983. Nature 303:401.

Stanton, L.W., Yang, J.-Q., Eckhardt, L.A., Harris, L.J., Birshtein, B.K., and Marcu, K.B. 1984. Proc. Natl. Acad. Sci. USA, in press.

Subak-Sharpe, J.H. 1967. Brit. Med. Bull. 23:161.

Activation of the c-myc Oncogene in B and T Lymphoid Tumors

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The cellular myc proto-oncogene, which is homologous to the transforming gene of the acute avian retrovirus MC29, is strongly implicated in the development of B lymphomas. Most murine plasmacytomas and human Burkitt lymphomas display translocations now known to represent recombination of the cellular myc gene with the immunoglobulin heavy chain constant region (C_H) locus. Since the findings by several groups that led to this important conclusion have been reviewed recently by Klein (1983), Perry (1983), and Leder et al. (1983), we will limit discussion here to our own recent data concerning the nature of the recombination event and its consequences for c-myc transcription. We also present evidence that c-myc can participate in oncogenesis within the T as well as the B lymphoid lineage.

NATURE OF c-myc TRANSLOCATIONS

Our previous work established that the predominant type of translocation in plasmacytomas and Burkitt lymphomas (15;12 and 8;14 respectively) represents a reciprocal exchange, in which, typically, one recombinant chromosome bears the body of the c-myc gene (exons 2 and 3) linked to a C_H gene and the other has the c-myc 5' flanking region linked to sequences that were 5' to the C_H gene. Blot data suggested that this held for 23 plasmacytomas (Cory et al., 1983b) and for at least 6 Burkitt lymphomas (Adams et al., 1983; Bernard et al., 1983). In all cases, the recombined c-myc and C_H genes appeared to be 'head to head', i.e. in opposite transcriptional orientation. Several different types of C_H region could apparently serve as a target, although C_α was highly preferred in the plasmacytomas.

To obtain further insights into the translocation mechanism, we have cloned and sequenced both chromosome junctions from four plasmacytomas (Gerondakis et al., 1984). The junction sequences have unexpectedly revealed that the exchange is not a precise cross-over, since deletions of variable size, ranging up to 1.6 kb, and extra nucleotides of unknown origin are a common feature. The deletions and a 106 bp duplication of c-myc sequences at the junctions in one tumor led us to propose that the translocation is initiated by staggered single-strand breaks in each chromosome. Subsequent excision of the single strands, or polymerization on them, prior to ligation to the other chromosome would account for the deletions and duplication observed.

Are the translocations related to normal immunoglobulin gene rearrangements? This notion is strongly supported by the apparent involvement of switch regions in at least 21 of 23 plasmacytomas (Cory et al., 1983b; Gerondakis et al., 1984) and in 4 Burkitt lymphomas (Bernard et al., 1983), although two other Burkitt breakpoints were nearer the J_H locus. These data argue that switch enzymes make most murine chromosome 12 (and human chromosome 14) scissions and raise the possibility that V-D-J joining enzymes might sometimes participate.

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The basis for scission of the c-myc-bearing chromosome is less clear. Blot hybridization analysis of 23 plasmacytomas revealed that c-myc breakpoints cluster strikingly within exon 1 and the first half of intron 1 (Cory et al., 1983b; Gerondakis et al., 1984) and a number of Burkitt 8;14 translocations also fall in this region (Bernard et al., 1983). This region is clearly not a pseudoswitch region, in man or mouse. Hybridization (Cory et al., 1983) and sequence analysis (Bernard et al., 1983) revealed no overall homology to any switch region, ruling out homologous recombination as the translocation mechanism. Indeed, the frequency of switch region sequence motifs in the myc translocation region is no greater than in any random sequence (Bernard et al., 1983). Nevertheless, the sequences of certain breakpoints do share short regions of homology with switch regions (Gerondakis et al., 1984). Hence the C_H switch enzymes may well make at least some chromosome 15 scissions. We surmise that the high preference for scissions within the c-myc gene (particularly in the mouse) largely reflects strong selection for altered myc regulation (see below).

THE CONSEQUENCES OF TRANSLOCATION FOR c-myc EXPRESSION

The first of the 3 exons comprising the c-myc gene represents an unusually long 5' untranslated region (Adams et al., 1983; Stanton et al., 1983; Bernard et al., 1983). Normal c-myc transcription yields mRNAs of 2.25 and 2.4 kb, reflecting two exon 1 promoters 160 bp apart. Different utilization of these promoters in certain cell lines suggests that they are under partially independent regulation (Bernard et al., 1983).

Tumors display either the normal c-myc transcripts or an altered mode. The altered mode is found in tumors where translocation has disrupted the normal transcriptional unit by scission within exon 1 or intron 1: 23 of 24 plasmacytomas we have examined (Cory et al.,1983b) fall into this category, as do 5 of 9 Burkitt lymphomas (Bernard et al., 1983). The altered mRNAs, which vary in size and relative amount in different tumors, bear sequences from within the 3' half of the first intron. They appear to initiate from cryptic promoters within intron 1, but splicing events could also be involved. It seems unlikely from the sequence of intron 1 that the new transcipts encode an altered polypeptide (Bernard et al., 1983). Although the altered transcriptional mode clearly is strongly favored in plasmacytomas, the existence of lymphomas with only the normal transcripts shows that the altered mode cannot be essential for neoplasia.

The major consequence of translocation appears to be induction of constitutive c-myc transcription. While exon 1 probes would not be expected to label c-myc RNAs transcribed from a 'decapitated' c-myc allele, it is highly significant that no c-myc RNAs were labelled by these probes in 12 of 13 such plasmacytomas or in 4 of 5 such Burkitt lines (Adams et al., 1983; Bernard et al., 1983). We infer that the untranslocated c-myc allele is silent and surmise that the mature normal B lymphocyte or plasma cell does not express c-myc. Nishikura et al. (1983) reached a similar conclusion from analysis of somatic cell hybrids. It seems reasonable to conclude, therefore, that constitutive c-myc expression during late B cell differentiation predisposes to malignancy.

The significant levels of myc mRNA we found in three of five lymphoblastoid cell lines (Bernard et al., 1983) presumably reflects activation by a different, unknown, mechanism. Myc expression in these lines, which are non-tumorigenic albeit 'transformed', is consistent with the notion that c-myc provides an 'immortalisation' function (Land et al., 1983; Ruley, 1983) that must be complemented by the fibroblast-transforming gene(s) also activated in these tumors (Lane et al., 1982).

It remains unclear how translocation induces constitutive c-myc expression and what role the C_H locus plays. The high preference for breaks within the c-myc gene raises the possibility that a negative regulatory region, such as a repressor binding site, or a site controlling chromatin structure or methylation, lies very close to the exon 1 promoters. Translocation would then release the c-myc coding region from its repression in mature lymphoid cells. On this model, an Ig locus might be the preferred fusion partner simply because of its predilection for breakage during normal Ig gene rearrangement. However, it also seems plausible that the Ig locus provides some positive lymphoid-specific transcriptional signal. Transcriptional activity per se cannot be the feature that attracts c-myc, or many other loci would be targets. Indeed, the Ig target itself need not be highly active transcriptionally, since the V_H gene required for abundant Ig transcription was not attached to several of the C_H targets we identified, such as those with the C_α gene in germline context (Cory et al.,1983b; Gerondakis et al., 1984). The known C_H locus enhancer, which lies between J_H and S_μ, would be linked to c-myc in three of the Burkitt lines we have studied, but not in four others, nor in any plasmacytoma so far described (Bernard et al., 1983). It is not ruled out, however, that other, as yet unidentified enhancers within the C_H locus contribute to c-myc activation.

RETROVIRAL INSERTION NEAR c-myc IN T LYMPHOMAS

Little has been known about the onc genes involved in T lymphomas, but many murine T cell leukemias, irrespective of their mode of induction, are trisomic for chromosome 15 (Klein, 1981). The possibility that c-myc was the chromosome 15 gene involved in at least a category of T cell neoplasia was raised by our observation that c-myc was rearranged in 3 of 18 murine T lymphomas (Adams et al., 1982). Two of the three lines were induced in BALB/c mice by a little studied 'slow' murine leukemia virus designated Soule MuLV (Stansly and Soule, 1962) and the third, Tikaut, arose spontaneously in a mouse of the highly leukemogenic AKR strain, in which retroviruses that are env gene recombinants between certain endogenous proviruses are implicated in leukemogenesis.

By cloning, we have now established that all three of these c-myc rearrangements correspond to proviral insertion (Corcoran et al., 1984). The inserts lie 0.7-1.3 kb 5' to c-myc and, significantly, all three would be transcribed away from c-myc, an orientation that rules out c-myc transcription from the promoter in the retroviral long terminal repeat (LTR). All three tumors contain the normal c-myc mRNAs; their level is about 5-fold higher than in most T lymphomas lacking c-myc rearrangement and some 10-fold higher than in normal thymocytes, and falls within the plasmacytoma range. Thus c-myc expression appears to have been stimulated by the inserts.

The results with Tikaut were particularly telling because the tumorigenicity of somatic cell hybrids between this AKR tumor line and normal cells correlated with the abundance of the chromosome with the rearranged myc gene (Spira et al., 1981; Cory et al., 1983a). We therefore used Southern blots to examine primary AKR T lymphomas. Seven of 31 had proviral inserts within the c-myc locus (Corcoran et al., 1984). Five mapped within a 2 kb region immediately 5' to c-myc exon 1, one within exon 3 - probably in the 3' untranslated region - and another, 24 kb 3' to the exon 1 promoters. Significantly, all the AKR myc-associated proviruses are of the recombinant, highly leukemogenic 'MCF' type. We conclude that a major role of the MCF virus in AKR leukemia is to induce expression of a critical oncogene. The c-myc gene is implicated in the genesis at about a quarter of these tumors and presumably in many of those induced by other 'slow' transforming retroviruses, judging by our results with Soule MuLV.

Significantly, none of the ten insertions we studied in T lymphomas is compatible with the 'promoter insertion' commonly found in B lymphomas induced by avian leukosis virus (Hayward et al., 1981). All would be compatible, however, with activation by an LTR enhancer, and indeed experiments with Dr. A. Dunn (Ludwig Cancer Institute) established that the LTR of Soule MuLV bears a functional enhancer. The clustering of the eight inserts upstream of c-myc could also reflect disruption of a putative c-myc regulatory region. It may be relevant that all three cloned inserts integrated within 11 bp of the octamer GTATACGT. Conceivably this sequence could represent part of a repeated c-myc control element.

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REFERENCES

Adams JM, Gerondakis S, Webb E, Mitchell J, Bernard O, Cory S ((1982) Transcriptionally active DNA region that rearranges frequently in murine lymphoid tumors. Proc Natl Acad Sci USA 79:6966-6970.

Adams JM, Gerondakis S, Webb E, Corcoran LM, Cory S (1983) Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc Natl Acad Sci USA 80:1982-1986.

Bernard O, Cory S, Gerondakis S, Webb E, Adams JM (1983) Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosomal translocation in B lymphoid tumors. EMBO J 2:2375-2383.

Corcoran LM, Adams JM, Dunn AR, Cory S (1984) Murine T lymphomas in which the cellular myc oncogene has been activated by retroviral insertion. Cell, in press.

Cory S, Adams JM, Gerondakis SD, Miller JFAP, Gamble J, Wiener R, Spira J, Francke U (1983a) Fusion of DNA region to murine immunoglobulin heavy chain locus corresponds to plasmacytoma-associated chromosome translocations. EMBO J 2:213-216.

Cory S, Gerondakis S, Adams JM (1983b) Interchromosomal recombination of the cellular oncogene c-myc with the immunoglobulin heavy chain locus in murine plasmacytomas is a reciprocal exchange. EMBO J 2:697-703.

Gerondakis S, Cory S, Adams JM (1984) Translocation of the myc cellular oncogene to the immunoglobulin heavy chain locus in murine plasmacytomas is an imprecise reciprocal exchange. Cell, in press.

Hayward W, Neel BG, Astrin (1981) Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 290:475-480.

Klein, G (1981) The role of gene dosage and genetic transpositions in carcinogenisis. Nature 294-313-318.

Klein G (1983) Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. Cell 32:311-315.

Land H, Parada L, Weinberg R (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two co-operating oncogenes. Nature 304:596-602.

Lane MA, Sainten, A, Cooper GM (1982) Stage-specific transforming genes of human and mouse B- and T-lymphocyte neoplasms. Cell 28:873-880.

Leder P, Battey J, Moulding C, Murphy W, Potter H, Stewart T, Taub R (1983). Translocations among antibody genes in human cancer. Science 222:766-771.

Nishikura K, Ar-Rushdi A, Erikson J, Watt R, Rovera G, Croce CM (1983) Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc Natl Acad Sci USA 80:4822-4827.
Perry RP (1983) Consequences of myc invasion of immunoglobulin loci: facts and speculation. Cell 33:647-649.
Ruley HE (1983) Adenovirus early region IA enables viral and cellular transforming genes to transform primary cells in culture. Nature 304:503-607.
Spira J, Wiener F, Babonits M, Gamble J, Miller J, Klein G (1981) The role of chromosome 15 in murine leukemogenesis. I. Contrasting behavior of the tumor vs normal parent-derived chromosomes no.15 in somatic hybrids of varying tumorigenecity. Int J Cancer 28:785-798.
Stansly PG, Soule HD (1962) Transplantation and cell-free transmission of a reticulum-cell sarcoma in Balb/c mice. J Natl Cancer Inst 29:1083-1097.
Stanton LW, Watt R, Marcu KB (1983) Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature 303:401-406.

The c-myc Gene Paradox in Burkitt's Lymphoma Chromosomal Translocation

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INTRODUCTION

The association of the human c-myc proto-oncogene on the long arm of chromosome 8 with the specific, consistent chromosomal translocations observed in Burkitt's lymphoma (BL) has been established by a variety of studies. This association involves, in about 90% of cases, the immunoglobulin heavy (H) chain locus on chromosome 14 (14q32) whilst in the so-called variant BL the light (L) chain loci are involved (in about 5% of cases the lambda (λ) light chain locus on 22q11 and the remaining 5% the kappa (κ) light chain on 2p12). In the predominant 8/14 translocations, the c-myc gene is included in the segment of chromosome 8 that translocates to chromosome 14 (Erikson et al. 1982; Davis et al. 1984) and the resultant proximity of c-myc and H chain constant region genes (in which the genes are arranged in the opposite transcription orientation) allows the orientation of the respective genes in relation to the chromosomal centromere (i.e. the c-myc gene is present on chromosome 8 with the 5' end towards the centromere). Recently, studies of both types of variant BL translocation (i.e. t8/22 and t2/8) have shown that the breakpoint in these cases occurs to the 3' side or downstream of the c-myc gene (Croce et al. 1983; Davis et al. 1984; Hollis et al. 1984).

The c-myc gene has three exons of which only the second two encode for a protein (Colby et al. 1983; Hamlyn and Rabbitts 1983; Watt et al. 1983). Studies on cloned DNA segments have shown that very frequently in mouse myeloma c-myc translocations the non-coding exon 1 (for which there is no assigned function) is lost from the translocated gene while it is usually retained in BL c-myc gene translocations (Adams et al. 1983; Hamlyn and Rabbitts 1983; Rabbitts et al. 1983; Stanton et al. 1983). This apparently conflicting situation between most BL translocations and those in mouse myelomas is one very puzzling aspect of the paradox of c-myc gene translocation. The other intriguing feature of the BL translocations is the great variability of the breakpoint with respect to the c-myc gene (illustrated in Fig. 1, on following page) and the rare occurrence of obvious damage to the BL translocated c-myc gene. We must therefore elucidate a general effect of the translocation on the c-myc gene in generating the putative oncogenic activity of this gene. In this paper we briefly describe results which indicate the possibility that sequence changes within the c-myc gene may be important, such as those found in exon 1, and we propose that such alterations (not apparent as gross c-myc gene changes) may affect the control of the c-myc gene.

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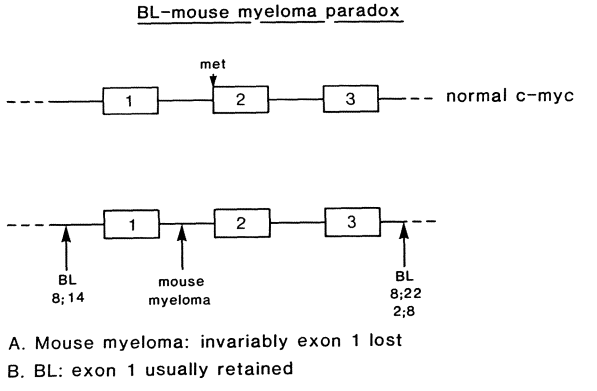

Fig. 1. The arrows showing breakpoints of the translocated c-myc gene only indicate the general region involved and are not intended to be specific positions.

Translocation in Variant t2/8 Burkitt's Lymphomas

Using in situ hybridisation we have recently demonstrated that the c-myc gene does not move from the abnormal chromosome 8 in BL with t2/8 (Davis et al., 1984). These results therefore show that in this case the breakpoint occurs on the 3' side of the c-myc gene as opposed to the 5' side as in t8/14. We wished to establish whether the K-L-chain locus was involved in the t2/8 translocation point, and, if so, how this locus was affected by the translocation. Accordingly, we used both Cκ and Vκ probes to carry out in situ hybridisation experiments with chromosomes from cells containing t2/8. A summary of the data on grain-counting from these hybridisations is shown in Table 1, which includes only the data for the pairs of chromosomes 2 and 8. With the HK101/80 Vκ probe there were 102 total grains in 14 cells analysed; with the λHK101 Vκ probe there were 231 total grains in 16 cells, and with the Cκ probe there were 113 total grains for 23 cells counted. The normal chromosome 2 shows positive hybridisation with both the Vκ probes (e.g. with HK101/80, 10 grains or 9.8% of the total) and the Cκ probe (7 grains or 6.2% of the total) which is consistent with the previous assignment of this locus to chromosome 2 (Malcolm et al., 1982; McBride et al., 1982). No significant grains were detected over either normal or abnormal chromosome 8 with any of the Vκ probes, which shows that the set of V genes detected in the experiment was not included in that part of chromosome 2 which translocated to chromosome 8. However the possibility that some undetected Vκ genes are translocated to chromosome 8 cannot be ruled out, especially since somatic cell hybrid experiments indicated that Vκ genes do move to chromosome 8 (Erikson et al., 1983). This is an intriguing difference in the two sets of data however as an identical probe (HK101/80 [Bentley and Rabbitts, 1983]) was utilised.

Our experiments with the Cκ probe (pUCR17Cκ - see Table 1 footnote) show that the Cκ gene does become translocated onto the $8q^+$ chromosome since we find significant grains of this long arm (11 grains or 9.7% of the total). These data show therefore that the Cκ gene moves into the $8q^+$ chromosome in t2/8, thus finally being positioned to the 3' side or downstream of the c-myc gene (see Fig. 2). Furthermore, it seems that the Vκ locus remains on the $2p^-$ chromosome with the breakage probably between Vκ and Cκ genes or within the Vκ locus itself (see caveat discussed above). The orientation of Vκ and Cκ genes in chromosome 2 may also be deduced from these results (Fig. 2); i.e. the Vκ genes (at least those detected in these hybridisations) are proximal to the centromere on the normal chromosome 2, while the Cκ genes are distal or nearer to the telomere. At present we cannot comment on the transcription orientations of these genes.

Table 1. Summary: in situ hybridisation data[1]

Cell line: JI t2/8

Probe: Vκ HK101/80		Probe: λHK101	
102 grains total/14 cells		231 grains total/16 cells	
2p	10	2p	13
$2p^-$	10	$2p^-$	14
8	0	8	1
$8q^+$	1	$8q^+$	0

Probe: Cκ pUC R17Cκ	
113 grains total/23 cells	
2p	7
$2p^-$	1
8	0
$8q^+$	11

1 The Vκ probes λHK101 and HK101/80 were previously described (Bentley and Rabbitts, 1981). The Cκ probe (pUCR17Cκ) was isolated as follows: a genomic library of Raji DNA (Hamlyn and Rabbitts, 1983) was screened with the human Cκ cDNA M13Cκ6 probe (Bentley and Rabbitts, 1983). A positively hybridising clone λR17 was analysed and found to contain the Cκ gene; a subclone was prepared from this in pUC8, which contained a 2.5 Kb EcoR1 fragment with the Cκ coding region plus immediate flanking sequences (pUCR17Cκ). Hybridisations were carried out as previously described (Davis et al., 1983) at 43°C for λHK101 and pUCR17Cκ, but 37°C for HK101/80.

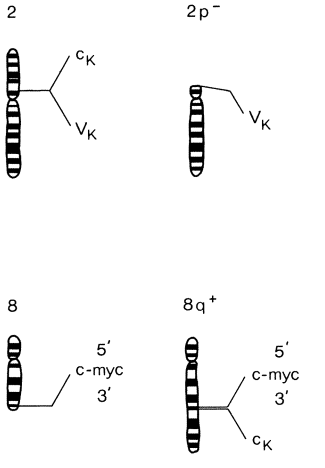

Fig. 2. Diagram of orientations of the various genes involved in translocations of chromosomes 2 and 8 in Burkitt's lymphoma.

Translocation Points can Occur at Great Distance from the c-myc Gene in BL

The type of experiment described in the previous section illustrates the variability in translocation point found in the different BL cell lines. In addition to this, it is also found that the translocation points do not always occur immediately adjacent to the c-myc gene but rather several kilobases from the gene. This phenomenon is illustrated in Fig. 3 where we have used two c-myc gene flanking segments to hybridise filters containing BL DNA digests.

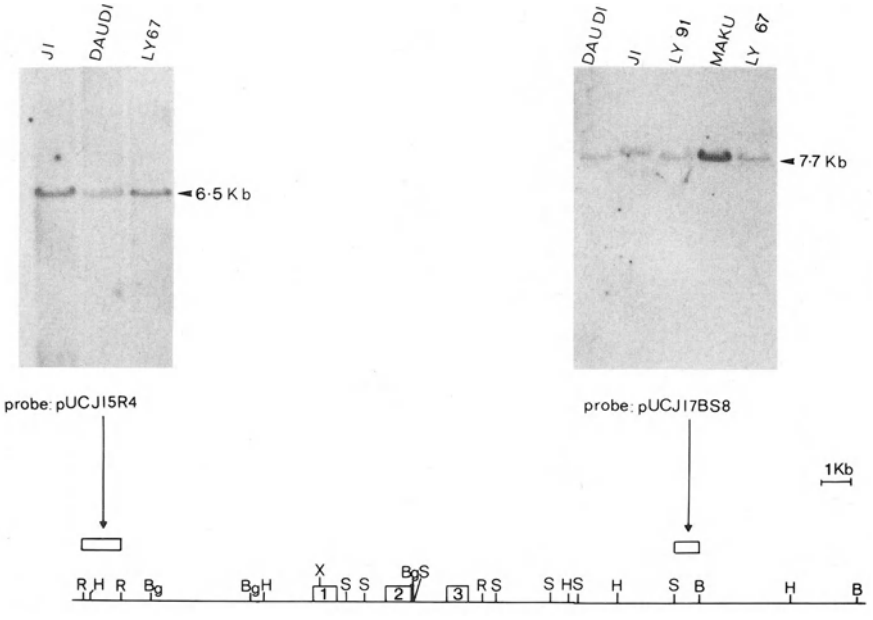

Fig. 3. Southern filter hybridisation of c-myc gene flanking probes with BL cellular DNA. The partial restriction of the c-myc gene is shown together with the location of probes used for the hybridisations. DNAs on the left-hand panel were digested with BglII and those on the right with SacI. R = EcoR1, H = HindIII, Bg = BglII (partial sites only), X = XhoI, S = SacI.

Clearly no evidence for gene rearrangement can be detected with either probe in cells carrying t8/14 (using pUC JI5R4) or t2/8 and t8/22 (using pUC JI7B58). This means that in Daudi cells the translocation point occurs at least 12 Kb upstream of the c-myc gene while in JI, LY91 (t2/8) MAKU and LY67 (t8/22) the breakpoint occurs at least 14 Kb downstream of the gene.

Sequence Changes in the c-myc Gene are a Clue to Oncogenic Activation of this Gene

We have previously reported substantial sequence alterations in the translocated c-myc gene of Raji (Rabbitts et al., 1983) in both exons 1 and 2. The latter would be expected to give rise to a substantially altered c-myc protein in this case. This appeared not to be so in the translocated gene of Daudi cells however as we found no coding sequence alteration in a cDNA clone (Rabbitts et al., 1983) which we presume to derive from the abnormal c-myc gene as this gene is preferentially expressed in BL cells (Bernard et al., 1983; Nishikura et al., 1983). However, we have now sequenced the exon 1 region of the Daudi clone (pUCcD1A) and discovered that, in addition to a 39-base duplication in this exon, there are 7.3% base differences in exon 1 compared with the normal c-myc gene derived from a genomic cloned DNA segment from Daudi cells. Table 2 outlines the base

Table 2. Sequence changes in BL-translocated c-myc gene[1]

Cell	Exon 1	Exon 2	Exon 3
Raji t8/14	7.0%	3.3%	0
Daudi t8/14	7.3%	0	0

1 The Raji exon 1 changes exclude deletions previously noted (Rabbitts et al., 1983).

differences found in the three exons of the translocated c-myc genes of Raji and Daudi cells. As a general rule coding region changes are therefore unlikely to be generally responsible for c-myc gene activation as there are no changes in Daudi cells (either coding or silent) in exons 2 or 3. However in both cases the sequences of exon 1 have substantial differences from the normal c-myc gene sequenced from the same cell lines; this indicates that sequence changes in this general region may be important for activation of c-myc in BL. Further, we have now a similar observation in the translocated c-myc gene from LY67 (t8/22).

Model: Loss of c-myc Gene Control Resulting from Mutational Changes

The observation of changes (presumably mutations introduced into the c-myc genes as a consequence of translocation) in exon 1 provides a possible explanation for the c-myc gene paradox. If we assume that exon 1 or an upstream region is the site of action of a repressor protein (a suggestion previously made by Leder et al. [1983]) that serves to tightly control c-myc gene transcription, this interaction could be altered (i.e. destabilised) by the sequence changes in the translocated c-myc gene in BL. Of course those BL cell lines, and the vast majority of mouse myelomas, in which exon 1 is lost by translocation from the coding part of the gene will also have this control region removed. Thus we can envisage that the most general way in which the c-myc gene is activated in the formation of Burkitt's lymphoma and mouse myeloma is by loss or damage of the exon 1 or upstream controlling regions resulting in constitutive transcription. This in turn would cause the constant presence of the c-myc protein in these malignant cells whereas normal cells would display controlled c-myc gene transcription. These models offer obvious predictions which can be readily tested. Such models as these do not of course preclude the possibility that in some cases other mechanisms may operate, such as the protein alteration in Raji (Rabbitts et al., 1983).

REFERENCES

Adams JM, Gerondakis, S, Webb E, Corcoran, L, Cory S (1983) Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. Proc Nat Acad Sci USA 80:1982-1986

Bentley DL, Rabbitts TH (1981) Human Vκ immunoglobulin gene number: implications for the origin of antibody diversity. Cell 24:613-633

Bentley DL, Rabbitts TH (1983) Evolution of immunoglobulin V genes. Evidence indicating that recently duplicated human Vκ sequences have diverged by gene conversion. Cell 32:181-189

Bernard O, Cory S, Gerondakis S, Webb E, Adams JM (1983) Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumours. EMBO J 2:2375-2383

Colby WW, Chen EY, Smith DH, Levinson AD (1983) Identification and nucleotide sequence of a human locus homologous to the v-myc oncogene of avian myelomcytomatosis virus MC29. Nature 301:722-725

Croce CM, Thierfelder W, Erikson J, Nishikura K, Finan J, Lenoir GM, Nowell PC (1983) Transcriptional activation of an unrearranged and untranslocated c-myc oncogene by translocation of a Cλ locus in Burkitt lymphoma cells. Proc Nat Acad Sci USA 80:6922-6926

Davis M, Malcolm S, Hall A, Marshall CJ (1983) Localisation of the human N-ras oncogene to chromosome 1 cen-p21 by in situ hybridisation. EMBO J 2:2281-2283

Davis M, Malcolm S, Rabbitts TH (1984) Chromosome translocation can occur on either side of the c-myc oncogene in Burkitt lymphoma cells. Nature, in press

Erikson J, Finan J, Nowell PC, Croce CM (1982) Translocation of immunoglobulin V_H genes in Burkitt lymphoma. Proc Nat Acad Sci USA 79:5611-5615

Erikson J, Nishikura K, AR-Rushdi A, Finan J, Emanuel B, Lenoir G, Nowell PC, Croce, CM (1983) Translocation of an immunoglobulin K locus to a region 3' of an unrearranged c-myc oncogene enhances c-myc transcription. Proc Nat Acad Sci USA 80:7581-7585

Hamlyn PH, Rabbitts TH (1983) Translocation joins c-myc and immunoglobulin γ1 genes in a Burkitt lymphoma revealing a third exon in the c-myc oncogene. Nature 304:135-139

Hollis GF, Mitchell KF, Battey J, Potter H, Taub R, Lenoir GM, Leder P (1984) A variant translocation places the λ immunoglobulin genes 3' to the c-myc oncogene in Burkitt's lymphoma. Nature 307:752-755

Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Steward T, Taub R (1983) Translocations among antibody genes in human cancer. Science 222:765-771

Malcolm S, Barton P, Murphy C, Ferguson-Smith MA, Bentley DL, Rabbitts, TH (1982) Localisation of human immunoglobulin kappa light chain variable region genes to the short arm of chromosome 2 by in situ hybridisation. Proc Nat Acad Sci USA 79:4957-4961

McBride OW, Hieter PA, Hollis GF, Swan D, Otey MC, Leder P (1982) Chromosomal location of human kappa and lambda immunoglobulin light chain constant region genes. J Exp Med 155:1480-1490

Nishikura K, AR-Rushdi A, Erikson J, Watt R, Rovera G, Croce CM (1983) Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc Nat Acad Sci USA 80:4822-4826

Rabbitts TH, Hamlyn PH, Baer R (1983) Altered nucleotide sequence of a translocated c-myc gene in Burkitt lymphoma. Nature 306:760-765

Stanton LW, Watt R, Marcu KB (1983) Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature 303:401-401

Watt R, Stanton LW, Marcu KB, Gallo RC, Croce CM, Rovera G (1983) Nucleotide sequence of cloned cDNA of human c-myc oncogene. Nature 303:725-728

Amplification, Expression and Rearrangement of c-myc and N-myc Oncogenes in Human Lung Cancer

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INTRODUCTION

Clinical Correlation of Lung Cancer Types

Human lung cancers can be divided into two major classes according to their clinical, histological, biochemical, and karyotypic properties: non-small cell lung cancer (NSCLC) (adenocarcinoma, epidermoid, large cell carcinoma) and "oat cell" or small cell lung cancer (SCLC) (Minna 1982; Gazdar 1980; Baylin 1980; Gazdar 1981a; Whang Peng 1982a). Of particular interest is a subset of SCLC, the morphological and biochemical variants (SCLC-V) (Gazdar 1981a; Radice 1982; Carney 1983). These variants can be seen histologically in approximately 6-15% of diagnostic biopsy specimens before any chemo- or radiotherapy treatment is given (Radice 1982; Hirsch 1983). At autopsy, approximately 30-40% of patients previously thought to have "pure" SCLC histologically will have these variant cells. Finally, patients with variant cells in their diagnostic biopsies have a fulminant course with inferior response to chemo- and radiotherapy and a much shorter survival than patients with "pure" SCLC (Radice 1982). Thus, clinically this SCLC-V group is quite significant.

Cell Biology of the Variant Class of Small Cell Lung Cancer

Because of these clinical correlates we compared the SCLC-V class with the non-variant or "classic" SCLC in a variety of assays. Tumor cell lines that grew out of the SCLC-V tumors had a less tightly clustered morphology, cloned with a 10 to 20 fold higher efficiency in soft agarose, and grew in mass culture with a 2-4 fold shorter doubling time than "classic" SCLC (Carney 1983). Thus, in vitro the SCLC-V lines behaved more aggressively than SCLC mirroring their behavior in patients. A variety of biochemical markers have been found in classic SCLC that reflect their amine precursor uptake and decarboxylation (APUD) properties (Gazdar 1980; Baylin 1980; Gazdar 1981a; Gazdar 1981b). SCLC-V tumor lines express some but not all of these markers. Specifically, they express high activities of the enzymes neuron specific enolase, and the BB isozyme of creatine kinase while they failed to produce, or expressed at much lower levels, L-dopa decarboxylase, peptide hormones such as bombesin-gastrin releasing peptide, and dense core (neurosecretory granules) (Gazdar 1981a). In addition, both classic and variant SCLC lines exhibit a cytogenetic abnormality (del 3p) (Whang Peng 1982a). Thus, the variant lines had some characteristics indicating SCLC-APUD lineage which clearly distinguished them from NSCLC but which also set SCLC-V tumors apart from classic SCLC.

SCLC-V lines frequently have double minute (DM) chromosomes or homogeneous chromosomal staining regions (HSRs) Whang Peng 1982a; Whang Peng 1982b). These changes have been associated with gene amplification and we reasoned they could occur in the SCLC-V lines because of drug resistance or increased expression of cellular proto-oncogenes related to malignancy. Recently, we reported that several established SCLC-V tumor lines have 20-40 fold c-myc DNA amplification and increased (30-70 fold) c-myc RNA expression (Little 1983). In contrast, the "classic" SCLC tumor lines did not exhibit c-myc amplification and failed

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to express detectable levels of c-myc mRNA by northern blot analysis. Comparing the amount of c-myc expression to B lymphoblastoid and Burkitt's lymphoma cells we found that c-myc amplified SCLC tumors expressed c-myc mRNA at levels 10-20 fold over those seen in the lymphoid cells. Thus, we concluded that activation of the c-myc oncogenes in SCLC is associated with gene amplification and that such activation of c-myc was in turn associated with dramatic changes in clinical and cell biologic behavior.

Because of the recent findings concerning c-myc promotor utilization and changes in the first exon of c-myc in Burkitt's lymphoma (Battey 1983; Taub 1984) we wished to see if similiar changes in c-myc genes occured in SCLC-V tumors where c-myc had been activated by a mechanism other than rearrangement. Also, in c-myc studies in a large number of SCLC and SCLC-V tumors we found some SCLC-V tumors that did not exhibit c-myc amplification but that had DM chromosomes or chromosomes with HSRs. Recent reports from two laboratories (Schwab 1983; Kohl 1983) reported on dramatic amplification of a gene with some sequence homology to the c-myc in human neuroblastomas and subsequently termed N-myc. We have found many similarities between human SCLC and human neuroblastomas (Baylin 1982) and thus it was natural to study this putative oncogene in human SCLC. Here we report that c-myc RNA made in tumor cells with c-myc amplification is transcribed preferentially from the 3' promotor (P2) and fails to show genetic changes in the first exon. In addition, we have found dramatic examples of amplification of the N-myc putative oncogene in SCLC-V cells not found to have c-myc amplification. Our evidence suggests that both the c-myc and other myc-related oncogene sequences may be involved in (or responsible for) the particular malignant phenotype of these SCLC-V cells.

S1 NUCLEASE MAPPING OF C-MYC mRNA TO STUDY PROMOTOR UTILIZATION AND FIRST EXON STRUCTURE

It has been reported previously that c-myc mRNA is transcribed from two active promoters each including the entire first exon of the c-myc onogene (Battey 1983; Taub 1984). In normal B lymphocytes or B lymphoblastoid lines the ratio of the utilization of the first (P1) to the second (P2) promotor is 1:3 to 1:4. In contrast, in Burkitt's lymphoma the P1:P2 promotor utilization ratio is 1:1 or greater showing an increase in the utilization of the first promotor. In addition, in some Burkitt's lymphoma lines genetic changes (insertions, deletions) have taken place in the first exon or promotor regions which are revealed by changes in the bands detected by S1 nuclease protection assays using probes specific for this region. Using S1 nuclease protection analysis of mRNA and a c-myc probe specific for the promoter region (probe A of Battey 1983) cloned into M13 for this purpose we found: 1.) no detectable c-myc transcription in "classic" SCLC lines which previously had been shown to lack c-myc DNA amplification or expression on Northern blots; 2.) abundant c-myc RNA expression in the c-myc amplified lines; 3.) promotor utilization in the c-myc amplified lines in a ratio (P1:P2) of 1:3 (that is "normal" or B lymphoblastoid-like promotor utilization); 4.) both P1 and P2 transcription units spanning all but 50 nucleotides of the first untranslated exon of c-myc show no evidence of insertion or deletion detectable by S1 analysis (4-5 bp insertions and deletions are easily detectable) 5.) "normal" promotor utilization ratios in a SCLC line (NCI-H146) that is intermediate between the variant and classic lines in morphologic and biochemical properties and which expresses 5-6 fold more c-myc RNA without c-myc DNA amplification. In Burkitt's lymphoma the c-myc gene is translocated and rearranged into an immunoglobulin locus associated with changes in promotor utilization and first exon structure and subtle changes in the regulation of c-myc RNA (Taub 1984). In contrast, in SCLC-V lines c-myc is often amplified resulting in expression of large amounts of c-myc mRNA without apparent changes in promotor utilization or the structure of the first

exon detectable by S1 analysis. Finally, recent studies of autopsy material have revealed that tumor c-myc amplification can take place in the patient without intervening cell culture.

AMPLIFICATION AND EXPRESSION OF THE N-MYC PUTATIVE ONCOGENE IN HUMAN SMALL CELL LUNG CANCER

After screening lung cancer lines for the presence of c-myc amplification we knew that all small cell lung tumors amplified for c-myc were biochemical and morphological variants. However, there were tumors that lacked L-dopa decarboxylase expression with or without morphologic changes and/or had DMs or HSRs. In addition, using a c-myc probe we detected a faint 2 kb Eco RI myc-related fragment in several of these cell lines when we used a second exon c-myc probe. Thus, following reports (Schwab 1983; Kohl 1983) that human neuroblastoma cells contained a 2 kb Eco RI amplified myc-related fragment we obtained the N-myc probe (Nb-1) from Drs. M. Bishop and M Schwab.

Tests of all of our SCLC and SCLC-V material with this probe revealed that the N-myc gene was dramatically amplified in the germline position in 4 SCLC tumor lines not amplified for c-myc. The biochemical and morphologic characterization of tumor cell clones containing amplified N-myc genes is under study. However, we know N-myc amplification occurred in tumor lines derived from patients before any treatment with chemo-or radioatherapy, and in early and later passages of the tumor cell lines. Northern blot analysis showed that the lines amplified for N-myc expressed a mRNA with N-myc homology that was not seen in N-myc non-amplified lines.

Detection of Myc-Related Oncogenes

Using the N-myc probe (Nb-1) in lines that are greatly amplified for c-myc, a faint band of reactivity in the c-myc germline position is seen indicating the homology (albeit distant) between N-myc and c-myc. The converse result is seen when a c-myc probe is used against an N-myc amplified line. Thus, it is possible to use a c-myc probe to detect an amplified N-myc gene and likewise, an N-myc probe to detect an amplified c-myc gene. We note that the use of oncogene probes to detect related sequences in human tumors when they are greatly amplified provides a novel approach to identifying new proto-oncogenes. While the biologic activity of N-myc has yet to be demonstrated its amplification in two tumor systems (human neuroblastoma and SCLC) and the correlation of N-myc amplification with increased malignant behavior of the SCLC-V lines suggests that it could have some properties of oncogenes and thus we term it a putative oncogene.

N-myc Amplification and Rearrangements in Direct Patient Tumor Samples

As part of the evidence that oncogene amplification plays a role in the clinical biology of tumors it is obviously critical to show that such amplification takes place in patient specimens in addition to tumor cell lines growing in culture. To answer this we have been harvesting and testing tumor and normal tissue at autopsy from lung cancer patients. In one informative case (patient JL) the patient had an extremely fulminant course with a rapidly growing tumor that quickly relapsed after intensive combination chemo- and radiotherapy. A tumor cell line from a bone marrow metastasis obtained at the time of diagnosis before any therapy, grew rapidly and cloned readily. This line exhibited dramatic N-myc amplification and increased expression of N-myc mRNA. In addition to amplification of the germline gene, a novel and equally amplified band was seen as 5.5 kb Eco RI fragment. At autopsy we harvested normal liver and

kidney and several discrete metastatic nodules from the liver. When probed for N-myc it was of interest to note that: 1.) the normal tissues did not have amplified N-myc sequences nor did they exhibit a new 5.5 kb Eco RI band; 2.) some liver metastases exhibited the equally amplified dual band pattern as the cell line; 3.) some of the liver metastases only exhibited the amplified N-myc band in the germ line location. From this experiment of nature we can conclude that: 1.) the amplification occured in the tumor in the patient; 2.) the amplification was not carried as a polymorphism by the patient's normal tissue; 3.) the novel 5.5 kb Eco RI band was not carried as a polymorphism. There appear to be several possibilities to explain the presence of two amplified N-myc fragments. The non-germline fragment could represent a rearranged N-myc gene or a new proto-oncogene with a N-myc-related sequence. Also the individual tumor cells could contain both or only one of the two amplified N-myc fragments. However, since the various metastatic deposits differ in their N-myc genes, it appears that the tumor seeded to distant sites at some early stage in the amplification sequence(s).

CONCLUSIONS AND DISCUSSION

It is apparent that the c-myc and myc-related sequences such as N-myc may play a role in the clinical and cellular biology of human SCLC. Indeed the frequency of these changes is striking. Currently we have studied a total of 25 SCLC lines of all types, each established from a different patient. At least 13 of these exhibit an amplification of a myc-related sequence. What the precise role of c-myc, N-myc and other myc-related oncogenes in the etiology, biology, and tumor progression of SCLC is at this point is not defined. Our simple working model is that such oncogene amplification is at a minimum associated with tumor progression. Whether such amplification alone could cause a lung cancer has not been shown. The recent results from gene transfection studies in embryo cells suggest that two or more oncogenes could act in concert to elicit malignant behavior (Land 1983). In fact it has been proposed that oncogenes could be divided into two or more complementary groups one of which was myc-like (and the gene product nuclear in location) while the other was ras-like (with the gene product located in the cytoplasm or membrane). In this regard, it is of interest to note that the promyelocytic leukemia line HL-60 exhibits both c-myc amplification and carries an activated ras family oncogene (Murray 1983). Thus, it is not unreasonable to speculate that more than one oncogene could be activated in human lung cancer cells.

Is there tissue specificity for myc-sequence oncogene amplification? C-myc amplification has been found in one human promyelocytic leukemia line (Collins 1982; Dalla-Favera 1982), one human colon line of possible neuroendocrine origin (Alitalo 1983), and in many human SCLC-V tumor lines, and in one lung adenocarcinoma line (Little 1983). These represent several tissue types. In contrast, N-myc amplification has only been seen in human neuroblastoma and SCLC. Despite the fact that neuroblastomas arise from the neuroectoderm and SCLC arises from the endoderm they share many properties in common such as expression of neuron specific enolase and their membrane protein phenotype (Marangos 1982; Baylin 1982). Thus, it is possible that certain oncogenes can only be amplified in tumors of defined lineage. The events that trigger oncogene amplification are unknown. While it is likely that disrupted events of DNA replication provide the basis for such change, it has not been excluded that such amplification may be a normal part of differentiation. Finally, in c-myc amplified lines large amounts of c-myc mRNA are expressed, and Dr. R. Eisenman has presented data at this meeting that such cells express large quantities of c-myc protein. The function of this protein, why large amounts cause abnormal cell behavior, and whether or not there are ways to therapeutically interupt such a process remain the crux of both cell biologic and clinical problems.

Chromosomal Aberrations in Murine Plasmacytomas

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INTRODUCTION

Murine plasmacytomas induced in Balb/c mice and its congenic strains contain one of the alternative translocations:rcpt (12:15) or rcpt (6:15).The former is referred to as the typical translocation because it was present in the majority of plasmacytomas including k and λ producers.The latter is only seen in a minority of k producers representing the group of variant plasmacytomas.There is no relationship between translocation type and inducing agent.Both aberrations were regularly present irrespectively of the mode of induction (pristane,pristane+A-MuLV or i.p implanted diffusion chamber).

High resolution banding studies have shown that both translocations are reciprocal.This was also confirmed for the typical translocation by molecular analysis of the reciprocal products on chromosomes 12 and 15 resp.The cytogenetic breakpoints both in typical and variant plasmacytomas were mapped to the interface of bands D2 and D3 on chr.15 and to the band F1 on chromosome 12 and to the band C on chr.6 resp.

Another relevant feature of PC-associated translocation is that in near-diploid tumor cells the translocation chromosome and its normal homologue are present in a single copy.In the tetraploid tumors the ratio is 2:2 indicating that the reciprocal exchange occured before the tetraploidization of the plasmacytoma cell.This is in line with the fact that the translocations are already found in primary (0 generation)tumors suggesting a causal relationship between translocation and the tumorigenic event.

PLASMACYTOMAS WITH PERICENTRIC INVERSION

Further cytogenetic studies on plasmacytomas induced in (Balb/c x AKR 6;15) x Balb/c backcross mice where the Balb/c chr.15 was replaced by an AKR-derived Rb 6;15 chromosome revealed a new type of

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Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J (1983) Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. Nature 305:245-248

Taub R, Moulding C, Battey J, Murphy W, Vasicek T, Lenoir GM, Leder P (1984) Activation and somatic mutation of the translocated c-myc gene in Burkitt Lymphoma cells. Cell 36:339-348

Whang-Peng J, Bunn PA Jr., Kao-Shan CS, Lee EC, Carney DN, Gazdar A, Minna JD (1982a) A nonrandom chromosomal abnormality, del 3p(14-23), in human small cell lung cancer (SCLC). Cancer Gen Cytogen 6:119-134

Whang-Peng J, Kao-Shan, CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, Portlock C, Minna JD (1982b) Deletion 3p(14-23), double minute chromosomes, and homogeneously staining regions in human small cell lung cancer. In: Schimke (ed) Gene Amplification, Cold Spring Harbor Laboratory Press, New York, pp 107-113

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translocation.In three out of ten tumors the AKR-derived Rb(6;15) fusion chromosome was solely responsible for the PC-associated translocation, by exchanging its two reciprocal segments.The biarmed chromosome has undergone a pericentric inversion leading to the same translocation and with the same breakpoints as previously observed in the "variant" Balb/c plasmacytomas where two separate 6 and 15 chrs. participated in the exchange.The fact that an usual intrachromosomal rearrangement led to the formation of the specific chromosomal aberration further supports its critical role in plasmacytogenesis.

Another important finding revealed by this study deserves special mention.No significant preferance was found for the Balb/c-derived chr.15 as the donor of the oncogene carrying chromosome compared to its AKR counterpart.This study already indicates that the 15 chr. segment originating from these two strains participate in the tumor associated translocation at random. This is further supported by our current experiments on another backcross series where the CBAT6T6-derived chromosome t(14;15) was introduced onto the Balb/c background. In two tumors inducedin the Balb/c t(14;15) mice with cytogenetically ditinguishable 15 chrs.(a normal 15 of Balb/c origin and a CBAT6T6--derived t(14;15) chromosome) the typical rcpt (12;15) was present. It arose,however,by the transposition of the distal segment of the t(14;15) chr. onto the Balb/c derived chr.12.

The lack of any demonstrable preference for the chr.15 region involved in the PC-associated translocation is in line with the concept that the oncogene is activated constitutively by the cis acting influence of the activeIg-H region in which case the strain of origin of the c-myc oncogene should not matter.

TRANSLOCATION NEGATIVE PLASMACYTOMAS

A new insight was gained concerning the role of c-myc and its orientation on chromosome 15 by the cytogenetic analysis of some exceptional translocation negative plasmacytomas.

In our previous studies on more than 7o pristane oil and pristane oil+A-MuLV induced plasmacytomas,we have encountered 5 translocation negative tumors.While some of them were near-diploid and the others near-tetraploid they appeared to lack any obvious chromosomal rearrangement.The question arose whether they have been generated by a different mechanism.

Conventional G-banding showed no detectable changes in chromosomes 6,12 and 15. Detailed analysis of some plates with preserved and

elongated chromosomes focused our attention on a regular size difference between the two chromosomes 15, however.Diploid tumor cells usually contained one chr.15 that was shorter than its normal homologue,whereas tetraploid tumor cells contained two shortened and two normal chrs.15. The difference in length could be related to a size difference in the major band 15D.

High resolution banding analysis of the normal and aberrant chr. 15 revealed that a rearrangement occurred within the major D band. The aberrant chromosome contained only a thin D band corresponding in size to either D1 or D3 sub-band of the normal homologue.The white and thin D2 sub-band that is intercalated between the D1 and D3 sub-bands on the normal 15 chromosome was removed by an interstitial deletion resulting in a smaller D band and a shorter chr.15.Southern blots of EcoRI DNA digests have shown the c-myc was rearranged in only one out of three plasmacytomas.Only the normal sized 2,4 kb myc RNA transcript was present in all three tumors, suggesting that the deletion breakpoints were outside the c-myc structural gene.

Since the HRB technique did not reveal any discernible minor "sub-bands" in D1 and D3, it was difficult to determine which of the dark bands was deleted in addition to the white D2 sub-band.However, a joint consideration of the cytogenetic and molecular data permits a tentative definition of the breakpoints and the band regions removed by the deletion.Moreover, it was also possible to map the orientation of the c-myc locus in relation to the centromere on chr.15 and to asses the orientation of the Ig-H locus on chr.12 indirectly.

CYTOGENETIC MAPPING AND ORIENTATION OF THE C-MYC LOCUS ON CHROMOSOME 15

Before presenting the details of this study it has to be emphasized that there is a considerable difference between the cytogenetic and molecular mapping of the PC-associated breakpoint on chromosome 15. The molecular breakpoints vary from tumor to tumor and cluster strikingly to a 1,1 kb region that spans the first exon and a part of the first intron of the c-myc gene.Thus, the breakpoints near the c-myc structural gene either sever the 5'exon of the gene or excise its 5'flanking region.

The variability of the molecular breakpoints is beyond the resolution power of the cytogenetic techniques.Therefore,the cytogenetic breakpoint is identical in all plasmacytomas that carry the

typical,variant or inverted translocation types.It is mapped in all tumors to the same sub-band,namely to the interface of bands D2 and D3

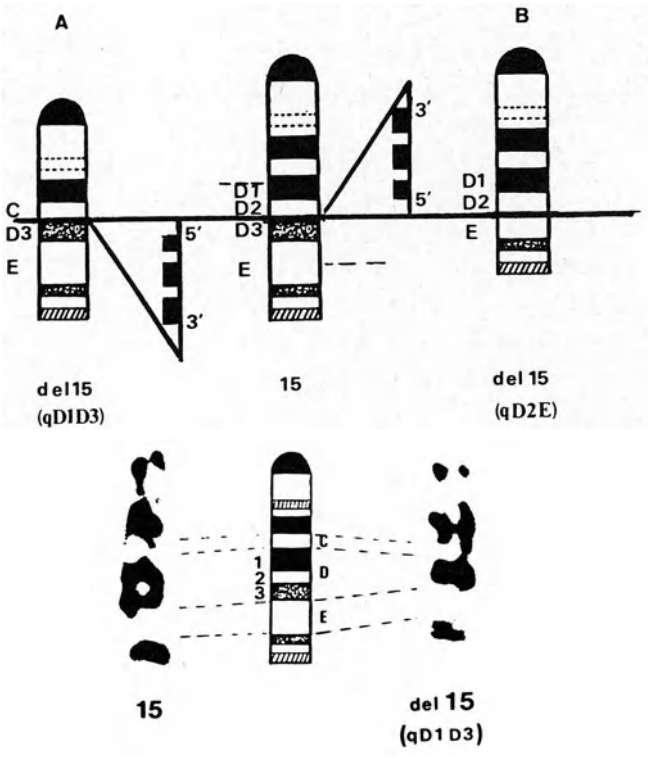
An interstitial deletion requires two breaks,followed by the loss of the interstitial piece and the joining of the centromeric and telomeric chromosomal segments.The cytogenetic mapping of the breakpoints flanking the deleted segment is determined by the orientation of c-myc locus in relation to the centromere.Since the molecular breakpoints cut through the first exon region,the orientation of the oncogene in relation to the cytogenetic breakpoint and centromere could be positioned according to two alternatives.

In alternative A,the first break occurs outside of the 5'exon of the oncogene through the interface of bands D2/3.This would be consistent with the normal sized c-myc transcript and also with the previously defined breakpoint on chr.15 in translocation plasmacytomas.Since the HRB revealed that the minor D2 band was deleted,the second break - proximal to the centromere - has to occur either on the interface of bands D2 and D1 or within the D1 band.This would suggest that the dark band and the deleted chromosome 15 arose from the joining of the D3 sub-band - the c-myc carrying chromosomal region - with the D1 sub-band or a part of the latter.

The other possibility is illustrated in alternative B.Here,the same two assumption are made: the first break occurs just outside the 5'exon of the c-myc gene and affects the interface of sub-band D2/3. It represents the centromere proximal break in this case. The second break would occur distally to the first,on the telomeric side resulting in a narrow dark band D1 - and a wide white band D2 + E.An opposite, centromere proximal deletion would remove the c-myc containing chromosomal segment.This would be unlikely,both in the view of the HRB pattern of the deleted chromosome and the high c-myc transcription in the tumors.

Two important conclusions emerge from the cytogenetic analyses of the deletion plasmocytomas.

One aspect relates to the activation mechanism of the c-myc oncogene in plsmacytomas.It was suggested that the translocation of the c-myc to an active Ig region may be responsible for the constitutive switch on the oncogene.The existence of the deletion plasmacytomas strongly suggests that c-myc can be activated by a rearrangement within a single chromosome.It is likely that the deletion disrupts the regulatory function of the 5'end of the gene and joins it to an actively transcribed chromatin region in D1 sub-band area of chromosome 15.In other words, the alteration of the normal function of c-myc by the break through its 5'end is the common event



in all types of plasmacytomas whereas the cellular mechanism that transposes it to an active chromosome region may be different.

The other point concerns the orientation of c-myc locus in relation to the centromere on chromosome 15 and the polarity of the Ig-H complex on chromosome 12. In this context, it must be remembered that: i) the translocation is reciprocal both in molecular and cytogenetic terms and ii) it recombines the 5'end of the c-myc to the 5' end of Ig-H. It follows that knowledge of the orientation of the c-myc on chr.15 in relation to the centromere will permit conclusions about the polarity of the Ig-H locus on chromosome 12.

The deletion plasmacytoma studies indicate that the orientation of the c-myc locus on chr.15 is centromere---5'---3'---telomere. That then implies that the orientation of the Ig-H loci on chromosome 12 can only be: centromere---C_h---V_h--telomere. This would also imply that the V2 and V3 exons of the c-myc are on chr.12 and the V_h on chr. 15. This is entirely in line with the translocation pattern and orientation of the c-myc and Ig-H in Burkitt lymphomas with rcpt(8;14) translocation.

Chromatin Structural Changes in the Putative Regulatory Region of c-myc Accompany the Translocation in a Burkitt Lymphoma

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SUMMARY

Several DNAase I hypersensitive sites mark the putative regulatory region immediately 5' of the myc gene. A sequence near at least one site binds to a protein(s) from nuclear extracts in vitro. Three patterns of chromatin structure exist, one associated with the translocated myc allele in a Burkitt lymphoma (BL 31), one associated with the non-translocated (germline) allele in the same Burkitt cell and one associated with the germline myc allele in non-malignant B cells. The non-translocated and transcriptionally silent myc allele in BL 31 shows only one strong hypersensitive site, a site which may mediate negative control over myc. The heavy chain immunoglobulin enhancer that is juxtaposed with myc on the translocated allele in BL 31 may be responsible for the chromatin structure on this deregulated allele. These data have novel implications for the activation of the myc oncogene by translocations.

INTRODUCTION

The chromosomal translocation of the myc oncogene is a consistent feature of all Burkitt lymphomas. Translocations of myc into the immunoglobulin loci in Burkitt lymphomas and murine plasmacytomas result in a general increase in myc transcription, but this increase is variable (Bernard 1983; Erikson 1983; Hamlyn 1983; Leder 1983; Maguire 1983; Taub 1984; Westin 1982). The actual perturbation of myc expression may indeed be a subtle one. Since myc transcription appears to be regulated by the cell cycle--it is under direct growth signal control in both fibroblasts and lymphocytes--translocations may, in fact, deregulate cell cycle control and thus cause expression of myc at the inappropriate time (Kelly 1983).

Another observation points to a loss of the normal control mechanism governing myc in Burkitt lymphomas. The nontranslocated myc allele is transcriptionally silent in Burkiktt lymphomas as well as in plasmacytomas (Bernard 1983; Stanton 1983; Taub 1984) and this has led to the prediction that the myc gene is under negative control (Leder 1983; Nishikura 1983). Thus, to understand how translocations affect myc expression it is critical to understand how myc is regulated. We, therefore, identified the presumed regulatory sequences near myc by DNAase I hypersensitivity studies (Siebenlist 1984).

DNAase I hypersensitivity is due to a discrete region on chromatin that is very sensitive to DNAase I (Stalder 1980; Wu 1980). Hypersensitive sites appear near many different DNA sequences which are known to be functionally important for gene expression, as is the case of the immunoglobulin kappa light chain and heavy chain enhancers (Mills 1983; Parslow 1983). In fact, hypersensitive regions may bind regulatory proteins (see Emerson 1984).

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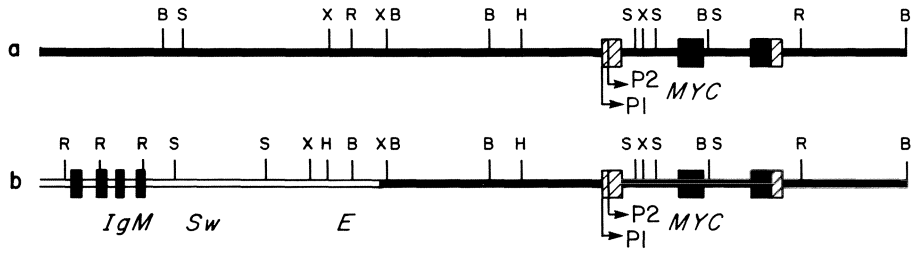


Fig. 1. The myc translocation in the Burkitt lymphoma BL 31. (a) Represents the non-translocated (germline) myc locus and (b) represents the translocated myc gene in BL 31, now juxtaposed with the mu constant region (IgM), the switch region (Sw), and the enhancer region (E). Solid bar indicates myc derived sequences, while open bar indicates immunoglobulin derived sequences. Exons are shown in boxes and P1 and P2 mark the two myc promoter start sites. Only some of the restriction sites are shown: B = BglI, S = SstI, X = XbaI, R = EcoRI, H = HindIII.

We will discuss here that the DNAase I hypersensitive sites immediately 5' of myc are located near sequences that we suspect on the basis of other data to be functionally important. We also will discuss the dramatic difference in chromatin structure between the translocated and the non-translocated allele in a Burkitt lymphoma, BL 31. The non-translocated allele features one strong hypersensitive site, a probable site for mediating negative transcriptional control of myc. The deregulation of the translocated myc allele is likely to be the result of the immunoglobulin heavy chain enhancer, juxtaposed with the myc gene in BL 31.

RESULTS AND DISCUSSION

In order to study the affect of a translocation on the chromatin structure of myc, we chose a Burkitt lymphoma in which the translocation point occurs at a considerable distance from the myc oncogene. As can be seen in Fig. 1, this less common situation occurs in BL 31. Here the myc gene is translocated into the IgM locus, with the crossover point occurring about 6 kb upstream of the first and untranslated myc exon. Also unusual, though not unique, is that myc is now juxtaposed with the immunoglobulin heavy chain enhancer.

DNAase I hypersensitive sites in this Burkitt cell and in the nonmalignant B cell line PF, an EBV transformed lymphoblastoid line, were determined essentially as described by Wu (1980) (for details see Siebenlist 1984). In this method, nuclei are digested with increasing amounts of DNAase I. Upon isolating and restricting the DNA, the DNAase I cutting sites (hypersensitive sites) can be visualized as subbands on genomic Southern blots, in addition to the original genomic restriction fragment. As is shown in Fig. 2 for the lymphoblastoid line PF and the Burkitt line BL 31, these subbands appear with increasing amounts of DNAase I, from left to right. PF cells contain two

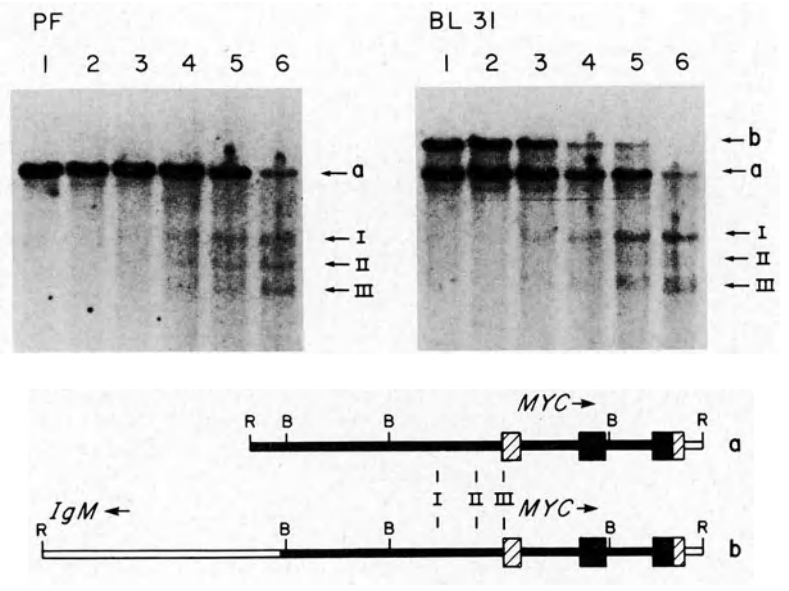


Fig. 2. DNAase I hypersenstiive sites near myc in PF and BL 31 cells. Nuclei of both cells were digested with increasing amounts of DNAase I (for details see Siebenlist 1984) and the isolated genomic DNA was restricted with EcoRI, electrophoresed, blotted onto nitrocellulose and hybridized to the SP probe. The hypersensitive sites are labelled I, II and III. (R = EcoRI, B = BglII, S = SmaI, P = PstI) For further description of map see Fig. 1.

germline myc alleles (fragment a in Fig. 2), whereas BL 31 cells have one translocated (b) and one germline (a) myc band. Clearly three (I, II and III) DNAase I hypersensitive sites emerge and their positions are indicated on the map in Fig. 2.

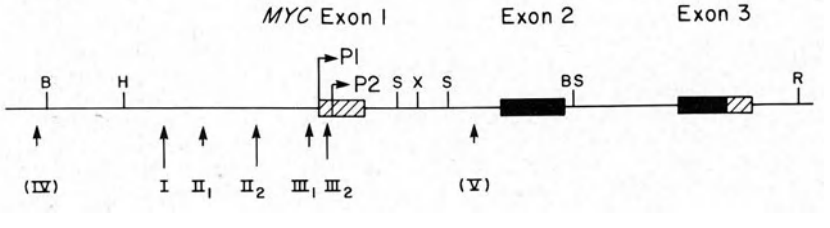


Fig. 3. Location of DNAase I hypersensitive sites. The sizes of the arrows reflect the approximate relative intensities of the hypersensitive sites in the lymphoblastoid line.

These hypersensitive sites were also mapped at a higher resolution by using a restriction digest that creates smaller subbands (see Siebenlist 1984). This not only allows a more accurate mapping, it also visualizes the subbands as stronger bands and resolves more bands. Figure 3 summarizes all the hypersensitive sites we have mapped. Sites I through III are consistently observed whereas sites IV and V are not. The consistently observed DNAase I hypersensitive sites I through III lie within a 2 kb region immediately 5' of the myc gene, a region we thus presume to contain regulatory sequences. Indeed all of these hypersensitive sites coincide with positions that we suspect on the basis of other data to be functionally important. This strengthens our notion that the DNAase I hypersensitive sites reflect regions critical to myc regulation.

To begin with, the very strong hypersensitive site I is located about 2 kb upstream of the P2 promoter start site, within a sequence region that is well conserved between mouse and man, as seen in a cross-species heteroduplex (Battey 1983). Such conservation is usually indicative of functional importance, and, as we will discuss below, this region is likely to mediate negative control of myc.

The hypersenstive sites III-1 and III-2 are located directly upstream of the two myc promoters P1 and P2, respectively. III-1 maps about 100 basepairs 5' of the P1 'TATA' box in a cystosine-rich stretch of DNA that is very homologous to the -100 region described by Dierks (1983), a region of functional significance for several genes. This sequence may therefore bind a more general transcription factor.

The relatively weak hypersensitive site II-1 lies just 5' of a sequence which is recognized by a protein(s) from nuclear extracts in vitro (see Siebenlist 1984). By comparing this sequence with another competing binding site located on the adenovirus terminal repeats, a conserved sequence emerges (TGA..G. AGCCAA). Interestingly, site II-2 lies next to a similar sequence. We speculate that the in vitro binding also occurs in vivo, resulting in a hypersensitive site II-1 and possibly also II-2. The DNA binding activity in nuclear extracts described here may be the nuclear factor 1, which has previously been shown to bind the adenovirus site and which is important for virus replication in vitro (Nagata 1983).

What is the chromatin structure of the two myc alleles in BL 31? The two alleles can be differentiated by employing a probe which hybridizes only to the non-translocated (germline) myc (a), but not to the translocated one, as seen in Fig. 4. The germline myc allele in BL 31 has only one hypersensitive site, I, and it is very intense when compared to the contribution from both chromosomes in PF; sites II and III are undetectable. We therefore propose that site I mediates the negative transcriptional control that appears to operate on the germline myc gene in all Burkitt cells and plasmacytomas where this could be analyzed (see Introduction). In one of several possible scenarios then, we imagine that the abnormally high production of myc from the translocated allele precipitates an increased activity in a trans-acting repressor which functions through site I on the germline myc allele (see Fig. 5). This, in turn, represses transcription of myc, possibly by preventing transcriptional factors from binding at site III and maybe even II.

How does the translocated myc allele escape repression in BL 31? Although this could not be shown directly, we can deduce very strong sites III-1 and

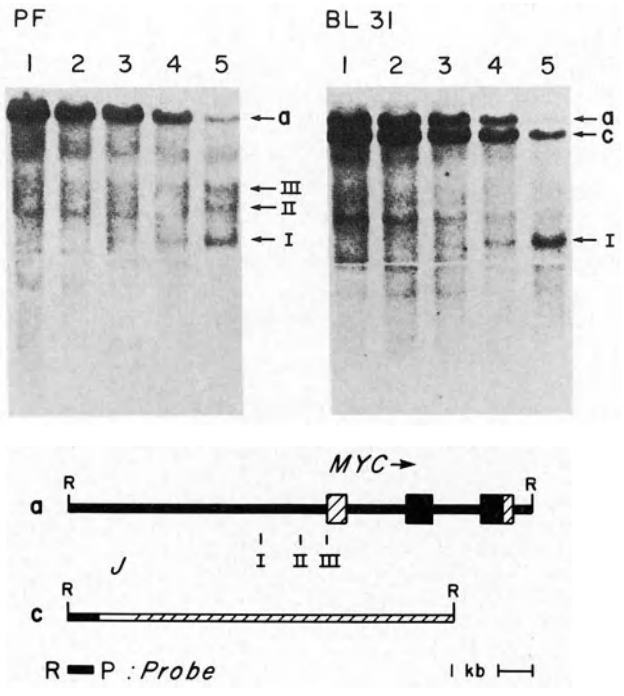
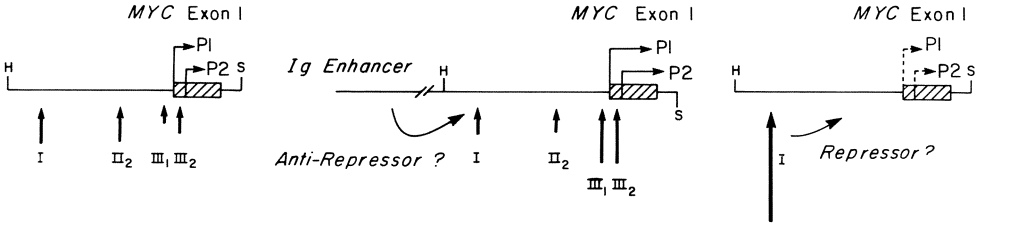


Fig. 4. The non-translocated myc allele in BL 31 has only one very intense DNAaseI hypersensitive site. The analysis was similar to the one described in Fig. 2, except that a different probe was used (R-P), which hybridizes to the germline myc allele (EcoRI: fragment a) and the reciprocal product of the translocation process (EcoRI: fragment c). J = immunoglobulin J region, hatched bar indicates an immunoglobulin rearrangement, R = EcoRI, P = PstI; for further description see Fig. 1.

III-2 for the translocated allele; it is further possible that site I is reduced. These chromatin changes suggest that the translocation interferes with the function of the hypothetical trans-acting repressor that acts through site I (see Figure 5). In the case presented here, BL 31, this may be due to immunoglobulin heavy chain enhancer, which is presumably functional, since it is itself associated with a hypersensitive site (see Siebenlist 1984). Interestingly, insertion of an ALV LTR 5' of the chicken myc gene changes the chromatin structure of that gene as well (Schubach 1984). Here, the enhancer may directly activate the promoters, possibly by allowing transcriptional factors to bind near sites III-1 and III-2. Of course other not yet identified elements either removed or introduced by the translocation could also contribute to the chromatin changes observed and thus lead to the deregulation of myc. These data are summarized in a model in Figure 5.

The presented data lead to a new interpretation of how translocations in general may deregulate the myc gene. We would like to suggest that the



Lymphoblastoid Cell Germline Gene, x 2 Chromosomes / Cell (P2 > PI)

Burkitt Cell Translocated Gene (Template for Most if Not All Transcription in BL31 P2≈PI)

Burkitt Cell Germline Gene (Little or No Transcription)

Fig. 5. Model. The non-translocated myc gene in BL 31 is dominated by a hypersensitive site (I) which is likely to mediate negative control (Repressor ?) over this transcriptionally silent gene. On the translocated myc allele, however, the immunoglobulin enhancer (Ig enhancer) might interfere with negative regulation (anti-Repressor), possibly by influencing sites III-1, III-2 and maybe also I (see text). This then leads to deregulated expression of the myc gene. The arrows indicate the strong and consistently observed hypersensitive sites and their lengths qualitatively represent their intensities. The hypersensitive site for the transcribed germline myc gene from the lymphoblastoid lines are shown for comparison.

structural alteration or elimination of site I might account for the observed loss of the normal control mechanisms governing this gene. Many translocations interrupt or eliminate this site and the untranslated first exon (Bernard 1983; Dalla-Favera 1983; Hayday 1984; Rabbitts 1984; Taub 1982; Taub 1984). In addition, it is possible that this region is mutated as a consequence of a translocation (Rabbitts 1983; Taub 1984). Of course, other mutational changes or elements may further affect the deregulation of the myc gene. In BL 31, site I is retained and most likely not mutated and here the strong dominant effect of the immunoglobulin enhancer may cause deregulation. Experiments testing these hypothesis are in progress.

REFERENCES

Battey J, Moulding C, Taub R, Murphy W, Stewart T, Potter H, Lenoir G, Leder P (1983) The human c-myc oncogene: structural consequences of translocation into the IgH in Burkitt lymphoma. Cell 34:779-789

Bernard O, Cory S, Gerondakis S, Webb E, Adams JM (1983) Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumors. EMBO J 2:2375-2383

Dalla-Favera R, Martinotti S, Gallo R, Erikson J, Croce C (1983) Translocation and rearrangments of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219:963-967

Dierks P, van Ooyen A, Cochran M, Dobkin C, Reiser J, Weissmann C (1983) Three regions upstream from the CAP site are required for efficient and accurate transcription of the rabbit beta-globin gene in mouse 3T6 cells. Cell 32:695-706

Emerson BM, Felsenfeld G (1984) Specific factor conferring nuclease hypersensitivity at the 5' end of the chicken adult beta-globin gene. Proc Natl Acad Sci USA 81:95-99

Erikson J, ar-Rushdi A, Drwinga HL, Nowell PC, Croce C (1983) Transcriptional activation of the translocated c-myc oncogene in Burkitt lymphoma. Proc Natl Acad Sci USA 80:820-824

Hamlyn PH, Rabbitts TH (1983) Translocation joins c-myc and immunoglobulin gamma-1 genes in a Burkitt lymphoma revealing a third exon in the c-myc oncogene. Nature 304:135-139

Hayday A, Gillies S, Saito H, Wood C, Wiman K, Hayward W, Tonegawa S (1984) Activation of a translocated human c-myc gene by an enhancer in the immunoglobulin heavy chain locus. Nature 307:334-340

Kelly K, Cochran B, Stiles C, Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. Cell 35:603-610

Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T, Taub R (1983) Translocations among antibody genes in human cancer. Science 222:765-771

Maguire RT, Robins TS, Thorgeirsson S, Heilman CA (1983) Expression of cellular myc and mos genes in undifferentiated B cell lymphomas of Burkitt and non-Burkitt types. Proc Natl Acad Sci USA 80:1947-1950

Mills F, Fisher M, Kuroda R, Ford A, and Gould H (1983) DNAase I hypersensitive sites in the chromatin of human mu Immunoglobulin heavy-chain genes. Nature 306:809-812.

Nagata K, Guggenheimer RA, Hurwitz J (1983) Specific binding of a cellular DNA replication protein to the origin of replication of adenovirus DNA. Proc Natl Acad Sci USA 80:6177-6181

Nishikura K, ar-Rushdi A, Erikson J, Watt R, Rovera G, Croce CM (1983) Differential expression of the normal and of the translocated human c-myc oncogenes in B cells. Proc Natl Acad Sci USA 80:4822-4826

Parslow T, Granner D (1983) Structure of a nuclease-sensitive region inside the immunoglobulin kappa gene: evidence for a role in gene regulation. Nucl Acids Res 11:4775-4792

Rabbitts TH, Hamlyn PH, Baer R (1983) Altered nucleotide sequence of a translocated c-myc gene in Burkitt lymphoma. Nature 306:760-765

Schubach W, Groudine M (1984) Alteration of c-myc chromatin structure by avian leukosis virus integration. Nature 307:702-708.

Siebenlist U, Hennighausen L, Battey J, Leder P (1984) Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell, submitted.

Stalder J, Larsen A, Engel J, Dolan M, Groudine M, Weintraub H (1980) Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNAase I. Cell 20:451-460

Stanton LW, Watt R, Marcu KB (1983) Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature 303:401-406

Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc Natl Acad Sci USA 79:7837-7841

Taub R, Moulding C, Battey J, Murphy W, Vasicek T, Lenoir G, Leder P (1984) Activation and somatic mutation of the translocated c-myc gene in Burkitt lymphoma cells. Cell 36:339-348

Westin EH, Wong-Staal F, Gelman EP, Dalla-Favera R, Papas T, Lautenberger JA, Eva A, Reddy EP, Tronick SR, Aaronson SA, Gallo RC (1982) Expression of cellular homologs of retroviral onc genes in human hematopoietic cells. Proc Natl Acad Sci USA 79:2490-2494

Wu C (1980) The 5' ends of Drosophila heat shock genes in chromatin are hypersensitive to DNAase I. Nature 286:854-860

Aberrant Rearrangements at the Murine Light Chain Locus in Plasmacytomas

M. Shapiro and M. Weigert

I. Introduction

Rearrangements at the κ locus can be analysed at the molecular level by using a variety of DNA probes. For example, a productive κ rearrangement (κ^+) will hybridize with probes from the constant (C_κ) region, the J_κ and the intervening sequence (IVS) region between J_κ and C_κ. It will not hybridize to probes upstream (U) of the J_κ region (Figure 1). The same applies to certain non-productive κ rearrangements (κ^-) that are a result of a mistake in the VJ joining process or due to the use of V_ψ genes. In plasmacytomas, rearrangements are detected that hybridize only with the C_κ probe or both the C_κ and IVS probes, but not with the J_κ probe. This suggests that rearrangement occurred in the IVS region. We have previously described such a rearrangement in the NZB myeloma PC7183 (1). This rearrangement hybridizes only with the C_κ probe and also contains DNA from chromosome 15 as well as DNA from the S_μ region. In this report we describe the structure of the reciprocal product and the relationship of both recombinants to the parental germline C_κ locus germline chromosome 15 loci.

II. Results

The κ^- rearrangement of PC7183 hybridizes only to the C_κ probe, not to the IVS, J_κ or U probes. However, another fragment in this tumor hybridizes with the IVS, J_κ and U probes and not with the C_κ probe. This putative reciprocal ($1/\kappa^-$) fragment was cloned and shown to contain DNA from the κ locus starting 5' of the J_κ genes and ending abruptly about halfway between $J_{\kappa 5}$ and C_κ. This segment of DNA is followed by DNA originating from the same region of chromosome 15 as that found in the κ^- rearrangement. No S_μ DNA is contained in the $1/\kappa^-$ clone.

Comparison of the recombination sites in both reciprocal products with the germline C_κ and chromosome 15 loci shows some features in common with other translocations and other examples of recombination. Most notable is a 14 bp deletion of C_κ sequence and a 1-2 bp deletion of chromosome 15. The size of the deletion here is ambiguous because the base at the site of recombination could have originated either from chromosome 15 or S_μ. Chromosomes 6 and 15 share a 5 bp homology at the site of recombination in the κ^- fragment. Another feature is that, within C_κ sequences, a YAGGTTG sequence (7/7 matches) lies 14 bp upstream of the recombination point in the $1/\kappa^-$ fragment. This sequence has been implicated in the normal IgCH switch process (2) and recently has been shown to be present in myc sequences near the point of myc/switch sequence recombination (3). In both cases YAGGTTG is found 8-16 bp upstream of the recombination.

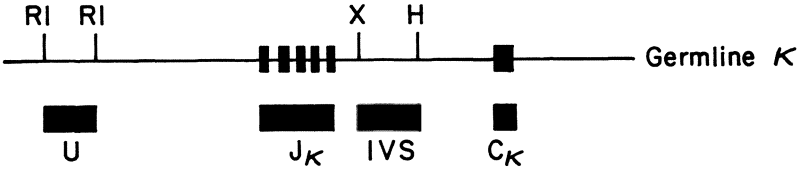


Figure 1

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III. Conclusions

The κ^- rearrangement in PC7183 is clearly an example of a tumor specific process. While PC7183 has not been karyotyped, it is clear a rcp (6:15) translocation has occurred. Translocations are very common in both mouse plasmacytomas and human Burkitt lymphomas. In the case of mouse plasmacytomas, the reciprocal translocation is usually between chromosomes 12 and 15, the heavy chain and myc bearing chromosomes. Occasionally variant translocations arise between chromosomes 6 and 15. While the myc oncogene apparently plays some role in plasmacytomagenesis, the segment of chromosome 15 involved in PC7183 is not within or near the myc exons. Restriction enzyme analysis shows no detectable rearrangement of the myc locus in PC7183. In most of the variant Burkitt lymphoma lines, the myc gene is also not rearranged and the translocation has occurred distant (at a molecular level) to the myc locus. These tumors still express high levels of myc mRNA. It is likely that PC7183 is in this category of translocation.

This translocation is complicated by the fact that the κ^- fragment contains S_μ DNA between the chromosome 15 and C_κ sequences. It is clear though, that both recombinant products could not have arisen due to 2 successive translocations. It is possible that S_μ, by virtue of its repetitive nature, transposed into chromosome 15. At a later time the translocation between chromosomes 6 and 15 could have resulted in the deletion of S_μ sequences as well as those from chromosome 6 and 15. This deletion could easily have left the $1/\kappa^-$ fragment without any S_μ DNA. It is well established that switch sequences are unstable due to their repetitive sequences (4).

A survey of plasmacytomas with known rcp (6:15) translocations reveals no rearrangement of the myc locus in 1/7 examples. This is similar to PC7183 and the majority of variant Burkitt lymphoma lines. However, based on their hybridization pattern, 5/7 contain κ^- and/or reciprocal fragments similar to those in PC7183. One of these rearranges the same fragment of chromosome 15 as in PC7183. The prevalence of this type of κ^- rearrangement in these tumors implies that the C_κ locus may be involved frequently in the rcp (6:15) translocations. This possibility is under further investigation.

IV. References

1. VanNess, B. G., Shapiro, M., Kelley, D. E., Perry, R. P., Weigert, M.: Aberrant rearrangement of the κ light-chain locus involving the heavy-chain locus and chromosome 15 in a mouse plasmacytoma. Nature 301, 425 (1983).

2. Marcu, K. B., Lang, R. B., Stanton, L. W., Harris, L. J.: A model for the molecular requirements of immunoglobulin heavy chain class switching. Nature 298, 87 (1982).

3. Dunnick, W., Shell, B. E., Dery, C.: DNA sequences near the site of reciprocal recombination between a c-myc oncogene and an immunoglobulin switch region. PNAS, USA 80, 7269 (1983).

4. Marcu, K. B., Arnheim, N., Banerji, J., Penncavage, N. A., Seperack, P., Lang, R., Miesfeld, R., Harris, L., Greenberg, R.: Studies on the nature and germline stability of DNA sequences flanking the mouse immunoglobulin heavy-chain constant-region genes. Cold Spring Harbor Symposia on Quantitative Biology 45, 899 (1980).

myc-Encoded Proteins of Chickens and Men

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INTRODUCTION

Recent work from many laboratories has demonstrated that the chromosomal locus containing the myc oncogene is the site of an extraordinary number of rearrangements. These rearrangements take the form of translocations, amplifications, and retroviral insertions and examples have been found in chickens, mice, and humans. In addition, such alterations in the myc locus appear to be associated with neoplasias of primarily lymphoid origin although myc amplification has also been observed in human neuroendocrine tumors and small cell lung carcinomas (see other papers in this volume).

Compared with the large amount of information pertaining to the structure of the myc locus and its transcriptional products, relatively little is known concerning the protein products encoded by myc. Within the last year however the difficulties encountered in obtaining antibodies against myc proteins have been largely overcome by use of antibodies raised against synthetic peptides corresponding to defined regions of the putative myc proteins (Hann et al. 1983; 1984) and antibodies raised against portions of myc proteins produced in bacterial expression vectors (Alitalo et al. 1983a). This work has resulted in the identification and localization of avian v-myc proteins in cells transformed by avian acute leukemia viruses and c-myc proteins in avian bursal lymphomas. In this communication we briefly summarize present ideas on the nature of avian myc proteins and outline our very recent findings on the proteins encoded by the human myc oncogene.

Avian v-myc and c-myc Encoded Proteins: Localization in the Nuclear Matrix/Lamin

The avian acute leukemia viruses which possess the myc oncogene produce their myc proteins as either a fusion product containing viral structural protein regions linked to myc protein regions (MC29 P110gag-myc; OK10 P200gag-pol-myc; CMII P90gag-myc) or as a protein not obviously linked to any retroviral encoded regions (MH2 p61/63v-myc; OK10 p62v-myc) (see Hayman 1983 for review). In addition the c-myc protein which is produced at high levels due to retroviral promoter insertion or enhancement has been identified by hybrid-selected translation and immunoprecipitation (Pachl et al. 1983; Hann et al. 1983; Alitalo et al. 1983a). When precipitated

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from avian bursal lymphoma cell lines this protein co-migrates with the OK10 p62 protein and has been termed p62c-myc. Thus far all v-myc and lymphoma c-myc proteins have been found to be phosphorylated.

One outstanding feature of both the fused and non-fused myc proteins is their predominant localization in the cell nucleus as shown by immunofluorescence and cell fractionation studies (Abrams et al. 1982; Donner et al. 1982; Hann et al. 1983; Alitalo et al. 1983a). Myc proteins are rapidly transported to the nucleus after synthesis as shown by pulse-labelling experiments, and transport does not appear to be immediately dependent on cellular DNA synthesis (Abrams and Eisenman, unpublished results). However despite this apparently rapid transit into the nucleus a variably small fraction of myc protein can also be found in the cytoplasm (Abrams et al. 1982; Bunte et al. 1982). Whether this cytoplasmic fraction represents a corresponding cytoplasmic role for myc or whether it derives from cells that are dying or undergoing mitosis is not yet clear.

Another potentially important property of myc proteins is their apparent affinity for DNA. Purified MC29 P110gag-myc eluted from anti-gag antibodies has been shown to associate with DNA in a filter binding assay (Donner et al. 1982). In addition non-gag linked v-myc and lymphoma c-myc proteins present in salt extracts from avian nuclei are retained on single- and double-stranded DNA cellulose columns and only eluted at 0.3-0.5M NaCl (Eisenman, Tachibana, Abrams and Hann, manuscript submitted). No evidence for binding to a specific sequence or class of sequences has been put forth. There is a possibility that the DNA binding observed in vitro is simply an ion exchange phenomenon due to the large number of basic amino acids at the carboxy-terminus of the protein and may not reflect the true function of the protein. Therefore, experiments have been carried out to determine whether myc proteins are associated with DNA in cells. Extraction of chromatin has shown association of only a small fraction of total nuclear P110gag-myc (Bunte et al. 1982). We have observed that digestion of nuclei with DNase I results in release of about 5-10% of total nuclear myc protein even at nuclease concentrations resulting in digestion of 99% of cell DNA. Further extraction of DNA/RNA depleted nuclei with high salt removes more myc protein but nonetheless leaves a significant amount behind with residual nuclear structures. These results have been obtained for both gag-linked and non-gag-linked v-myc proteins and for lymphoma c-myc proteins. These biochemical studies have been supported by immunofluorescence analysis and strongly suggest that myc proteins are associated with the nuclear matrix/lamin complex (Eisenman et al. manuscript submitted).

Human myc Proteins

Our success in preparing antisera reactive with avian myc proteins by using synthetic peptides as immunogens prompted us to attempt to identify human myc proteins in the same manner. We therefore synthesized a peptide corresponding to the twelve carboxy-terminal amino acids of human c-myc. This peptide (peptide hu-myc 12C) differs only at positions 2 and 10 from MC29 v-myc (counting from the carboxy-terminus) and at position 2 from chicken c-myc (see

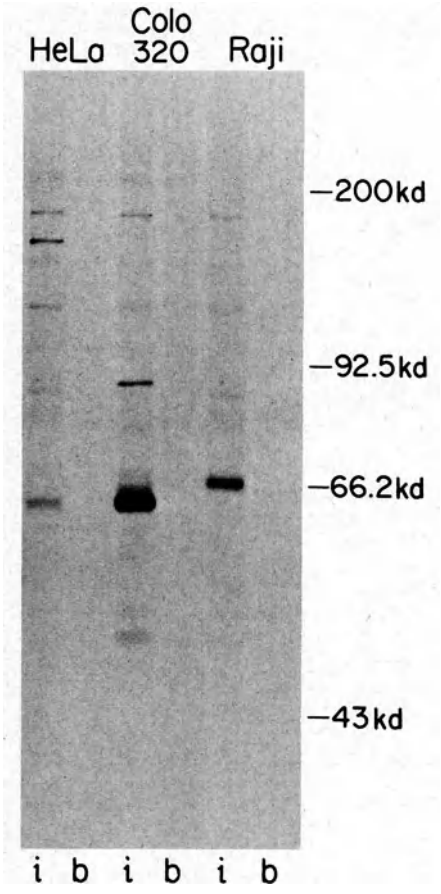

Figure 1. Analysis of anti-hu-myc 12C immunoprecipitates from human cell lines. Approximately 10^7 cells were labelled for 60 minutes with 35S-methionine and lysed in buffer containing 0.5% SDS containing equal quantities of acid precipitable radioactivity. The cleared lysates were then treated with either anti-hu-myc 12C (lanes i) or an anti-hu-myc 12C preparation that had been previously incubated with peptide hu-myc 12C (lanes b). The immunocomplexes were absorbed to fixed Staph. aureus, washed extensively and dissolved in 5% SDS electrophoresis sample buffer. The samples were analyzed on a 10% polyacylamide slab gel. A radioautograph of the dried gel is shown.

Bernard et al. 1983; Watson et al. 1983 for comparison of these sequences). Rabbit antibodies against peptide hu-myc 12C were affinity purified and found to react, albeit poorly, with avian myc proteins. However when used in immunoprecipitation reactions with lysates prepared from 35S-methionine labelled human cells anti-hu-myc 12C precipitated a major protein of 64kd and a minor protein of 67kd. This is shown in Fig. 1 for HeLa cells which contain low levels of myc mRNA (Saito et al. 1983) and for Colo 320, a cell line derived from a neuroendocrine tumor and possessing amplified myc DNA sequences and high levels of myc mRNA (Alitalo et al. 1983b). Preincubation of anti-hu-myc 12C antibodies with

peptide hu-myc 12C effectively blocks precipitation of the p64/67 proteins suggesting that precipitation of these proteins occurs through antibody recognition of the peptide sequence (Fig. 1 compare lanes i with lanes b).

Further evidence that p64/67 are encoded by the human myc gene comes from hybrid-selected translation experiments. We used a human c-myc cDNA clone bound to nitrocellulose filters to hybridize cytoplasmic RNA extracted from several human cell lines containing high levels of myc RNA. The bound RNA was released from the washed filters and used to program an in vitro translation system. The primary specific translation products were found to be both p64 and p67 and both proteins were precipitated from the translation lysate with anti-hu-myc 12C. This indicates that not only are both proteins likely to be encoded by the myc gene but that they may be translated from two distinct mRNAs (Hann and Eisenman, manuscript in preparation).

We have observed p64 and p67 in a wide variety of human cells. The highest levels of these proteins are found in cell lines containing amplified myc DNA. The lowest levels have been observed in normal human lung cells and several T cell derived lines while about 2-3 fold higher levels are seen in EBV immortalized lymphoblastoid cell lines and in Burkitt lymphoma lines. The single exception to our finding of p64/p67hu-myc in human cells has been the Raji Burkitt's lymphoma line where we observe a single band at 66kd whose precipitation is blocked by incubation of anti-hu-myc 12C with the immunizing peptide (Fig. 1). The presence of a specifically precipitated polypeptide having a mobility distinct from myc proteins in other cell lines probably reflects the large number of mutations in the coding exons as well as deletions in the non-coding exon of the translocated myc allele in the Raji line (Rabbitts et al. 1983).

We have observed several interesting features of these proteins. Both p64 and p67 are phosphorylated and both are located predominantly in the nucleus of the transformed cells examined. In addition pulse-chase experiments do not reveal any precursor-product relationship between the two proteins as would be expected if they were products of separate mRNAs. Interestingly both proteins appear to be rapidly turned over with half-lives on the order of 30-45 minutes. The rapid decay would seem likely to be a reflection of a tight cellular control over these proteins. Rapid turnover has also been observed for the v-myc proteins produced by MC29 and OK10 (Eisenman et al. 1980; S.R. Hann unpublished). Several lines of indirect evidence have linked avian v-myc expression with cellular hyperproliferation (Royer-Pokora et al. 1978; Palmieri et al. 1983; Land et al. 1983; P. Neiman, C. Wolf and G. Cooper, manuscript in preparation). In addition recent evidence has suggested a relationship between myc expression and cell cycle control (Kelly et al. 1983; Campisi et al. 1984) If so then the stabilization, deregulation, or overproduction of myc proteins might be expected to have important consequences for cell growth control.

ACKNOWLEDGEMENTS

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REFERENCES

Abrams HD, Rohrschneider LR, Eisenman RN (1982) Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell 29:427-439

Alitalo K, Ramsay G, Bishop JM, Pfeifer SO, Colby WW, Levinson AD (1983a) Identification of nuclear proteins encoded by viral and cellular myc oncogenes. Nature 306:274-277

Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM (1983b) Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80:1707-1711

Bernard O, Cory S, Gerondakis S, Webb E, Adams JM (1983) Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosome translocation in B lymphoid tumors. EMBO J 2:2375-2383

Bunte T, Greiser-Wilke I, Donner P, Moelling K (1982) Association of gag-myc proteins from avian myelocytomatosis virus wild-type and mutants with chromatin. EMBO J 1:919-927

Campisi J, Gray HE, Pardee AB, Dean M, Sonenshein GE (1984) Cell cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell 36:241-247

Donner P, Greiser-Wilke I, Moelling K (1982) Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature 296:262-266

Eisenman RN, Linial M, Groudine M, Shaikh R, Brown S, Neiman PE (1980) Recombination in the avian oncoviruses as a model for the generation of defective transforming viruses. Cold Spring Harbor Symp Quant Biol 44:1235-1247

Hann SR, Abrams HD, Rohrschneider LR, Eisenman RN (1983) Proteins encoded by the v-myc and c-myc oncogenes: Identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. Cell 34:789-798

Hann SR, Bender MA, Eisenman RN (1984) Analysis and purification of v-myc proteins using anti-peptide antibodies. In: Cancer Cells 2/Oncogenes and Viral Genes. GF VandeWoude, AJ Levine, WC Topp and JD Watson eds. Cold Spring Harbor Laboratory, New York

Hayman MJ (1983) Avian acute leukemia viruses. Current Topics in Microbiology and Immunology 98:109-126

Kelly K, Cochran BH, Stiles CD, Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603-610

Land H, Parada LF, and Weinberg RA (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304:596-602

Pachl C, Schubach W, Eisenman R, Linial M (1983) Expression of c-myc RNA in bursal lymphoma cell lines: Identification of c-myc-encoded proteins by hybrid-selected translation. Cell 33:335-344

Palmieri S, Kahn P, Graf T (1983) Quail embryo fibroblasts transformed by four v-myc-containing virus isolates show enhanced proliferation but are non-tumorigenic. EMBO J 2:2385-2389

Rabbitts TH, Hamlyn PH, Baer R (1983) Altered nucleotide sequences of a translocated c-myc gene in Burkitt lymphoma. Nature 306:760-765

Royer-Pokora B, Beug H, Clavier M, Winkhardt H-J, Friis RR, Graf T (1978) Transformation parameters in chicken fibroblasts transformed by AEV and MC29 avian leukemia viruses. Cell 13:751-760

Saito H, Hayday A, Wiman K, Hayward WS, Tonegawa S (1983) Activation of the c-myc gene by translocation: A model for translational control. Proc Natl Acad Sci USA 80:7476-7480

Watson DK, Psallidopoulos MC, Samuel KP, Della-Favera R, Papas TS (1983) Nucleotide sequence analysis of human c-myc locus, chicken homologue, and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. Proc Natl Acad Sci USA 80:3642-3645

Properties of the myc-Gene Product: Nuclear Association, Inhibition of Transcription and Activation in Stimulated Lymphocytes

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SUMMARY

Using a variety of myc-specific antibodies against bacterially expressed viral and human cellular myc genes and against synthetic peptides, we have characterized viral and cellular myc-gene products and obtained the following results:

(1) Immunofluorescence analysis of MC29-transformed fibroblasts sequentially treated with detergent, nucleases and salt indicates that part of the p110gag-myc remains insoluble suggesting its association with the so-called nuclear matrix.

(2) Immune-affinity purified p110gag-myc protein inhibits transcription of the Adenovirus 2 major late promoter (Ad2 MLP) two- to fourfold if added to in vitro transcription systems.

(3) The c-myc gene product expressed in avian lymphoma (RP9) cells was identified as a protein of 55,000 molecular weight, p55c-myc, similar to the avian viral p55v-myc protein of OK10 cells.

(4) Myc-specific antibodies gave rise to nuclear fluorescence in HeLa cells and precipitated two proteins of about 62,000 and 49,000 molecular weights which were competed out by the appropriate antigens.

(5) Mitogen-stimulated lymphocytes exhibit two novel proteins of similar size detected by myc-specific antibodies.

INTRODUCTION

The avian myelocytomatosis viruses code for transforming proteins which are either fused to viral structural proteins, e.g. p110gag-myc, or translated from subgenomic messenger RNAs as is the case with OK10, which codes for a p55v-myc protein (Moelling et al. 1984). Definition of its molecular weight varies in different laboratories from 57,000 to 62,000 (Alitalo et al. 1983; Hann et al. 1983). The

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human cellular myc-gene product has been identified as a molecule of 49,000 daltons (Giallongo et al. 1983) which corresponds best to its predicted size. The normal cellular homologue of the viral myc gene, c-myc, is expressed in avian lymphoma cells through the indirect activation by the LTR-promoter of avian leukemia viruses (Hayward et al. 1981). Recently, activation of myc-gene transcription has been described in stimulated mouse lymphocytes (Kelly et al. 1984).

Here we report on some properties of the myc-gene product concerning its intracellular localization, the in vitro effect of the myc protein on transcription, its size in avian lymphomas and human cells and its novel appearance in stimulated human lymphocytes.

MATERIALS AND METHODS

Preparation of cells, radioactive labeling, lysis, immunoprecipitation procedure has been described previously (Bunte et al. 1982,1983). Immune-affinity column chromatography was performed essentially as described (Donner et al. 1982, 1983). Antibodies against synthetic peptides have been published (Moelling et al. 1984). Cloning of viral genes and expression in bacteria has been reviewed in Moelling (1984). Stimulation of lymphocytes was performed as described by Kelly (1983).

RESULTS

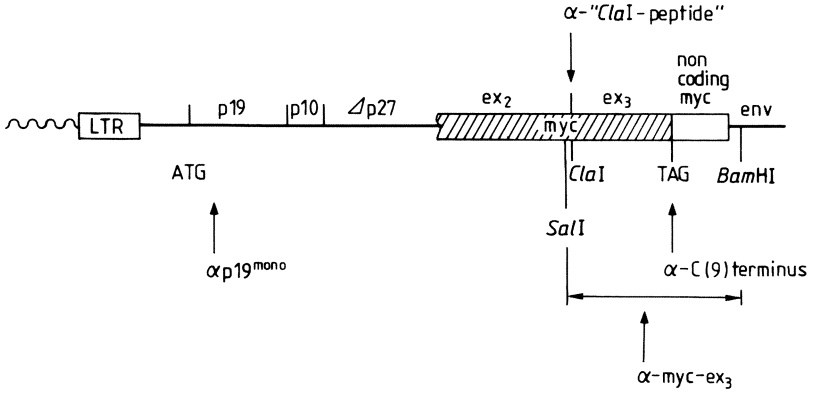

Fig. 1 shows schematically the genome of the MC29 virus. The coding exons of the myc gene are indicated by ex_2 and ex_3. The arrows point out to regions against which antibodies have been prepared: αp19mono is a monoclonal antibody, α"ClaI-peptide" is an oligopeptide of 16 amino acids which maps around the ClaI restriction site, α-C_9 terminus indicates the antibody against an oligopeptide consisting of 9 amino acids of the carboxy-terminal end, α-myc-ex_3 is an antibody prepared against a bacterially expressed protein representing $exon_3$ and part of $exon_2$.

Figure 1 schematically shows the viral myc gene from the MC29 virus. The four arrows indicate the regions against which antibodies were prepared. Besides the structural protein p19, against which monoclonal antibodies are available (Greiser-Wilke et al. 1981), a carboxy-terminal peptide consisting of nine amino acids (C_9), and an internal peptide indicated as "ClaI-peptide" have been synthesized and used for antibody production. Furthermore, the SalI-BamHI region has been expressed in E.coli and was used for antibody production.

Subcellular Localization of the Viral myc-Gene Product

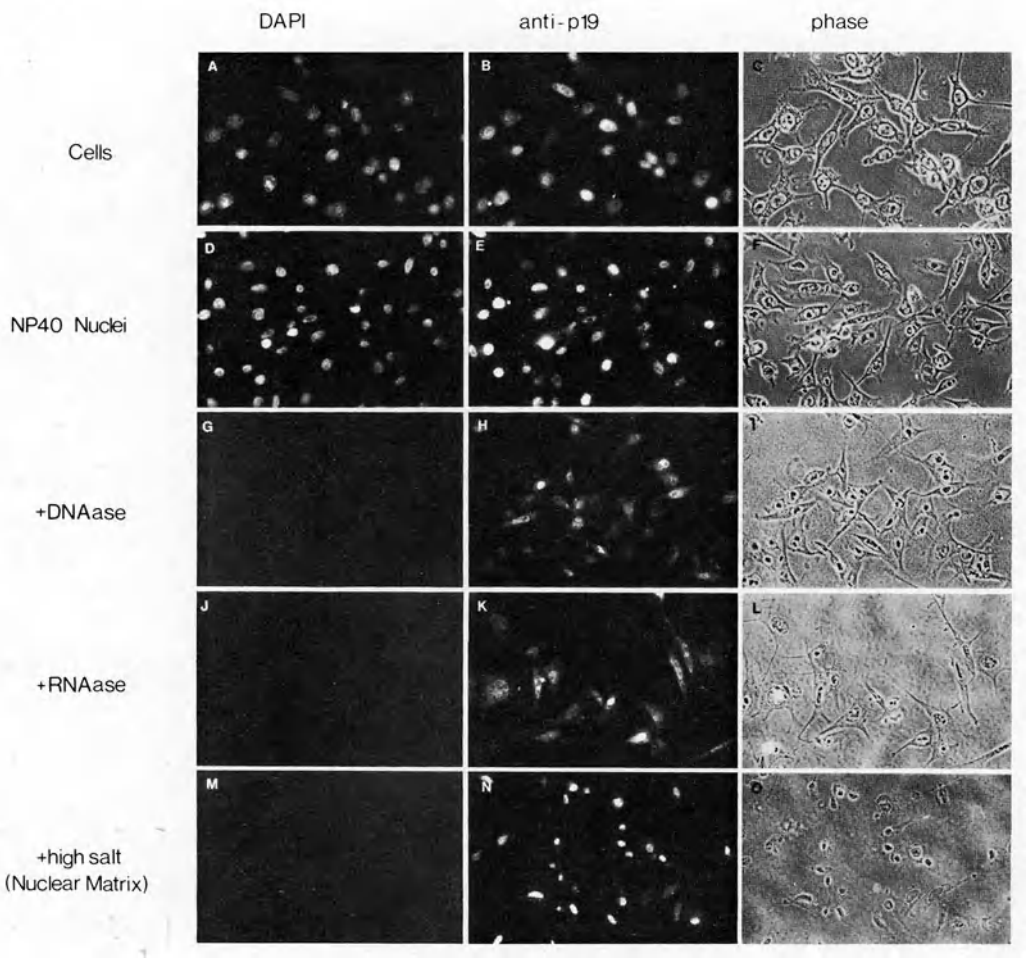

Fig. 2. Fluorescence analysis of MC29-transformed quail nonproducer fibroblasts (MC29-Q8-NP) for DNA and p110gag-myc. Fibroblasts were fractionated and processed for fluorescence microscopy as described (Staufenbiel and Deppert 1983). The same cells and nuclear structures were analyzed for DNA with the intercalating dye DAPI, for p110gag-myc with monoclonal antibody against p19 followed by FITC-coupled anti-mouse immunoglobulin. Cells and nuclear structures were viewed with phase contrast. A,B,C: whole cells, D,E,F: NP40 nuclei, G,H,I: DNase treatment, J,K,L: RNase treatment, M,N,O: nuclear matrix.

With monoclonal antibodies against p19 or anti-gag antibodies about 60% of the p110gag-myc protein was identified in the nucleus as MC29-transformed fibroblasts (Donner et al. 1982; Abrams et al. 1982). About 20% of the nuclear myc-gene product was found associated with a chromatin preparation (Bunte et al. 1982). For further localization studies MC29-transformed quail fibroblasts were grown on cover slips and sequentially treated with detergent, DNase, RNase and high salt according to published procedures (Staufenbiel and Deppert 1983). After each treatment an immunofluorescence analysis was performed to identify the p110gag-myc protein. A parallel treatment with DAPI allowed to identify DNA and phase contrast microscopy revealed the state of the cells after each treatment. The results are shown in Fig. 2. Some removal of p110 was achieved by DNase treatment (compare the differences in intensities of fluorescence between E and H). A portion of p110 resisted to all treatments (N) indicating association of p110 with the so-called nuclear matrix.

Effect of the Purified myc-Gene Product on In Vitro Transcription

The purified viral myc-gene products p110gag-myc as well as p55myc have been shown to bind to double-stranded DNA in vitro (Donner et al. 1982; Bunte et al, manuscript submitted). Assuming a regulatory role for the myc-gene product, it might exert its function on the DNA in vivo either through activation or inhibition of gene expression. To test these possibilities in vitro, the purified p110gag-myc protein was added to an in vitro transcription system consisting of a HeLa whole cell extract and an Adenovirus-2-specific DNA template which contains the major late promoter (Ad2 MLP) as described by Hen et al. (1982). Increasing amounts of purified p110 protein was added to the system and transcription of the Ad2 MLP was analyzed by gel electrophoresis. The effect of p110gag-myc on RNA transcription was compared with that of a control protein, Pr76gag, prepared in an identical fashion. Protein-free buffer and a bovine-serum-albumin standard served as controls (Fig. 3, controls 1 and 2). The effect of p110 was dose-dependent and resulted in a two- to fourfold reduction of transcription (Fig. 3). Replacement of the whole cell extract by an S-100 derived fraction (Davison et al. 1983) resulted in the same effect.

Deletion of the Ad2 MLP upstream at nucleotides -34 to -97 did not alter the inhibitory effect, indicating that inhibition by p110 is independent of the presence of upstream sequences of this promoter (data not shown).

The inhibitory effect of p110 on transcription is independent of the time of addition of p110 to the reaction mixture. Cell extract and DNA were preincubated, then the ribonucleotides were added to start transcription. p110 or Pr76 were added before or after the onset of transcription and resulted in inhibition (Fig. 3, bottom).

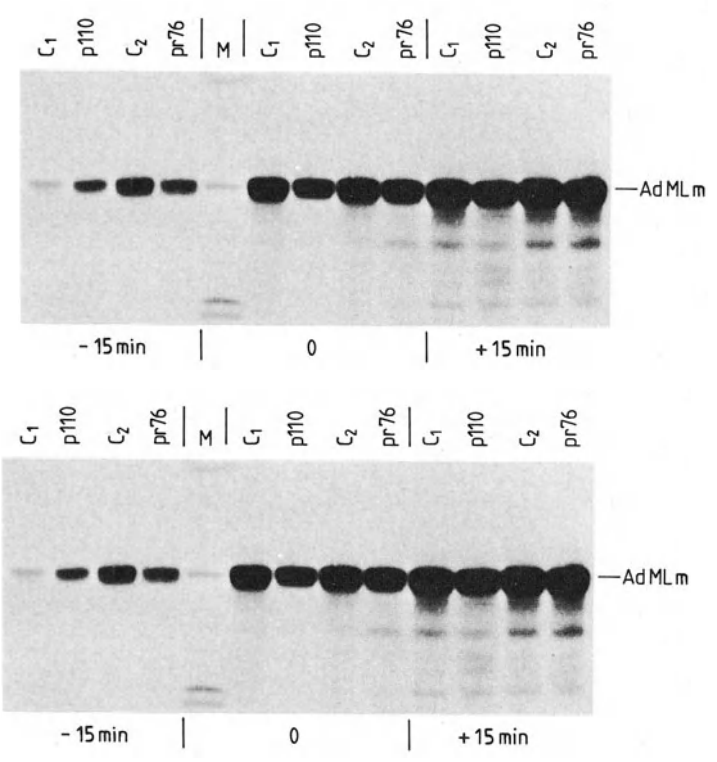

Fig. 3. In vitro transcription of the Adenovirus 2 major late promoter, deletion mutant -97 (Ad2 MLP) (Hann et al 1983) in HeLa whole cell extract in the presence of purified p110gag-myc and Pr76gag. Top: 0.1, 0.2, 0.4 µg of protein were added to extracts with 0.1 µg corresponding to 5fold molar excess of protein over DNA. The ^{32}P-labeled RNA transcript was analyzed by gel electrophoresis and autoradiography. Controls 1 and 2 correspond to buffer and buffer supplemented with bovine serum albumin (BSA), respectively. Bottom: Transcription was analyzed with addition of p110 at various time points before and after initiation of complex formation. Whole cell extract and DNA were mixed and transcription was initiated by the addition of ribonucleotides at time 0. At time points -15 min, 0, and +15 min, p110 protein was added to the reaction mixtures and incubation performed for 30 min at 24°C. Pr76, buffer and BSA served as controls (C_1, C_2, respectively).

Analysis of the c-myc-Gene Product

Only when myc-specific antibodies became available, the myc-gene product unlinked to the gag portion was detectable in cells transformed by MC29-related viruses. The protein has been described in avian cells with molecular weights of 57,000 (Alitalo et al. 1983), 62,000 (Hann et al. 1983) and 55,000 daltons (Moelling et al. 1984). In mammalian cells a 48,000 molecule has been observed (Giallongo et al. 1983) which corresponds to the size predicted from the gene. Whether these differences are attributable to different marker proteins, degradation effects, or to differences in the sera used, is still unclear.

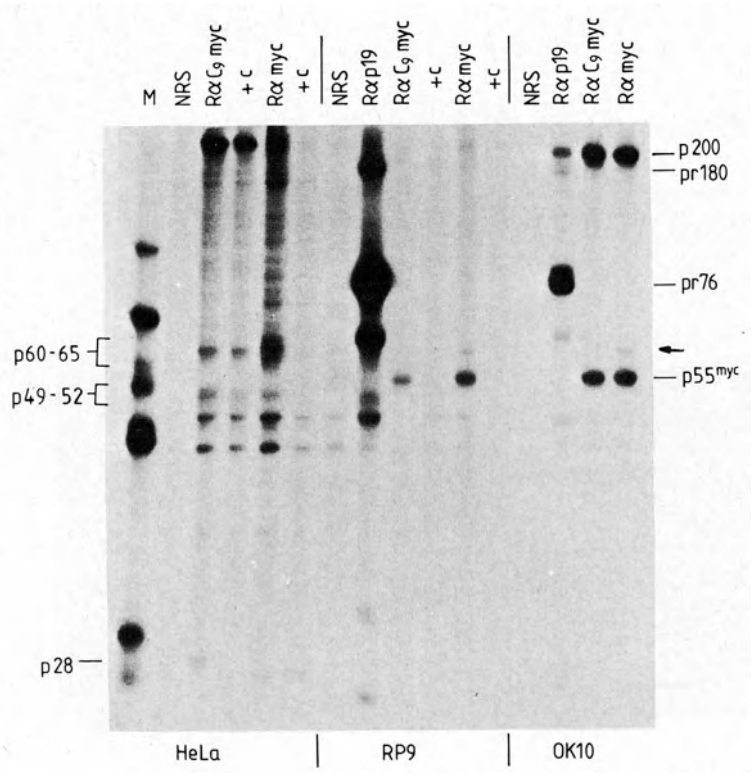

Fig. 4. Radioimmunoprecipitations were performed with HeLa cells, RP9 avian lymphoma cell line and OK10-transformed quail fibroblasts. Cells were labeled with ^{35}S-methionine for 2 hrs, lysed in RIPA buffer and treated with sera and Staph. aureus for indirect immunoprecipitation. Proteins were analyzed by gel electrophoresis and autoradiography. NRS: normal rabbit serum, α-C_9-myc: antiserum against the 9 carboxy-terminal amino acids of the viral myc, α-myc: antibody against the bacterially expressed viral myc protein as indicated in Fig. 1, Rαp19: antibody against p19. +C indicates competition with 10 µg of the respective peptide or protein against which the sera were prepared. p200 represents the gag-myc-pol protein of OK10, Pr180 the gag-pol precursor, Pr76 the gag precursor, p55 the v-myc protein. The arrow (←) points to the 62,000 molecular weight cellular myc-related protein.

Using anti-myc sera against the bacterially expressed viral myc gene (α-myc), a myc-specific protein of 55,000 molecular weight, p55c-myc, is activated in lymphoma cells (RP9) through the action of the LTR of a leukosis virus. Its size is similar to p55v-myc coded for by OK10 (Fig. 4). The carboxyl terminus of the normal cellular myc gene differs in two out of 9 amino acids, therefore the anti-C_9 viral peptide antibody recognizes the viral p55v-myc more efficiently than the p55c-myc (Fig. 4). In RP9 as well as in OK10 cells the α-myc antibody recognizes small amounts of a 62,000 molecular weight protein which is competed out by the myc protein. It therefore also appears to be myc-specific.

In HeLa cells the anti-C_9 peptide antibody precipitates a 62,000, a 49,000 and to less extent a 28,000 molecular weight protein. α-myc antibodies recognize a protein around 60,000 to 65,000 daltons and a protein of 49,000 daltons. Competition with the appropriate peptides was performed in parallel with HeLa, RP9 and OK10 cells. These two proteins correspond to the proteins predicted by Gazin et al (1984), the larger one being predicted by a possible open reading frame in exon 1. Purification of the human-c-myc protein from HeLa cells by immune-affinity chromatography also resulted in a complex polypeptide pattern. Processing by proteases cannot be excluded here either (Moelling et al, unpublished observation). Anti-myc antibodies give rise to nuclear fluorescence (Fig. 5).

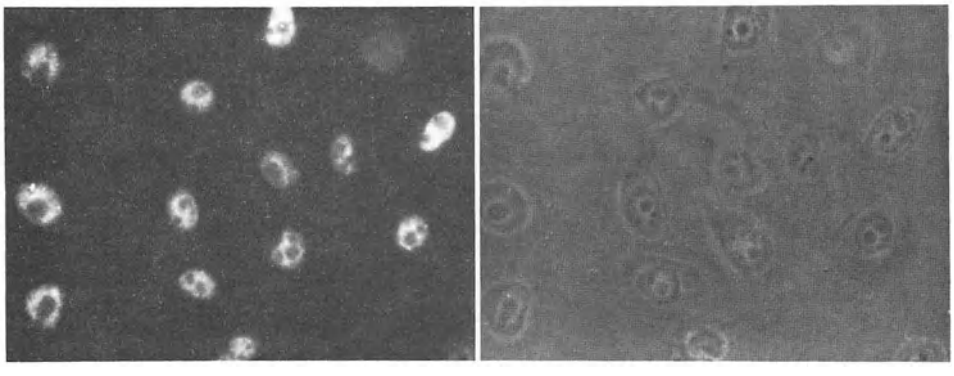

Fig. 5. Indirect immunofluorescence analysis of HeLa cells using Rα myc -specific antibodies at a dilution of 1:40. A phase contrast picture of the identical cells is shown for comparison. Details of the procedure have been described (Donner et al. 1982).

Mitogenic Stimulation of Human Lymphocytes

One possible function of the myc-gene product is to immortalize cells. Therefore its expression may correlate with growth. To test this immortalization function of myc, lymphocytes offer a suitable system. They allow a comparison of quiescent and actively dividing cells which can be stimulated to grow by mitogens. Human lymphocytes were cultured in the presence of ^{35}S-methionine for 4 hrs at 37°C with and without mitogenic stimulation by Concanavalin A (ConA). Subsequently the cells were lysed and treated with anti-myc serum for indirect immunoprecipitation. The anti-myc serum precipitates two proteins in stimulated lymphocytes which are absent from unstimulated ones (Fig. 6). The proteins resemble those detected in actively dividing HeLa cells (Fig. 4) and have molecular weights of 49,000 and 62,000. Normal rabbit serum and antibody against $ß_2$ microglobulin were used for control and standardization (Fig. 6).

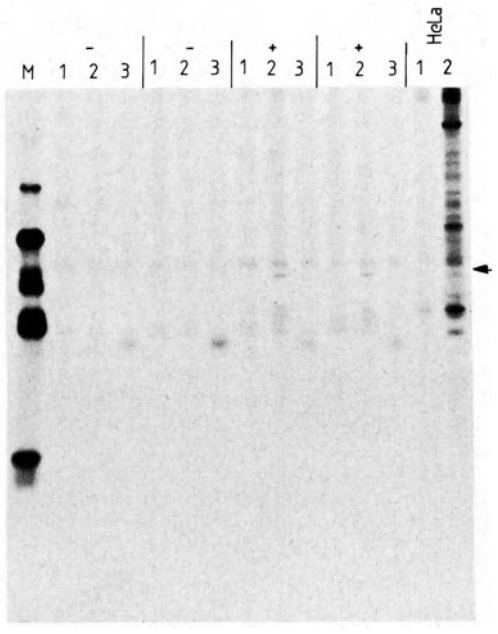

Fig. 6. 10^7 human lymphocytes from a normal donor were incubated with ^{35}S-methionine for 4 hrs in the absence (-) and presence (+) of Concanavalin A (20 µg/ml). Cells were lysed and immunoprecipitated with NRS, Rα-myc serum (see legend to Fig. 4) and a β-2 microglobulin antibody which served as internal standard. M indicates molecular weight markers. 1, 2, and 3 indicate NRS, Rα-myc and antibody against microglobulin respectively.

DISCUSSION

The myc-gene product has been shown to interact with DNA in in vitro assay (Donner et al. 1982). Immunofluorescence analysis reported here suggests that a fraction of p110gag-myc may also be associated with DNA in vitro, since DNase treatment reduces its fluorescence. Furthermore, a portion of the myc-gene product appears to be associated with a high salt- and detergent-resistant structure defined as nuclear matrix. A more quantitative analysis of these observations is in progress. Association of the myc-gene product with nuclear matrix has recently also been reported by Eisenman et al. (1984). Nuclear matrices are considered to be important for gene activity and gene regulation (Ciejek et al. 1983). Since the myc-gene structure predicts a bifunctional molecule, one domain, the carboxy-terminal one, is thought to be responsible for DNA-binding whereas the other one may be associated with the matrix (Moelling 1984). The DNA-binding site maps around the ClaI cleavage site (Bunte et al, manuscript submitted).

The myc-gene product is assumed to play a role in gene expression. Two models have been suggested, myc may either activate genes or, alternatively, repress gene expression, possibly in an autoregulatory fashion. We report here one experiment in favor of the repressor model. The purified myc-gene product inhibits in vitro transcription. Since the assay is very complex, details remain to be elucidated.

Kelly et al. (1984) also lend support to such a model from cycloheximide studies which result in superinduction of c-myc transcripts which would be consistent with the idea of a labile myc repressor protein. On the other hand, some recent experiments seem to suggest that myc activates gene expression (for summary see Newmark 1983).

In spite of significant amino acid changes between the viral myc-gene product and its cellular homologues in chicken and human cells, cross-reactivity was observed with all the myc-specific antisera described here. The antibodies give rise to nuclear fluorescence in HeLa cells (Fig. 5). They identify a human myc-gene product(s) of 60,000 to 65,000 molecular weights. Smaller proteins are also detectable. Whether they are the results of uncontrolled proteolytic events needs to be proven. This question is of particular importance, since a human cellular myc-gene product of 48,000 daltons has been published (Giallongo et al. 1983). Clearly, the viral myc-gene product, p55v-myc, from MH2 transformed cells, is smaller than the one in HeLa cells. However, a faint band of 62,000 daltons was also present in MH2 cells as well as avian lymphoma cells (indicated by arrow in Fig. 4). This could present the normal cellular homologue, the c-myc gene product, which may be also expressed in viral transformed cells. The partially purified HeLa cell protein(s) bind(s) to DNA in vitro (Moelling et al. unpublished observation).

Recently, Kelly et al. (1984) described the induction of c-myc transcripts in stimulated lymphocytes. Analyses of stimulated human lymphocytes with myc antibodies confirm this observation on the level of protein expression as is shown in Fig. 5. Pulse-chase experiments or cycloheximide treatment should allow to define whether the myc-gene product is a labile molecule.

We have recently also expressed a portion of the human cellular myc-gene product in bacteria and raised antibodies against it. This should recognize the human myc protein(s) more specifically.

REFERENCES

Abrams HD, Rohrschneider LR, Eisenman RN (1982) Nuclear localization of the putative protein of avian myelocytomatosis virus. Cell 29: 427-439

Alitalo K, Ramsay G, Bishop JM, Ohlsson-Pfeiffer S, Colby WW, Levinson AD (1983) Identification of nuclear proteins encoded by viral and cellular myc oncogenes. Nature 306:274-277

Bunte T, Greiser-Wilke I, Donner P, Moelling K (1982) Association of gag-myc proteins from avian myelocytomatosis virus wild-type and mutants with chromatin. EMBO J 1:919-927

Bunte T, Greiser-Wilke I, Moelling K (1983) The transforming protein of the MC29-related virus CMII is a nuclear DNA-binding protein whereas MH2 codes for a cytoplasmic RNA-DNA binding polyprotein. EMBO J 2:1087-1092

Ciejek, E.M., Tsai MJ, O'Malley BW (1983) Actively transcribed genes are associated with the nuclear matrix. Nature 306:607-609

Davison BL, Egly JM, Mulvihill ER, Chambon P (1983) Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature 304:680-686

Donner P, Bunte T, Greiser-Wilke I, Moelling K (1983) Decreased DNA-binding ability of purified transformation-specific proteins from deletion mutants of the acute avian leukemia virus MC29. Proc Natl Acad Sci USA 80:2861-2865

Donner P, Greiser-Wilke I, Moelling K (1982) Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature 296:262-266

Eisenman R, Hann S, Abrams H, Tachibana C, Rohrschneider L (1984) The myc oncogene encodes a class of nuclear proteins: possible association with the nuclear matrix. In: Levine A, van de Woude G (eds) Cell proliferation and cancer, vol 11. Cold Spring Harbor Press (in press)

Gazin C, Dupont de Dinechin S, Hampe A, Masson J-M, Martin P, Stehelin D, Galibert F (1984) Nucleotide sequence of the human c-myc locus: provocative open reading frame within the first exon. EMBO J 3:383-387

Giallongo A, Appella E, Riccardi R, Rovera G, Croce CM (1983) Identification of the c-myc oncogene product in normal and malignant B cells. Science 222:430-432

Greiser-Wilke I, Owada MK, Moelling K (1981) Isolation of monoclonal antibodies against the avian oncornaviral protein p19. J Virol 39: 325-329

Hann SR, Abrams H, Rohrschneider LR, Eisenman RN (1983) Proteins encoded by v-myc and c-myc oncogenes: identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. Cell 34:789-798

Hayward WS, Neel BC, Astrin SM (1981) Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 290:475-480

Hen R, Sassone-Corsi P, Corden J, Gaub MP, Chambon P (1982) Sequences upstream from the T-A-T-A- box are required in vivo and in vitro for efficient transcription from the adenovirus serotype 2 major late promoter. Proc Natl Acad Sci USA 79:7132-7136

Kelly K, Cochran BH, Stiles CD, Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35:603-610

Moelling K (1984) Fusion proteins in retroviral transformation. In: Weinhouse S, Klein G (eds) Advances in cancer research, vol 42. Academic Press, New York (in press)

Moelling K, Bunte T, Greiser-Wilke I, Donner P, Pfaff E (1984) Properties of avian viral transforming proteins gag-myc, myc and gag-mil. In: Levine A, van de Woude G (eds) Cell proliferation and cancer, vol 11. Cold Spring Harbor Press (in press)

Newmark P (1983) The rasmatazz of cancer genes. Nature 305:407-471

Staufenbiel M, Deppert W (1983) Different structural systems of the nucleus are targets for SV40 large T antigen. Cell 33:173-181

Introduction to Long-Term Cultures of Normal B Lymphocytes

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Over the last 15 years, the pathways involved in the maturation of cells within the B lymphocyte lineage have become increasingly well understood. It is now apparent that sIg^+ B cells derive from a large pool of immature precursors that may constitute 25% or more of normal adult bone marrow (1). Their progeny are seeded to peripheral lymphoid tissues at rates sufficient to replace 5% to 10% of all mature B cells each day (2,3). Upon reaching the periphery, the majority of immunocompetent cells die rapidly with a T 1/2 = 24 hr (4,5) unless they are salvaged by antigen-specific or nonspecific stimuli. Even with continuing stimulation, it appears that the life span of these rescued cells would not exceed that required for an additional 30 to 50 rounds of replication (6). Within the broadly-sketched outline of B cell growth and clonal senescence, there exists a multitude of sequential decision points that contribute to the extensive phenotypic and functional heterogeneity observed within the pool of peripheral B lymphocytes.

Recently, a number of new systems have been developed that have the potential for substantially enhancing our understanding of this heterogeneity. They include, first, the identification of cells that may reflect some of the earliest stages of commitment to B cell differentiation. It was previously shown that two subsets of B cell precursors, termed pre-B cells, could be identified on the basis of differential expression of cell-surface antigens and rearrangements of Ig heavy and light chain genes. Large pre-B cells are $Lyb\text{-}2^+$ $Ly\text{-}5(B220)^+$ ThB^- and have undergone rearrangements of both Ig heavy chain genes whereas Ig light chain genes in these cells are not rearranged. Their progeny, termed small pre-B cells, are $Lyb\text{-}2^+$ $Ly\text{-}5(B220)^+$ ThB^+ and exhibit rearrangement of light as well as heavy chain genes (7). Cells with rearrangements of a single Ig heavy chain gene that could be the progenitors of large pre-B cells are yet to be defined. On the basis of phenotypic studies of murine lymphomas and bone marrow and spleen cells from newborn mice, Holmes and Morse suggest that these progenitors, provisionally termed pro-B cells, may be $Lyb\text{-}2^+$ $Mac\text{-}1^+$. They also suggest that there maybe discrete $Ly\text{-}1^+$ and $Ly\text{-}1^-$ pathways of B cell differentiation.

Second, Hardy, et al., describe the functional attributes of both normal and neoplastic B lymphoid cells that express the cell-surface marker, Ly-1. Cells with the Ig^+ $Ly\text{-}1^+$ phenotype were initially defined as a subset of B cell lymphomas (8). Their current studies show that some $Ly\text{-}5(B220)^+$ ThB^- pre-B cell lymphomas are $Ly\text{-}1^+$ and that at least one (NFS-5) can "mature" to become progressively $sIgM^+$, $sIgK^+$, ThB^+ and $BLA2^+$. They also indicate that $Ly\text{-}1^+$ B cells may predominate among those cells producing antibodies to "self" determinants including thymocytes, ss-DNA, bromelain-treated red cells, and Ig. Thus, although $Ly\text{-}1^+$ B cells represent less than 3% of all normal B cells, they may play an important role in the development

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of autoimmune diseases and may also affect the function of other subsets of sIg^+ cells (9).

Third, Witte, Braun and Morse, et al. describe the characteristics of non-transformed B lymphoid cells that can be maintained in long term cultures. Analyses of the Braun-Morse lines from a variety of inbred strain show remarkably that all the lines are $Ly\text{-}1^+$ and express lambda light chains. These results suggest that $Ly\text{-}1^+$ λ^+ cells may have a unique potential for long term growth or, alternatively, that they are less susceptible than other cells to B cell-derived factors, described by Braun, that inhibit B cell proliferation. The cultures described by Kurland and Witte are much more heterogeneous including both mature B cells and their precursors. Their cultures also contain cells that can reconstitute the B cell lineage in vivo. Both types of culture systems may prove to be of major importance for dissecting the events involved in the normal differentiation and neoplastic transformation of cells within the B lymphocyte lineage.

References

1. Coffman, R.L., Weissman, I.L.: A monoclonal antibody that recognizes B cells and B cell precursors in mice. J. Exp. Med. 153, 269-279 (1981).
2. Osmond, D.G., Nossal, G.J.V.: Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labelling. Cell Immunol. 13, 132-141 (1974).
3. Osmond, D.G.: Generation of B lymphocytes in bone marrow. In: B Lymphocytes in the Immune Response. Cooper, M., Mosier, D.E., Scher, I., and Vitetta, E. (eds.), Elsevier-North Holland, N.Y., 1979, pp. 63-66.
4. Freitas, A.A., Countinho, A.: Very rapid decay of mature lymphocytes in the periphery. J. Exp. Med. 154, 994-999 (1981).
5. Freitas, A.A., Rocho, B., Forni, L., Countinho, A.: Population dynamics of B lymphocytes: demonstration of high turnover rates in the central and peripheral lymphoid organs. J. Immunol. 128, 54-60 (1982).
6. Williamson, A.R., Askonas, B.A.: Senescence of an antibody forming cell clone. Nature 238, 337-342 (1972).
7. Coffman, R.L.: Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. Immunol. Rev. 69, 5-23 (1982).
8. Lanier, L.L., Warner, N.L., Ledbetter, J.A., Herzenberg, L.A.: Expression of Lyt-1 antigen on certain murine B cell lymphomas. J. Exp. Med. 153, 998-1003 (1981).
9. Okumura, K., Hayakawa, K., Tada, T.: Cell-to-cell interaction controlled by immunoglobulin genes. Role of $Thy\text{-}1^-$, $Lyt\text{-}1^+$, Ig^+ (B') cell in allotype-restricted antibody production. J. Exp. Med. 156, 443-453 (1982).

Characteristics of Continuous in vitro Lines of BALB/c and NZB B Cells

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A. Introduction

The normal heterogeneity of cells in the B lymphocyte lineage greatly complicates studies of the mechanisms involved in B cell maturation and function and of events that induce their neoplastic transformation. Until quite recently, the only homogeneous populations of B lineage cells available for these types of studies have been lymphomas and plasmacytomas with phenotypic and functional characteristics of normal B cells arrested at distinct maturational stages (1,2). Extrapolations of results obtained in these systems to normal B cells has been shown to be valid in many instances and, although they certainly have their limitations, transformed lines will continue to be useful for both phenotypic and genotypic characterizations of specific differentiative stages.

The utility of fully malignant lines in studies of transformation events is well documented (reviewed in ref. 3). However, because these cells may display only those features required for maintenance of the transformed state, rather than its induction, they may be less than adequate models for analyses of early steps in what may be a multistage process resulting in neoplasia (4,5).

From these considerations, it is clear that studies of B cell biology and oncology could be significantly advanced by analyses of continuous lines of normal B lymphocytes. Recently, several laboratories have established conditions under which long-term cultures of non-transformed murine (6-8) and human (9) B lineage cells can be obtained. Using techniques similar to those described in one of these reports (8), cultures of BALB/c and NZB B cells have been established and maintained in culture for six months. The initial characterizations of these B cell lines are described in this report.

B. Materials and Methods

Mice. Female BALB/cAnN, NZB/BlN and DBA/2N mice were obtained from the colonies of the National Institutes of Health. Mice were 2 to 3 months of age at the time of sacrifice.

B cell cultures. Single cell suspensions of spleens were enriched for surface immunoglobulin (sIg)-positive cells by adherance to anti-Ig-coated petri dishes (10). Adherant cells were cultured in 24-well Linbro plates at cell densities of 1.25×10^6 to 10^7 cells/well in a highly enriched medium previously described in detail (11) and supplemented with 1 µg human transferrin/ml. Cultures (2 ml) were fed twice a week by removing 1 ml of spent medium and replacing it with 1 ml of fresh medium. Some cultures were fed with 50:50 mixtures of fresh medium and supernatants containing a B cell-derived growth factor(s) from cultures of NFS-1.2 (Davidson et al., submitted).

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Flow microfluorometry (FMF). Single cell suspensions of normal spleen or cultured lymphocytes were prepared and stained for FMF as described (10,12). Antibodies included: a) FITC-labeled monoclonal antibodies specific for Thy-1.2, Ly-1, Ly-2, Ly-5(B220), ThB, IgM and IgD; b) FITC-labeled goat antibodies specific for mouse Fab, IgG_1, and IgG2; c) FITC-labeled rabbit antibodies to xenotropic murine leukemia virus (MuLV)-related cell surface antigens (XenCSA); and d) unlabeled monoclonal antibodies specific for $I-A^d$, $H-2^d$, Fc receptors, and H-11 that were sandwiched with FITC-labeled goat anti-mouse IgG2 or rabbit anti-rat Ig. The specificities of these reagents for normal and malignant hematopoietic cells are described in detail elsewhere (2,10,13,14).

Proliferative and reverse plaque-forming cell (RPFC) responses. BALB/c and NZB B cell lines (10^4 cells/well) and T cell-depleted DBA/2 spleen cells (10^5 cells/well) were cultured alone or with lysopolysaccharide (LPS; 20 μg/ml), dextran sulfate (DexS; 20 μg/ml), LPS plus DexS, or rabbit $F(ab)_2$ anti-mouse IgM heavy chain (anti-μ; 20 μg/ml) in 200 μl of medium in flat bottom microculture wells. For proliferative responses, cells were pulsed for 5 hr with 1 μC ^{3}H-thymidine before harvest. Results are presented as counts per minute (cpm) and are the geometric means of triplicate cultures. For RPFC assays, the number of antibody-secreting cells was determined using S. aureus protein A-coupled sheep red blood cells and a polyvalent rabbit anti-mouse Ig (15). Results are presented as PFC/culture and are the means of triplicate cultures.

C. Results

Establishment of B cell lines. BALB/c and NZB spleen cells, enriched for sIg^+ cells by adherence to anti-Ig coated plates, were cultured in Linbro plates using a highly enriched medium (11). Half the culture medium was replaced with fresh medium twice a week. After 3 to 4 weeks of progressive cell loss, lymphoblasts appeared at the edges of some wells and proliferated rapidly such that the cultures could be progressively split and expanded to bulk cultures by 6 weeks. One line was selected for each of the strains and they have been maintained in continuous culture for 6 months. The cells grow as monomorphic non-adherent populations with no evidence of residual adherent cells. Both BALB/c and NZB B cell lines have been successfully frozen in dimethylsulfoxide and recovered unchanged in culture.

Young adult (6 week old) mice inoculated intraperitoneally with $5x10^6$ cells from these lines show no evidence of lymphadenopathy or splenomegaly after 3 months. Spleens and lymph nodes from some of the recipients were examined at 3-1/2 weeks after inoculation to determine if any cells with surface markers characteristic of the lines (see below) could be detected by FMF; no such cells were found.

Phenotypic characteristics. The cell-surface phenotypes of the BALB/c and NZB B cell lines were determined by FMF using a large panel of monoclonal and xenoantibodies (Table I).

Table I. Cell-Surface Phenotypes of BALB/c and NZB B Cells*

Cells	IgM	IgD	Kappa†	Lambda†	IgG1	IgG2	Ia	H-2	ThB
BALB/c	+++	++	-	++	-	-	+	+++	+++
NZB	+++	++	-	++	-	-	(+/-)	+++	+++

	Ly-5(B220)	FcγR	Thy-1	Ly-1	Ly-2	H-11	XenCSA
BALB/c	+++	++	-	+++	-	+++	+++
NZB	++	++	-	++	-	+++	++

*Reactivity on the cell lines compared with staining intensity on normal cells as indicated by: -, non-reactive; +, reactive but with lower intensity than normal cells; ++, reactive with intensity equal to normal cells; +++, reactive with intensity greater than normal cells.

†Personal communication - R. Hardy, K. Hayakawa, L.A. Herzenberg.

Both lines were strikingly similar for most surface markers in that they were IgM^+ IgD^+ λ^+ $H\text{-}2^+$ ThB^+ $Ly\text{-}5(B220)^+$ FcR^+ $Ly\text{-}1^+$ $H\text{-}11^+$ and $XenCSA^+$ but $Thy\text{-}1^-$ $Ly\text{-}2^-$ K^- $IgG1^-$ and $IgG2^-$. They differed significantly only for expression of Ia with BALB/c cells showing levels of expression nearly equal to normal B cells whereas NZB cells expressed little if any of this determinant. Both cell lines thus resemble those described by Braun in being IgM^+ λ^+ and $Ly\text{-}1^+$ but differed from his lines in that they were IgD^+ and uniformly FcR^+ (8). It should also be noted that both cell lines express high levels of H-11, a determinant not found on normal B cells (14) and that the BALB/c cell line express high levels of XenCSA which is normally expressed at low levels by lymphocytes of this strain (12).

Proliferative responses. BALB/c and NZB B cell lines and T cell-depleted DBA/2 spleen cells were tested for their responses to stimulation with LPS, DexS, LPS+DexS, and anti-μ after 2, 3-1/2 and 5 days in culture (Table II).

Table II. Proliferative Responses of BALB/c and NZB B Cell Lines and DBA/2 B Cells

		Treatment of Cells*				
Day	B Cells	0	Anti-μ	LPS	DexS	LPS+DexS
2	DBA/2	1.1	57.5	53.6	4.2	66.9
	BALB/c	37.3	2.4	84.5	92.3	123.4
	NZB	10.1	2.8	28.7	17.2	40.2
3-1/2	DBA/2	1.3	15.8	97.8	8.2	86.9
	BALB/c	100.7	0.5	123.4	180.9	33.6
	NZB	29.4	0.6	60.2	60.8	29.7
5	DBA/2	0.8	3.2	19.6	8.5	14.8
	BALB/c	155.3	0.6	100.0	254.2	8.7
	NZB	119.3	0.7	98.9	144.9	8.2

*Numbers indicate geometric means (cpmx10^3) of triplicate cultures for ^{3}H-thymidine uptake.

Although interpretation of these results is complicated by the rapid proliferation exhibited by unstimulated BALB/c and NZB B cells, several findings are readily apparent. First, although normal DBA/2 spleen cells responded strongly to stimulation with anti-μ with a maximal response at day 2, proliferation of both B cell lines was essentially completely suppressed by this treatment. Second, normal DBA/2 spleen cells gave low proliferative responses after stimulation with DexS whereas both B cell lines responded well above control levels to this stimulus. Third, the day 2 proliferative response of both cell lines to LPS was above background levels, but by day 5, LPS-treated cells were dividing less rapidly than untreated controls. Finally, the combination of LPS+DexS produced an early proliferative response that was followed at days 3-1/2 and 5 by growth inhibition.

RPFC responses. The numbers of antibody-secreting cells induced by treatment of the B cell lines and normal DBA/2 B cells with anti-μ, LPS, DexS, and LPS+DexS were determined for cells cultured in the presence or absence of irradiated normal spleen cells as feeder cells. RPFC formed by the BALB/c line were very small and not easily quantitated whereas RPFC formed by the NZB line could be read without difficulty. The data in Table III compare the numbers of RPFC obtained with the NZB B cell line and normal DBA/2 B cells.

Table III. Antibody-Secreting Cells Produced in Response to Treatment of the NZB B Cell Line and DBA/2 B Cells with LPS, DexS, and LPS+DexS*

		Culture Conditions+							
		- Feeders				+ Feeders			
Day	B Cells	0	LPS	DexS	LPS+ DexS	0	LPS	DexS	LPS+ DexS
2	DBA/2	0.4	46.1	0.2	60.2				
	NZB	<0.1	0	0	0.2	0.2	0.2	0	0.1
3-1/2	DBA/2	0.3	243.2	1.2	210.3				
	NZB	<0.1	0	0.1	0.2	0.1	2.6	0.7	]2.7
5	DBA/2	0.9	237.3	12.7	244.7				
	NZB	<0.1	3.8	0	3.8	0	3.6	0.4	3.2

*Numbers indicate the numbers of RPFCx10^{-2} (geometric mean of triplicate cultures).

+B cells were cultured in the absence (- feeders) or presence (+ feeders) of irradiated (3000 R) syngeneic spleen cells (5x10^4 cells/well).

The results demonstrated a high proportion of DBA/2 B cells differentiated to antibody-secreting cells after stimulation with LPS or LPS+DexS in the absence of feeder cells whereas less than 4% of the number of NZB B cells initially placed in culture registered as RPFC at 5 days after stimulation. Addition of irradiated feeder cells to the NZB B cell cultures produced some increases in the numbers of RPFC detected particularly after 3-1/2 days of culture.

Parallel studies of Ig secretion by these cells showed that DBA/2 B cells differentiated to produce both IgM and IgG whereas the NZB and BALB/c B cell lines produced only IgM (data not shown).

D. Discussion

The data presented in this report demonstrate that the continuous cell lines obtained from cultures of B cell-enriched BALB/c and NZB normal spleen cells have a number of features in common with most normal B cells but that other characteristics of these lines are expressed only by restricted populations of normal B cells or B cell lymphomas. The characteristics shared with the majority of normal adult B cells include expression of cell-surface IgM, IgD, H-2, FcγR, ThB, Ly-5(B220) and Ia determinants (Table I).

By comparison, less than 5% of B cells from normal mice express the lambda class of Ig light chains (16) whereas both cell lines are uniformly lambda-positive (Table I). It is noteworthy that other B cell lines obtained under similar conditions all expressed lambda light chains (8) and that the frequency of lambda expressed by

stimulated normal pre-B cells was higher than expected for most B cells (17). The cell lines described here (Table I) and by Braun (8) were also uniformly Ly-1$^+$, a phenotype restricted to ~3% of B cells from normal mice and up to 13% of B cells from NZB mice (18,19). In conjunction with earlier studies which showed that the majority of pre-B cell and B cell lymphomas that were grown in primary cultures were Ly-1$^+$ and that some expressed lambda light chains (ref. 2 and Hardy et al., this volume) these studies suggest that Ly-1$^+$ lambda$^+$ B cells and their precursors may have an unusual capacity for growth in vitro.

The relations of these B cell lines to restricted populations of normal B cells is further evidenced by several observations. First, treatment with anti-μ almost completely inhibits their growth whereas normal B cells proliferate rapidly in response to this treatment (Table I). Second, the cell lines sustain a proliferative response after treatment with DexS that is not observed with normal B cells (Table II). Third, in contrast to normal B cells, only a small proportion of cells from these lines can be induced to differentiate to Ig-secreting cells (Table III). Finally, induction of differentiation to the state of Ig secretion results only in the production of IgM by the cell lines and not IgM plus IgG as for normal B cells.

The normality of these cell lines is open to question. Although they do not expand or apparently even persist in immunocompetent hosts, their continued growth in vitro and after freezing is clearly at odds with patterns of clonal senescence among normal B cells (20,21). In this respect, the NZB and BALB cell B cell lines may be the lymphoid equivalent of NIH 3T3 cells - immortality has been achieved but another step is required for true transformation. Viewed in this light, continous cultures of B lymphoid cells may provide invaluable insights into the early events involved in B cell neoplasia as well as providing unique opportunities to study B cell differentiation using homogeneous cell populations.

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References

1. Warner, N.L., Daley, M.J., Richey, J., and Spellman, C.: Flow cytometry analysis of murine B cell lymphoid differentiation. Immunol. Rev. 48, 197-243 (1979).
2. Morse, H.C. III: Tumors of B lymphocytes and retroviruses. In: Mechanisms of B Cell Neoplasia. Melchers, F., Potter, M., and Weigert, M. (eds.), Workshop at the Basel Institute for Immunology, 15-17 March 1983, Roche, Basel, Switzerland, 1983, pp. 108-124.
3. Mechanisms of B Cell Neoplasia. Workshop at the Basel Institute for Immunology, Roche, Basel, Switzerland, (1983).
4. Land, H., Parada, L.F., and Weinberg, R.L.: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 304, 596-602 (1983).
5. Ruley, H.E.: Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 304, 602-606 (1983).

6. Whitlock, C.A., and Witte, O.N.: Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci., U.S.A. 79, 3608-3612 (1982).
7. Aldo-Benson, M., and Scheiderer, L.: Long-term growth of lines of murine dinitrophenyl-specific B lymphocytes in vitro. J. Exp. Med. 157, 342-347 (1983).
8. Braun, J.: Spontaneous in vitro occurrence and long-term culture of murine B lymphoblast cell lines. J. Immunol. 130, 2113-2116 (1983).
9. Sredni, B., Sieckmann, D.G., Kumagai, S., House, S., Green, I, and Paul, W.E.: Long-term culture and cloning of non-transformed human B lymphocytes. J. Exp. Med. 154, 1500-1560 (1981).
10. Morse, H.C. III, Davidson, W.F., Yetter, R.A., and Coffman, R. L.: A cell-surface antigen shared by B cells and $Ly2^+$ peripheral T cells. Cellular Immunol. 70, 311-320 (1982).
11. Davidson, W.F.: Immunologic abnormalities of the autoimmune mouse, Palmerston North. J. Immunol. 129, 751-758 (1982).
12. Morse, H.C. III, Chused, T.M., Boehm-Truitt, M., Mathieson, B. J., Sharrow, S.O., and Hartley, J.W.: XenCSA: Cell surface antigens related to the major glycoproteins (gp70) of xenotropic murine leukemia viruses. J. Immunol. 122, 443-454 (1979).
13. Fredrickson, T.N., Langdon, W.Y., Hoffman, P.M., Hartley, J.W., and Morse, H.C.III: Histologic and cell-surface antigen studies of hematopoietic tumors induced by Cas-Br-M murine leukemia virus. J. Natl. Cancer Inst. 72, 447-454 (1984).
14. Ledbetter, J.A., and Herzenberg, L.A.: Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 63-90 (1979).
15. Lamers, M.C., Heckford, S.E., and Dickler, H.B.: Hybridoma 2.4G2: Dissociation of the B lymphocyte triggering activity from the monoclonal anti-Fe IgG receptor antibody. Molec. Immunol. (in press) (1984).
16. Kessler, S., Kim, K.J., and Scher, I.: Surface membrane κ and λ light chain expression on spleen cells of neonatal and maturing normal and immune-defective CBA/N mice: the κ:λ ratio is constant. J. Immunol. 127, 1674 (1981).
17. Nishikawa, S.-I., Kina, T., Gyotoku, J.-I., and Katsura, Y.: High frequency of λ gene activation in bone marrow pre-B cells. J. Exp. Med. 159, 617-622 (1984).
18. Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A.: The "Ly-1 B" cell subpopulation in normal, immunodeficient, and autoimmune mice. J. Exp. Med. 157, 202-218 (1983).
19. Manohar, V., Brown, E., Leiserson, W.M., and Chused, T.M.: Expression of Lyt-1 by a subset of B lymphocytes. J. Immunol. 129, 532-538 (1982).
20. Möller, G.: Regulation of cellular antibody synthesis. Cellular 7S production and longevity of 7S antigen-sensitive cells in the absence of antibody feedback. J. Exp. Med. 127, 291-305 (1968).
21. Williamson, G.R., and Askonas, B.A.: Senescence of an antibody forming cell clone. Nature 238, 337-342 (1972).

Development of Cells of the B-Lymphocyte Lineage in Longterm Culture

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INTRODUCTION

The objective of this paper is to give a brief overview of our laboratory's present activities utilizing a culture system which supports the longterm growth of cells restricted to the B-lymphocyte lineage. These studies are aimed at understanding the fundamental mechanisms involved in the normal development of B-lymphocytes. This in vitro system can overcome some difficulties associated with heterogeneous cell populations and interpretations of studies utilizing immortalized B cell lymphoma lines.

LONGTERM CULTURE OF B-LYMPHOID CELLS

The requirement for an adherent bone marrow stromal cell population for the establishment of longterm hematopoietic cell cultures has been demonstrated by Dexter and colleagues (Dexter et al 1977). In the Dexter system an association between self-renewing hematopoietic stem cells and the stromal cells accounts for the prolonged maintenance of in vitro granulopoiesis and erythropoiesis. Although cells capable of repopulating the lymphoid compartments of lethally irradiated mice are maintained in the Dexter cultures, these cells fail to develop in vitro into identifiable pre-B cells and B-lymphocytes (Schrader JW and Schrader S 1978; Jones-Villeneuve and Phillips 1980). By modifying the conventional Dexter system to provide conditions more appropriate for lymphoid cells, we have successfully established long-term murine bone marrow cultures which routinely and reproducibly contain B-lymphoid cells (Whitlock and Witte 1982). The culture method is described in detail elsewhere (Whitlock et al 1984) and is shown schematically in Fig. 1.

The hallmark of the B-lymphoid cultures is the early depopulation or crisis phase. As illustrated in Fig. 1, this is characterized by the near total cell death of all the nonadherent hematopoietic elements, and the coincident development of an adherent stromal cell layer. The adherent layer consists of fibroblasts, macrophages, adipocytes and dendritic-reticular cells, and is similar to the stromal cells present in the Dexter system. However, unlike the Dexter system, there is no evidence of sustained granulopoiesis or erythropoiesis, and by two weeks of culture no identifiable members of the myeloid cell lineage can be detected. The subsequent phase of the cultures is the appearance above the adherent layer of multiple foci of round nonadherent cells which progressively increase in size and number and are easily harvested for functional and biochemical analysis.

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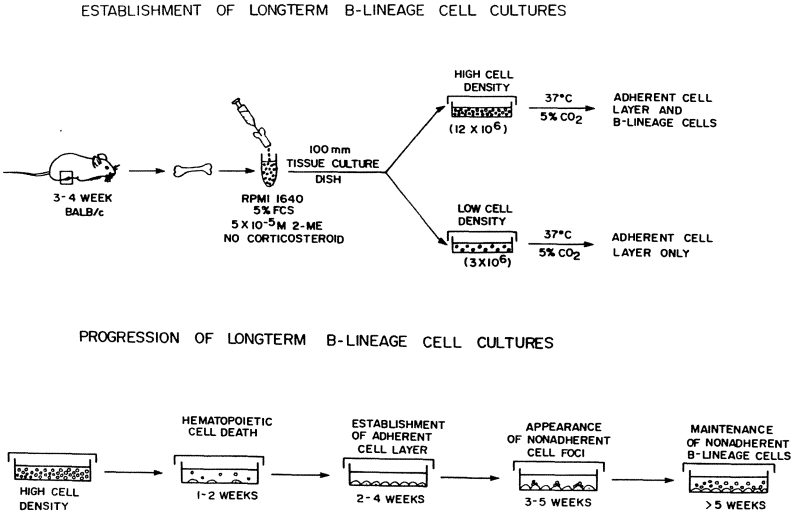

Fig. 1. Schematic representation of the method of longterm B-lymphoid cell culture and the observable changes which accompany the progression of the cultures.

CELLULAR PHENOTYPES PRESENT IN LONGTERM CULTURE

We have accumulated considerable evidence which indicates that the nonadherent cell populations comprise a variety of B-lymphoid cell phenotypes. Nearly all the cells are lymphoid in morphology. The majority are $Cmu^{+}SIg^{-}$ pre-B cells, a minor population of SIg^{+} B-lymphocytes, and a subpopulation of lymphoid cells which do not contain cytoplasmic or surface-bound immunoglobulin and lack the pre-B cell and B-lymphocyte associated B220 (Kincade et al 1981) cell surface antigen (Kurland unpublished). Neither Thy 1.2 positive T-lymphocytes, nor committed granulocyte-macrophage (GM-CFC) (Kurland et al 1978) nor erythroid (BFU-E) (Kurland et al 1980) progenitor cells have been detected in the longterm cultures following the crisis phase (Kurland unpublished).

We believe that these null cells may represent B-lymphoid cells ancestral to the pre-B cell stage. This is supported by our success in obtaining from the mass B-lymphoid cultures several clones of cells which do not synthesize immunoglobulin, but have undergone a nongermline rearrangement in their immunoglobulin heavy chain genes (Whitlock et al 1983). A much higher frequency of pre-B cell and B-lymphocyte clonal lines have been established from the mass cultures (Whitlock et al 1983). DNA analysis of the mass cultured lymphoid cells reveals germline as well as multiple immunoglobulin mu heavy chain gene rearrangements (Treiman and

Witte, unpublished); whereas, each of the clonal pre-B and B-lymphocyte lines demonstrate two discrete and mutually exclusive gene rearrangements (Whitlock et al 1983). Evidence that these immunoglobulin gene rearrangements can be productive has been confirmed at the protein level. Analysis by two-dimensional isoelectric focusing-SDS polyacrylamide gel electrophoresis of metabolically labeled immunoglobulin reveals multiple species of mu heavy chain and light chain immunoglobulins which are separable in the absence of post-translational glycosylation (Whitlock et al 1983).

The majority of the B-lymphoid cells in the longterm cultures have therefore rearranged the immunoglobulin mu heavy chain gene on one or both of their chromosomes, and have differentiated to the pre-B cell stage, as well as a subfraction into biochemically distinct populations of polyclonal B-lymphocytes. There is however evidence for the persistence within the mass B-lymphoid cultures of cells whose immunoglobulin genes still remain in the germline configuration. In the absence of any identifiable cells of the other hematopoietic lineages, we have interpreted this as evidence for the existence of ancestral hematopoietic cells which have not yet entered the B-lymphocyte lineage. Direct confirmation of this requires the clonal analysis of these putative B-lymphoid stem cells and their developmental progession at both the DNA and protein levels.

FEEDER LAYER DEPENDENCE OF LONGTERM CULTURED B-LYMPHOID CELLS

Polyclonality of B-lymphocytes in vitro has been maintained for periods over 6 months with no evidence for spontaneous transformation or growth independent of the adherent feeder layer (Whitlock and Witte 1982). As shown in Fig. 1, adherent layers can be established without the subsequent emergence of nonadherent B-lymphoid cells, by initiating the cultures at a reduced cell density. We have routinely prepared feeder layers from the bone marrow of BAB-14 mice which are syngeneic with BALB/c, except for the immunoglobulin mu heavy chain gene derived from C57BL/6 mice (Nottenberg and Weissman 1981). BALB/c B-lymphoid cells passaged on the BAB-14 feeder layers are periodically analyzed for the EcoRI restriction fragment polymorphism in the constant region of the immunoglobulin mu heavy chain gene which differs between these two mouse strains (BALB/c 11.5kb; BAB-14 13.5kb). In this way we have determined that sustained maintenance of in vitro B-lymphopoiesis occurs without the direct contribution of an adherent stromal cell.

LONGTERM CULTURES CONTAIN TARGETS FOR A-MuLV TRANSFORMATION

Clonal pre-B cell and B-lymphocyte cell lines can be recoverd from freezing and expanded on donor-host marked feeder layers. This has been used to obtain large numbers of feeder layer dependent, yet clonally derived, cells at discrete stages of differentiation for a variety of purposes. These clones have been used as defined target cells for in vitro transformation by infection with Abelson murine leukemia virus (A-MuLV) (Whitlock et al 1983). These studies have shown that infection with A-MuLV can induce the

differentiation of pre-B cells into B-lymphocytes capable of synthesizing immunoglobulin light chains. Clonal Abelson transformed cell lines have been obtained from the mass B-lymphoid cells and, like those found after infection of fresh bone marrow with A-MuLV, have the phenotypes of pre-B cells and B-lymphocytes. Once transformed by A-MuLV, the cells are capable of growth independent of the adherent feeder layer and give rise to lymphoid tumors which cause death early after in vivo injection (Whitlock et al 1983). In contrast, neither growth autonomous of the adherent layer, nor the production of lymphoid tumors in vivo, have been typically seen by the culture adapted B-lymphoid cells in the absence of A-MuLV infection.

B-LYMPHOID GROWTH FACTOR PRODUCTION BY MASS POPULATIONS OF ADHERENT BONE MARROW CELLS AND ADHERENT CELL CLONES.

When either the mass B-lymphoid cells or the clonal pre-B and B-lymphocyte lines are removed from the adherent feeder layers, proliferation ceases and cell death ensues within 24-48 hours. We have exploited this dependence upon the adherent layer to provide an assay for the identification of B-lymphoid growth factors. A short-term assay based on the incorporation of ^{3}H-TdR into proliferating B-lymphoid target cells has been developed by Debra Robertson in our laboratory, who has shown that media conditioned by the adherent cells contain growth stimulatory activity (Robertson and Witte in preparation). To identify which of the adherent cells contributes to the elaboration of the growth activity, and to aid in its isolation and purification, we have successfully obtained clones of rapidly dividing adherent cells which retain growth factor production. The morphology of the adherent cell clones resemble dendritic cells. Biochemical characterization of the growth activity obtained in serum free medium conditioned by the adherent cell clones is presently under investigation.

LONGTERM CULTURED B-LYMPHOID CELLS RECONSTITUTE THE B-LYMPHOCYTE LINEAGE IN VIVO

We have recently completed a series of experiments which have shown that cells removed from the longterm cultures can reconstitute the B-lymphocyte lineage of genetically immunedeficient mice resulting in the restoration of normal humoral immunity (Kurland et al, submitted). In these experiments, longterm cultured BALB/c B-lymphoid cells were passaged into sublethally irradiated (CBA/N x BALB/c) $F_1\sigma^{\male}$ mice. Hemizygous and homozygous CBA/N mice possess a defective X-chromosome linked gene which leads to a series of B-lymphocyte abnormalities (Amsbaugh et al 1972; Scher et al 1975). Among these are the complete absence (Kincade 1977) of a functional population of B-lymphocytes which clone in soft agar (Colony Forming Units-B-lymphocyte, CFU-B) (Metcalf et al 1976; Kurland et al 1977) and unresponsiveness to TNP-Ficoll and other Type II T-independent antigens (Mosier et al 1977).

Four-six weeks following in vivo passage of the cultured cells, both CFU-B and TNP-plaque forming cells were found to

engraft the lymphoid organs of the defective recipients. By 12 weeks these functional populations of B-lymphocytes continued to increase and did not appear to be replaced by defective host B-lymphocytes. In vivo immunization with TNP-Ficoll induced the secretion of anti-TNP hemagglutinating antibody into the sera of recipients, indicative of an intact humoral immune response (Kurland et al, submitted).

STEM CELLS OF THE LONGTERM B-LYMPHOID CULTURES ARE DISTINCT FROM MULTIPOTENTIAL HEMATOPOIETIC STEM CELLS

A rare cell population which has been shown to be distinct from multipotential hematopoietic stem cells as defined by the spleen colony forming unit (CFU-S) assay (Till and McCulloch 1961) can be recovered from the bone marrow contents of lethally irradiated recipients soon after reconstitution. When placed back in vitro and after a period of adaptation, this cell population undergoes extensive self-renewal and differentiates along the B-lymphocyte pathway. In this way, secondary longterm cultures of B-lymphoid cells have been established from primary cultured cells which have been passaged through the animal (Kurland et al submitted). The secondary cultures contain the same phenotypes as the primary cultured cells used as donor inocula. DNA analysis has confirmed that they contain the same 11.5 kb EcoRI restriction fragment polymorphism in the constant region of the immunoglobulin mu heavy chain gene as the primary BALB/c cultured cells (Kurland et al submitted).

These findings suggest that both the initiation of the long-term cultures and the repopulation of the B-lymphocyte lineage of immunedeficient mice occurs without any significant contribution by CFU-S. The identification of the stem cell of the longterm B-lymphoid cell cultures is unknown, but we suspect it to be contained within the subpopulaton of cultured cells which have not yet rearranged their immunoglobulin mu heavy chain gene and which lack the B220 cell surface antigen.

FUTURE DIRECTIONS

The present work has defined the establishment of a longterm culture system which supports normal B-lymphopoiesis as it occurs in vivo. We hope to utilize this system to obtain clonal cell lines of the most ancestral cells of the B-lymphocyte lineage and to follow their progressive differentiation in vitro and following passage in vivo. This will allow an analysis of the critical steps in the committment of hematopoietic cells to the B-lymphocyte lineage, the precise role of early immunoglobulin gene rearrangements, the expression of these genes at the RNA and protein levels, and the role of early acting B-cell growth factors which may operate at defined stages of B-lymphocyte development. We hope to take these clonal cells through the terminal stages of B-lymphocyte differentiation in concert with known B-cell differentiation factors and specific antigens, in order to examine questions of the diversity of antigen specificity.

REFERENCES

Amsbaugh DF, Hansen CT, Prescott B, Stashak PW, Barthold DR, Baker PJ (1972) Genetic control of the antibody response to type III pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. J Exp Med 136:931-949.

Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the in vitro proliferation of hemopoietic stem cells in vitro. J Cell Physiol 91:335-340.

Jones-Villeneuve EV, Phillips RA (1980) Potentials for lymphoid differentiation by cells from longterm cultures of bone marrow. Exp Hemat 8:65-76.

Kincade PW, Lee G, Watanabe T, Sun L, Scheid MP (1981) Antigens displayed on murine B-lymphocyte precursors. J Immunol 127:2262-2268.

Kincade PW (1977) Defective colony formation by B lymphocytes from CBA/N and C3H/HeJ mice. J Exp Med 145:249-263.

Kurland JI, Kincade PW, Moore MAS (1977) Regulation of B lymphocyte clonal proliferation by stimulatory and inhibitory macrophage derived factors. J Exp Med 146:1420-1433.

Kurland JI, Bockman R, Broxmeyer HE, Moore MAS (1978) Limitation of excessive myelopoiesis by the intrinsic modulation of macrophage derived prostaglandin E. Science 199:552-555.

Kurland JI, Meyers PA, Moore MAS (1980) Synthesis and release of erythroid colony- and burst-promoting activities by purified populations of murine peritoneal macrophages. J Exp Med 151:839-852.

Metcalf D, Wilson JW, Shortman K, Miller JFAP, Stocker J (1976) The nature of cells generating B lymphocyte colonies in vitro. J Cell Physiol 88:107-116.

Mosier DE, Zitron M, Mond J, Ahmed A, Scher I, Paul WE (1977) Surface immunoglobulin D as a functional receptor for a subclass of B-lymphocytes. Immunol Rev 37:89-104.

Nottenberg C, Weissman IL (1981) Cu gene rearrangement of mouse immunoglobulin genes in normal B cells on both the expressed and non-expressed chromosomes. Proc Natl Acad Sci 78:484-488.

Scher I, Steinberg AD, Bernig AK, Paul WE (1974) X-linked B-lymphocyte immune defect in CBA/N mice. II. Studies of the mechanism underlying the immune defect. J Exp Med 142:637-649.

Schrader JW, Schrader S (1978) In vitro studies on lymphocyte differentiation. I. Longterm in vitro culture of cells giving rise to functional lymphocytes in irradiated mice. J Exp Med 148:823-828.

Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal bone marrow cells. Radiat Res 14:213-222.

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

Whitlock CA, Zeigler SF, Treiman LJ, Stafford JI, Witte ON (1983) Differentiation of cloned populations of immature B cells after transformation with Abelson murine leukemia virus. Cell 32:903-911.

Whitlock CA, Robertson D, Witte ON (1984) Murine B cell lymphopoiesis in longterm culture. J Immunol Meth (in press).

Witte ON, Whitlock CA, Robertson D, Kurland JI, Swansen SK., Treiman JL, Denis K, Stafford, JI (1984) Growth regulation of murine B-lymphoid cells with and without the v-abl oncogene. In: Levine A, Vandewoude G (eds) The Cancer Cell. Cold Spring Harbor Conferences on Cell Proliferation and Cancer Vol II, Cold Spring Harbor, New York.

Coexpression of Lyb-2 and Mac-1 by Murine Lymphomas and Subpopulations of Normal Spleen and Bone Marrow Cells

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A. Introduction

The earliest indication of commitment to the B lymphocyte pathway of differentiation is probably the functional rearrangement of a single immunoglobulin (Ig) heavy chain gene. The phenotypic characteristics of these B cell progenitors have not been determined, but their progeny, termed pre-B cells, are known to have rearrangements of both heavy chain genes and to express cell surface antigens Lyb-2 and Ly5(B220) (1,2). Two subpopulations of pre-B cells can be distinguished based on difference in size, rearrrangement of Ig light chain genes, and expression of the cell surface antigen, ThB. Early pre-B cells are large, have no Ig light chain rearrangements and are ThB^- whereas their progeny are smaller, have rearranged at least one of their light chain genes, and are ThB^+ (1).

Analysis of lymphomas established in culture demonstrated that one cell line, NFS 70, was Lyb-2^+ Ly-5(B220)$^+$ ThB^- and thus phenotypically resembled a large pre-B cell (W.F. Davidson et al., submitted). However, this line also expressed Mac-1, a cell-surface antigen reportedly restricted to macrophage-granulocyte lineages (3). In light of the observation that an Abelson-MuLV induced pre-B lymphoma could be induced by 5-azacytidine to express characteristics of mature macrophages (4), the results of this study were interpreted to suggest that the normal precursors of pre-B cells might co-express surface antigens previously thought to be restricted to either the B cell or macrophage-granulocyte differentiation series (Davidson, et al., submitted). In this report, we describe the results of two experimental approaches designed to evaluate this possibility. First, the expression of Lyb-2, Mac-1, Ly-5(B220) and ThB was tested on several lymphomas which had been adapted to culture, including some induced by Abelson MuLV. Second, spleen and bone marrow cells from neonatal mice were analysed for co-expression of Lyb-2 and Mac-1 using two-color flow microfluorometry (FMF). The results of these analyses showed that several Abelson plasmacytoid lymphomas and the B cell tumor BCL_1, co-expressed Lyb-2 and Mac-1 and that cells of this phenotype constituted a significant proportion of normal newborn bone marrow and spleen.

B. Materials and Methods

Flow Microfluorometry - Single cell suspensions of bone marrow and spleen from NFS/N mice (obtained from colonies at the National Institutes of Health, Bethesda, MD) and the lymphomas were prepared and stained for FMF analysis as previously described (5). Cells were reacted with fluorescein (Fl)-labeled monoclonal antibodies specific for cell surface antigens Thy-1.2, Ly-1.2, Ly-5(B220), ThB, and Mac-1 as previously described (6). In addition, cells were

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reacted with monoclonal antibodies specific for antigens Lyb-2.1 (clone 10.D2 from Dr. D. Mosier, Fox Chase Institute for Cancer Research, Philadelphia, PA) and Lym-20 (clone K9/361 from U. Hammerling, Memorial Sloan-Kettering Cancer Research Center, New York, NY) and with alloantisera directed against surface antigens Lyb-2.2 and Ly-17.1 (provided by Dr. F.W. Shen, Memorial Sloan-Kettering Cancer Research Center, New York, NY). These reagents were counterstained with a Fl-labeled anti-mouse IgG2. An affinity purified goat anti-mouse immunoglobulin was used labeled directly with fluorescein. For two color analysis, Fl-labeled Mac-1 and biotin-labeled Lyb-2.1, counterstained with Texas Red avidin, were used. Biotin-labeled Leu-1 (Becton Dickinson, Sunnyvale, CA) was used as a isotype control in the two color analysis and the anti-Fc receptor specific monoclonal 2.4G2 (provided by Dr. J.C. Unkeless, The Rockefeller University, New York, NY) was included in the staining protocol to rule out Fc receptor binding. FMF analyses were performed on a fluorescence activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA) fitted with a logarithmic amplifier. Non-viable cells were electronically gated from analysis by light scatter (for two color) and by exclusion of propidium iodide (for lymphoma analysis).

Cell Cultures - Abelson MuLV - induced plasmacytoid lymphomas (ABPL) and lymphosarcomas (ABLS) were adapted to culture from ascites of Balb/c mice provided by J.F. Mushinski and M. Potter (7). The *in vitro* Abelson MuLV-induced lymphoma 2M3 was provided by N. Rosenberg. *NFS* lymphomas were induced *in vivo* by inoculation of newborn mice with extracts of lymphomatous *spleens* from mice infected with Cas-Br-M ecotropic MuLV (manuscript in preparation). The pre-B lymphoma 70Z/3 was provided by M. Kuehl. BCL_1 was adapted to culture from a tumorous mouse provided by W. Paul. Lymphomas were grown in a highly enriched medium as previously described (8) with the addition of 1 μg/ml of human transferrin. Single-cell cloning was performed as previously described (9).

C. Results

FMF Analysis of B Cell Lymphomas - The results of FMF analyses on 5 Abelson lymphomas and 5 non-Abelson lymphomas are shown in Table I. All 10 lymphomas were positive for Lyb-2 and Ly-17, indicating their commitment to the B cell lineage. Four lymphomas (ABPL2-F5, ABPL69, ABPL1-H5 and NFS 70) were also positive for the monocyte-macrophage antigen Mac-1. Of these, two (NFS 70 and ABPL1-H5) were also Ly-5(B220)$^+$. Five lymphomas were Mac-1$^-$ and these all expressed Ly-5(B220). NFS 105 was also ThB$^+$ in addition to being Ly-5(B220)$^+$. Surprisingly, the *in vitro*-adapted lymphoma BCL_1 was surface Ig$^+$ Ly-5(B220)$^+$ ThB$^+$ *Lyb-2$^+$* and Mac-1$^+$. All but two lymphomas (ABPL2-F5 and 2M3) were Ly-1$^+$.

Table I. Phenotypic Analysis of Lymphomas Adapted to *in vitro* Culture

Strain	Virus	Cell Line	Thy-1.2	sIg	Ly-5 B220	Ly-17.1 Ly-20.2	ThB	Mac-1	Lyb-2	Ly-1
Balb/c	Abl+Mol	ABPL2-F5	-	-	-	+	-	+	+	-
"	"	ABPL69	-	-	-	+	-	+	+	+
"	"	ABPL1-H5	-	-	+	+	-	+	+	+
"	"	ABLS-19	-	-	+	+	-	-	+	+
"	"	2M3	-	-	(+)	+	-	-	+	-
NFS/N	Cas-Br-M	NFS-70	-	-	+	+	-	+	+	+
"	"	NFS-112	-	-	+	+	-	-	+	+
"	"	NFS-105	-	-	+	+	+	-	+	+
BDF_1	MNU*	70Z/3	-	-	+	+	-	-	+	+
Balb/c	-	BCL_1	-	+	+	+	+	+	+	+

*Methyl nitrosurea induced.

FMF Analysis of NFS Spleen and Bone Marrow - To determine if there is a normal counterpart to the lymphomas which co-express Lyb-2 and Mac-1, spleen and bone marrow cells from newborn and 7-day old NFS/N mice were examined for expression of these antigens by simultaneous two color FMF analysis. The single parameter analysis of bone marrow for Lyb-2.1 showed that while the frequency of Lyb-2^+ cells in newborn marrow was greater than in 7 day marrow, the intensity of fluorescence was much lower in newborn mice (Figure 1a and Table II).

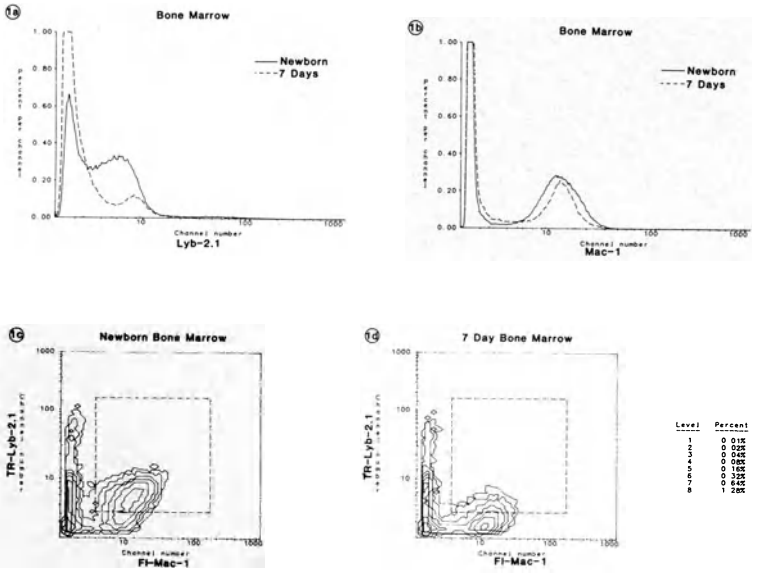

Fig. 1. FMF analysis of bone marrow from newborn and 7 day NFS/N mice stained with fluorescein-labeled Mac-1 and biotin-labeled Lyb-2.1 (counterstained with Texas Red avidin).

The frequency of Mac-1^+ cells also declined with age (Figure 1b and Table II). Four populations were recognizable with two color analysis of newborn bone marrow (Figure 1c). These populations were Lyb-2^- Mac-1^-; Lyb-2^+ Mac-1^-; Lyb-2^- Mac-1^+; and Lyb-2^+ Mac-1^+. The Lyb-2^+ Mac-1^+ double-staining population constituted 35% of newborn bone marrow as determined by integration of the area outlined by the dotted lines. By 7 days, however, this population comprised only 5% of the total bone marrow population (Figure 1d). Similar analysis of newborn and 7 day spleen showed that 20% of newborn spleen cells were Lyb-2^+ Mac-1^+ and only 4% of 7 day spleen cells were of this phenotype (Table II).

Table II. Early B Cell Differentiation

	Speculation		Established Pathway		
	Cell Type				
Differentiation Marker	Pro-B		Large Pre-B	Small Pre-B	Lymphocyte
Ly-17	+	+	+	+	+
Mac-1	+	+/-	-	-	-
Lyb-2	+	+	+	+	+
Ly-5(B220)	-	+/-	+	+	+
ThB	-	-	-	+	+
sIg	-	-	-	-	+
Ly-1	+	+	+	+	+
	-	-	-	-	-
Lymphomas	ABPL2-F5 ABPL4	ABPL1-H5 ABLS-19 2M3 NFS 70	70Z	NFS105	BCL_1
Newborn					
Bone Marrow	35%				
Spleen	20%				
7-day					
Bone Marrow	5%				
Spleen	4%				

D. Discussion

Lyb-2 is a cell surface alloantigen which is expressed on surface Ig^+ cells (10) and on all adult bone marrow cell that become surface Ig^+ after in vitro culture (11). This antigen has been shown to be associated with blast transformation of B lymphocytes (12) which may explain its apparent restriction to the B cell lineage. Mac-1 is a cell surface antigen which is expressed by mouse macrophages, polymorphonuclear leukocytes and natural killer cells (3) and is probably the complement type 3 (CR3) receptor which binds C3bi (13). The co-expression of these antigens by 5 lymphomas suggested that cells of this phenotype may exist in the normal animal. FMF analysis of newborn mouse bone marrow and spleen showed that 35% and 20%, respectively, of these cells were Lyb-2^+ Mac-1^+. Although the differentiative potential of this cell population has not been determined, the co-expression of Lyb-2 and Mac-1 with Ly-5(B220) on ABPL1-H5 suggests that Lyb-2^+ Mac-1^+ cells may be the precursors of the Ly-5(B220)$^+$ large pre-B cells of early B cell development. We have provisionally termed this Lyb-2^+ Mac-1^+ cell a pro-B and indicate its relation to the established pathway of B cell differentiation in Table II. The lymphomas with phenotypes corresponding to each stage of differentiation and the frequency of the Lyb-2^+ Mac-1^+ cell type in normal tissues is also indicated. Another intriguing possibility is that co-expression of B and myeloid lineage-associated antigens may be indicative of a precursor common to both of these lineages. This is evidenced by the apparent sensitivity of both B and myeloid lineage cells to Abelson MuLV-induced transformation (14,15) and the induction of lines with macrophage characteristics

by treatment of an Abelson pre-B lymphoma with 5-azacytidine (4).

It should be noted that all but two of the 10 lymphomas examined were Ly-1$^+$. This surface antigen, originally thought to be T cell restricted, has been identified on a subpopulation of normal B cells (16,17). This Ly-1$^+$ B cell comprises only a small percentage of spleen cells of normal mice but is increased in frequency in NZB mice and accounts for a large proportion of the cells which spontaneously secrete immunoglobulin *in vitro* (17). The expression of Ly-1 by pre-B cell lymphomas has recently been reported (R.R. Hardy, et al., this volume and W.F. Davidson, et.al., submitted). The expression of Ly-1 on otherwise phenotypically identical lymphomas (i.e. ABPL2-F5 and ABPL69; and 2M3 and NFS 112), suggests that if the differentiative scheme presented in Table II proves correct, Ly-1 may be expressed early in B cell differentiation but that distinct Ly-1$^+$ and Ly-1$^-$ lineages may exist.

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References

1. Coffman, R.L.: Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. Immunol. Rev. 69, 5-23 (1982).
2. Kincade, P.W., Lee, G., Watanabe, T., Sun, L., and Scheid, M.P.: Antigens displayed on murine B lymphocyte precursors. J. Immunol. 127, 2262-2268 (1981).
3. Springer, T.G., Galfre, D.S., Secher, D.S., and Milstein, C.: Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. 9, 301-306 (1979).
4. Boyd, A.W. and Schrader, J.W.: Derivation of macrophage-like lines from the pre-B lymphoma ABLS 8.1 using 5-azacytidine. Nature 297, 691-693 (1982).
5. Morse, H.C. III, Chused, T.M., Boehm-Truitt, M., Mathieson, B.J., Sharrow, S.O. and Hartley, J.W.: XenCSA: Cell surface antigens related to the major glycoproteins (gp70) of xenotropic murine leukemia viruses. J. Immunol. 122, 443-454 (1979).
6. Fredrickson, T.N., Langdon, W.Y., Hoffman, P.M., Hartley, J.W., and Morse, H.C. III: Histologic and cell surface antigen studies of hematopoietic tumors induced by Cas-Br-M murine leukemia virus. J. Natl. Cancer Inst. 72, 447-454 (1981).
7. Mushinski, J.F., Potter, M., Bauer, S.R., and Reddy, E.P.: DNA rearrangement and altered RNA expression of the c-myb oncogene in plasmacytoid lymphosarcomas. Science 220, 795-798 (1983).
8. Davidson, W.F.: Immunologic abnormalities of the autoimmune mouse, Palmerston North. J. Immunol. 129, 751-758 (1982).
9. Davidson, W.F., Fredrickson, T.N., Rudikoff, E.K., Coffman, R.L., Hartley, J.W., and Morse, H.C. III: A unique series of lymphomas related to the Ly-1$^+$ lineage of B lymphocyte differentiation. Submitted.
10. Sato, H. and Boyse, E.A.: A new alloantigen expressed selectively on B cells: The Lyb-2 system. Immunogenetics 3, 525-531 (1976).

11. Kincade, P.W., Lee, G., Scheid, M.P. and Blum, M.D.: Characterization of murine colony-forming B cells. II. Limits to in vitro maturation, Lyb-2 expression, resolution of IgD^+ subsets and further population analysis. J. Immunol. 124, 947-953 (1980).
12. Subbarao, B. and Mosier, D.E.: Induction of B lymphocyte proliferation by monoclonal anti-Lyb-2 antibody. J. Immunol. 130, 2033-2037 (1983).
13. Beller, D.I., Springer, T.A., and Schreiber, R.D.: Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. J. Exp. Med. 156, 1000-1009 (1982).
14. Risser, R.: The pathogenesis of Abelson virus lymphomas of the mouse. Biochem. et Biophys. Acta. 651, 213-244 (1982).
15. Raschke, W.C.: Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15, 261-267 (1978).
16. Manohar, V., Brown, E., Leiserson, W.M., and Chused, T.M.: Expression of Lyt-1 by a subset of B lymphocytes. J. Immunol. 129, 532-538 (1982).
17. Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A.: The "Ly-1 B" cell subpopulation in normal, immunodeficient and autoimmune mice. J. Exp. Med. 157, 202-218 (1983).

Ly-1 as a Differentiation Antigen on Normal and Neoplastic B Cells

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INTRODUCTION

A small subpopulation of splenic B cells carries the Ly-1 antigen, previously considered restricted to T cells (Hardy et al., 1982; Manohar et al.). The cells in this "Ly-1 B" subpopulation express other B lineage cell surface antigens, are detectable in spleen, are enriched in peritoneal washouts, are rare in (or absent from) lymph nodes and bone marrow and appear early during development (Hayakawa et al., 1983; Hayakawa et al., 1984). Thus Ly-1 B cells constitute a significant fraction of the Ig^+ cells in spleens of newborn (3-5 days) mice, but decrease to a minor population (1-2 percent of total lymphocytes) in adults.

Certain autoimmune mouse strains (NZB) have increased levels of Ly-1 B and this enlarged population is responsible for the large amounts of IgM secreted *in vitro* in the absence of exogenous antigen (Hayakawa et al., 1983). Moreover, such secreted IgM contains autoantibody, binding to thymocytes or ssDNA (Hayakawa et al., 1984). In normal strains such as BALB/c, the PFC generated in response to exogeneous antigens (such as SRBC, DNP-KLH and TNP-Ficoll) do not derive from Ly-1 B; however an autoantibody elicited by injection of LPS that lyses bromelain-treated mouse erythrocytes does come exclusively from Ly-1 B (Hayakawa et al., 1984).

Several years ago, Lanier noted the presence of Ly-1 on several B lymphomas and suggested that these might represent the transformation of a rare normal cell type. Recent studies with B cell tumor lines at various stages of differentiation (Davidson et al.) suggest that the Ly-1 marker is expresed on at least some B cells from pre-B to antibody forming cells. One of these lines, NFS-5, appears to be a pre-B cell that expresses Ly-1 and spontaneously differentiates to a $Ly\text{-}1^+, IgM^+$ population *in vitro*. Another, NFS-1.0, appears similar by surface phenotype to the Ly-1 B population in NZB mice and this tumor can be triggered with LPS to make bromelain plaques. Curiously, many of these Ly-1 B cell lines express the lambda light chain, normally very rare in mice.

LY-1 EXPRESSION ON B CELLS IN NORMAL TISSUES

Quantitation of the number of Ly-1 bearing B cells in most normal tissues is complicated by the fact that the level of Ly-1 is quite low (compared with the level on T cells) and by the relative rarity of such cells. We have utilized three color immunofluorescence on a modified dual-laser Fluorescence Activated Cell Sorter (FACS) to quantitate the levels of three surface antigens on a cell-by-cell basis (Parks et al.; Hardy et al., 1983; Hardy et al, 1984). Thus we can stain cells simultaneously with antibodies specific for Ly-1, Ly-2 (an isotype-matched

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reagent control) and some B cell marker; cells that are Ly-1$^+$, Ly-2$^-$ should include all Ly-1 B cells in the cell suspension under study.

This analysis is carried out by staining bone marrow or spleen cell suspensions (from which erythrocytes have been lysed) with biotin-coupled (bi-) anti-Ly-1 (revealed by Texas Red-avidin), allophycocyanin-coupled (apc-) anti-Ly-2 and several different fluorescein-conjugated (Fl-) monoclonal antibodies specific for various B cell differentiation antigens. In addition, to avoid nonspecific dead cell staining we employ the viability dye, propidium iodide (Dangl and Herzenberg). Forward and right angle scatter are also recorded for each cell. The reagents employed in this study are sumarized in Table 1.

Table 1. Monoclonal antibody reagents used in these studies.

Clone	Determinant	Isotype	Conjugate	Reference
331.12	IgM	rat IgG2b	Fl	Kincade
53-7.8	Ly-1	rat IgG2a	bi	Ledbetter
53-6.7	Lyt-2	rat IgG2a	bi, apc	Ledbetter
M1/70	MAC-1	rat IgG2b	Fl	Springer
RA3-6B2	Ly-5(B220)	rat	Fl, bi	Coffman
49-H4	ThB	rat IgG2c	Fl, bi	Eckhardt
10-3.6	IA(Ial7)	mouse IgG2a	Fl	Oi
MK-D6	IA(d)	mouse IgG2a	Fl	Kappler
10-4.22	IgD	mouse IgG2a	Fl, bi	Oi
53-10.1	BLA-1	rat IgG2c	Fl	Hardy, 1984
30-E2	BLA-2	rat IgG2b	bi	Hardy, 1984
187.1	Kappa	rat IgG2c	Fl	Yelton

As expected from previous two-color studies, about 2-4 percent of spleen cells in 4 week old mice are Ly-1$^+$, Ly-2$^-$, IgM$^+$. Furthermore, these cells express high levels of ThB, but rather heterogenous levels of Ly-5(B220). Most Ly-1 B cells in spleen are thus IgM$^+$, ThB$^+$, Ly-5(B220)$^+$. Similar analyses of bone marrow suspensions demonstrate that while IgM$^+$ cells expressing Ly-1 are very rare, ThB$^+$ cells expressing Ly-1 are somewhat less rare, and that about 10 percent of the Ly-5(B220)$^+$ cells also express Ly-1. Staining for the macrophage-associated antigen defined by the MAC-1 monoclonal antibody is essentially at background in the Ly-1 B population in either spleen or bone marrow. These results are sumarized in Table 2.

Table 2. Antigen expression on Ly-1$^+$/Ly-2$^-$ cells in 4 week old NFS mice.

Tissue	Antigen	% of total cells (50K) exp 1	exp 2	Level of expression
Bone Marrow	Ly-5(B220)	0.48	2.50	low
	ThB	0.40	1.60	high
	IgM	0.18	1.00	heterogenous
	MAC-1	0.10	0.08	
Spleen	Ly-5(B220)	3.8		low/high (50/50)
	ThB	3.6		high
	IgM	3.0		high
	MAC-1	0.13		

This data, while not conclusive, suggests that the Ly-1$^+$,IgM$^+$ cells we have studied previously in spleen express the Ly-1 antigen as early as the Ly-5(B220)$^+$ pre-B stage. Indeed, although the numbers are quite small, the trend of more ThB$^+$/Ly-1$^+$ cells than IgM$^+$/Ly-1$^+$ cells and more Ly-5(B220)$^+$/Ly-1$^+$ cells than ThB$^+$/Ly-1$^+$ cells follows a B cell differentiation scheme described by Coffman for the bulk of the B cells in bone marrow. The significant point that we make here is that a functionally distinct population of splenic B cells (Ly-1 B) is phenotypically distinct from the bulk of bone marrow cells as early as the pre-B cell stage.

LY-1 EXPRESSION IN B CELL NEOPLASMS

The likely presence of Ly-1 on some pre-B cells and our previous demonstration of Ly-1 on certain antibody secreting cells suggests that the Ly-1 antigen marks a subpopulation of B cells at several different stages of differentiation. In order to investigate this more thoroughly, we have examined the phenotype of a number of B cell lines that can be grown in cell culture. We present the results of a series of Ly-1 bearing cell lines in table 3. As can be readily seen, this list includes cell lines previously typed as representing several distinct B cell differentiation stages.

Table 3. Ly-1 bearing B cell tumors and long term cultured cell lines.

Tumor or Cell Line		Characteristics	Light Chain
70Z/3	Pre-B;	IgM$^-$, Ly-1$^+$ --------> IgM$^+$, Ly-1$^+$ (LPS)	Kappa
NFS-5	Pre-B;	IgM$^-$, Ly-1$^{+/-}$ --------> IgM$^+$, Ly-1$^+$ (in vitro)	Kappa
WEHI-231	Early-B;	IgM$^+$, Ly-1$^+$, IA$^-$, IgD$^-$	Kappa
CH-1	B cell;	IgM$^+$, Ly-1$^+$, IA$^+$, IgD$^-$	Lambda
BCL_1	B cell;	IgM$^+$, Ly-1$^+$, IA$^+$, IgD$^+$	Lambda
NFS-1.0	Ly-1 B;	IgM$^+$, Ly-1$^+$, IA$^+$, IgD$^+$ inducible to make BrmRBC plaques	Lambda
NZB NB	Ly-1 B;	IgM$^+$, Ly-1$^+$, IA$^-$, IgD$^-$ inducible to make BrmRBC plaques	Lambda
BALB/c NB	Ly-1 B;	Similar to NZB NB	Lambda

Certain of these cell lines have rather low levels of Ly-1 (WEHI-231) and we have noted some variability in Ly-1 expression with different isolates of another (70Z/3). However all of these lines stain above background (defined by isotype controls) for Ly-1.

LPS-INDUCED DIFFERENTIATION TO BROMELAIN PFC IN A LY-1 B TUMOR

Of all the lines examined, one (NFS-1.0) showed very low, but detectable reactivity with bromelain-treated mouse red blood cells. This line was cultured for 4 days in medium containing 10 micrograms/ml LPS and

then examined for surface phenotype and for bromelain PFC. Although the surface levels of IgM and Ly-1 were essentially unchanged from control cultures, there was a very large increase in the frequency of bromelain PFC (see table 4). This result demonstrates that the NFS-1.0 cell line is very similar to the Ly-1 B population in normal and NZB mice, not only in surface phenotype, but also in responsiveness (to LPS) and in specificity (for bromelain mouse RBC).

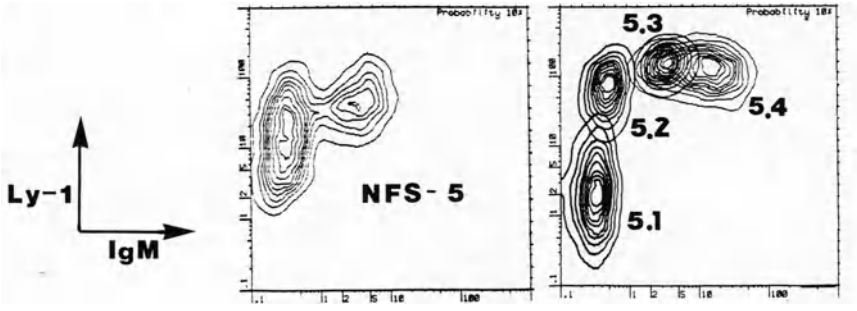
Table 4. The NFS-1.0 cell line is inducible with LPS to make bromelain PFC.

Cell Line	PFC/10^5 when cultured without LPS	with LPS
NFS-1.0	10	3000
70Z/3	<1	<1

IN VITRO DIFFERENTIATION OF A LY-1 PRE-B CELL LINE

Examination of one of the "pre-B" Ly-1 bearing cell lines (NFS-5) showed considerable heterogeneity in the level of Ly-1 expression and, in addition, a small subpopulation of IgM^+ cells. Two color staining for IgM and Ly-1 demonstrated that the IgM^- population was very heterogenous for Ly-1 level (over at least a 100-fold range) whereas the IgM^+ population was uniformly high for Ly-1 expression (see figure 1). Subsequent FACS cloning of the three phenotypic Ly-1/IgM populations of NFS-5 has yielded large numbers of cells that stably are $Ly\text{-}1^-/IgM^-$, $Ly\text{-}1^+/IgM^-$ and $Ly\text{-}1^+/IgM^+$. Unexpectedly, we find that the $Ly\text{-}1^-/IgM^-$ cell line spontaneously becomes $Ly\text{-}1^+/IgM^+$ after culture for several weeks in medium containing horse serum, but not in medium containing FCS. The transition is even faster for the $Ly\text{-}1^+/IgM^-$ cell line.

Figure 1. Two color analysis of various NFS-5 isolates.



Recently the $Ly\text{-}1^+/IgM^+$ NFS-5 line has been shown to express yet another surface marker combination after culture in LPS. These cells were cultured in LPS for 4 days and then stained with a monoclonal antibody specific for Kappa light chain. The $Kappa^+$ cells were cloned into LPS-containing medium and several clones were isolated. Analysis of these clones demonstrated that although all clones were Kappa positive (with higher surface levels of IgM than the parent culture), they exhibited variable levels of a B cell differentiation antigen know as ThB (Eckhardt and Herzenberg). All prior cultures of NFS-5 were uniformly negative for ThB. The various stages of NFS-5 are sumarized in table 5.

Table 5. NFS-5 differentiation stage phenotypes.

Cell Line	Ly-1	IgM	Kappa	BLA-1	BLA-2	ThB	IA	IgD
NFS-5.1	+/-	-	-	+	-	-	-	-
NFS-5.2	++	-	-	+	-	-	-	-
NFS-5.3	+++	+	+/-	+	+	-	-	-
NFS-5.4	+++	++	+	+	+	+	-	-

LY-1 EXPRESSION ON "NORMAL" B CELL LINES

There have been numerous attempts to culture "normal" B cells from the bone marrow or spleen of various mice strains, but most have been unsuccessful. Recently, there have been several reports of successful cultivation of either bone marrow (Whitlock and Witte) or splenic (Braun) B cells. Davidson, following the protocol of Braun has established several splenic B cell lines and has observed that all are $Ly\text{-}1^+$ (see report in this volume). We have investigated two of these lines, one derived from BALB/c and one from NZB, for the expression of a number of B cell surface antigens. The results are summarized in table 3. Both of these lines have identical surface phenotypes for all markers examined to date, and, with the exception of IA and IgD, are very similar to the NFS-1.0 tumor line. Curiously, both cell lines express lambda light chains, a rare class (about 5 percent of IgM^+ cells in spleen) in mouse.

CONCLUSIONS

It appears that the expression of Ly-1 on a subpopulation of B cells occurs as early as the $Ly\text{-}5(B220)^+$, IgM^- pre-B stage in bone marow. This finding is corroborated by the demonstration of Ly-1 on pre-B type tumors (Davidson et al, 1984). One unusual specificity of splenic Ly-1 B (for bromelain-treated mouse erythrocytes) is also found with one of the Ly-1 bearing tumors (NFS-1.0). Finally, one Ly-1 bearing pre-B tumor shows regulated expression of a number of B cell differentiation antigens *in vitro*. The sequence of surface antigen expression in this line suggests that Ly-1 is a very early marker on cells of the Ly-1 B lineage.

The presence of Ly-1 on several "spontaneous" B cell lines established by long term culture of whole spleen is not wholely unexpected, considering early findings that Ly-1 B cells in spleen survived *in vitro* much better than $Ly\text{-}1^-$ B cells (Hayakawa et al, 1983). On the other hand, the finding that many of these Ly-1 B tumors and lines express the lambda light chain is surprising. Although there is some enrichment for lambda light chain expression in splenic Ly-1 B (compared with $Ly\text{-}1^-$ B cells), most Ly-1 B cells have kappa light chains (unpublished observations). Perhaps the expression of lambda light chain confers on the Ly-1 B cell a susceptibility to relatively unrestrained growth.

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REFERENCES

Braun J (1983) Spontaneous in vitro occurence and long-term culturing of murine B lymphoblastoid cell lines. J Immunol 130: 2113-2116

Coffman RL (1983) Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. Immunol Rev 69: 5-23

Dangl JL, Herzenberg LA (1982) Selection of hybridomas and hybridoma variants by the fluorescence activated cell sorter. J Immunol Methods 52: 1-14

Davidson WF, Fredrickson TN, Rudikoff EK, Coffman RL, Hartley JW, Morse HC III (1984) A unique series of lymphomas related to the Ly-1$^+$ lineage of B lymphocyte differentiation. J Immunol (submitted)

Eckhardt LA, Herzenberg LA (1980) Monoclonal antibodies to ThB detect close linkage of Ly-6 and a gene regulating ThB expression. Immunogenetics 11: 275-291

Hardy RR, Hayakawa K, Haaijman J, Herzenberg LA (1982) B cell subpopulations identifiable by two-color fluorescence analysis using a dual-laser FACS. In: Thorbecke GJ, Leslie GA (eds) Immunoglobulin D: Structure and Function (Annals of the New York Academy of Science, Vol. 399) p. 112

Hardy RR, Hayakawa K, Parks DR, Herzenberg LA (1983) Demonstration of B cell maturation in X-linked immunodeficient mice by simultaneous three-color immunofluorescence. Nature 306: 270-272

Hardy RR, Hayakawa K, Parks DR, Herzenberg LA, Herzenberg LA (1984) Murine B cell differentiation lineages. J Exp Med (in press)

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

Hayakawa K, Hardy RR, Herzenberg LA, Steinberg AD, Herzenberg LA (1984) Ly-1 B: A functionally distinct B cell subpopulation. In: Tada T (ed) Progress in Immunology (5th Internat'l. Congress of Immunology, Kyoto, Japan; August, 1983) Academic Press (in press)

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 Nat'l Acad Sci USA (in press)

Kappler JW, Skidmore B, White J, Marrack P (1981) Antigen inducible, H-2-restricted interleukin-2-producing T cell hybridomas. J Exp Med 153: 1198-1214

Kincade PW, Lee G, Sun L, Watanabe T (1981) Monoclonal rat antibodies to murine IgM determinants. J Immunol Methods 42: 17-26

Lanier LL, Warner NL, Ledbetter JA, Herzenberg LA (1981) Expression of Lyt-1 antigen on certain murine B cell lymphomas. J Exp Med 153: 998-1003

Ledbetter JA, Herzenberg LA (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol Rev 47: 63-90

Manohar VE, Brown E, Leiserson WM, Chused TM (1982) Expression of Lyt-1 by a subset of B lymphocytes. J Immunol 129: 532-538

Oi VT, Jones PP, Goding JW, Herzenberg LA, Herzenberg LA (1978) Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. Current Topics in Microbiol and Immunol 81: 115-129

Parks DR, Hardy RR, Herzenberg LA (1984) Three-color immunofluorescence analysis of mouse B lymphocyte subpopulations. Cytometry (in press)

Springer T, Galfre G, Secher DS, Milstein C (1979) Mac-1: a macrophage differentiation antigen identified by a monoclonal antibody. Eur J Immunol 9: 301-306

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

Yelton DE, Desaymard C, Scharff MD (1981) Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. Hybridoma 1: 5-11

Propagation of B Lymphocytes in Vitro

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INTRODUCTION

Several differentiative states of the mature B lymphocyte are well known, including clonal expansion, antibody secretion, heavy chain class switching, and somatic mutation. However, the precise sequence of these differentiative events, and the mechanism by which immunoregulatory cells and mediators influence this sequence remains uncertain. This is due in large part to the paucity of suitable clonal B cell lines which may be propagated while retaining differentiative potential (Howard, 1981; Sredni, 1981; Whitlock, 1982).

This paper summarizes a simple culture strategy which yields long-term B lymphocyte cell lines similar to a distinct B cell population in vivo. In addition, a novel self-regulatory mediator produced by B cells is described, which may normally interfere with the propagation of B cell clonal expansion.

Establishment of B Cell Lines

We have recently described a new culture strategy for producing long-term lines of murine B lymphocytes (Braun, 1983). Briefly, T cell-depleted, Ficoll-Hypaque-purified splenic lymphocytes were cultured at high density (1 to 5 x 10[6] per ml) in standard culture medium (RPMI 1640, 10% fetal calf serum, 5 x 10^{-5} M 2-mercaptoethanol, 10 mM HEPES); culture fluid was replaced with fresh medium at approximately three-day intervals.

During the initial two weeks, cell number and viability rapidly declined, yielding a population composed predominantly of adherent cells. Subsequently, cultures of non-adherent lymphoblasts appeared, which grow to confluency within an additional two to three weeks. These lymphoblasts display a rapid growth rate (doubling time equals eighteen to twenty-four hours), and may be serially passaged for more than one year. They are clonable in soft agar or by limiting dilution; however, they are not tumorogenic in syngeneic mice.

A summary of immunocytochemical and immunoprecipitation studies is listed in Table 1. The phenotype is generally characteristic of B lymphocytes but with certain unusual features: the absence of delta heavy chain and kappa light chain expression; and the presence of Ly1, a conventional "helper" T lymphocyte antigen.

Recently, the existence of an in vivo population of Ly1+ B lymphocytes has been documented by functional (Okumura, 1982) and flow cytometry (Hayakawa, 1983; Manohar, 1982) studies. This B cell subpopulation expresses high levels of membrane IgM, low levels of membrane IgD, and includes an increased frequency of cells utilizing the lambda light chain isotype (Hayakawa, 1983). Thus, this population may represent the in vivo equivalent of the long-term B cell lines established by our culture strategy.

Ly1+ B lymphocytes are also distinguished by a novel allotype-specific helper activity (Okumura, 1982), spontaneous autoantibody production in models of murine

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lupus (Manohar, 1982; Hayakawa, 1983; Hardy [this volume]), and their predominance in certain B cell neoplastic states (Lanier, 1981; Wang, 1980). Accordingly, the differentiative capacity and regulatory influence of long-term B cell lines is currently under investigation.

Table 1. Phenotype of long-term B cell lines

Positive	
Immunoglobulin:	Membrane IgM-lambda
MHC:	Class I (K, D) and Class II (I-A)
Other:	Ly-1; Fc-gamma receptors, C3M receptors
Negative	
Immunoglobulin:	Delta, gamma, alpha heavy chains; kappa light chains; secreted Ig
Other:	Thy1, Lyt-2

Self-Regulation of B Cell Growth

In the course of characterizing the splenic precursor of these long-term B cells, we were surprised to find that large, low-density B cells—the predominantly spontaneously proliferative cell fraction—actually inhibited the growth of B cells in vitro. This observation suggested the possibility that proliferative B cells produced an inhibitor of B cell growth.

To test this idea, T cell-depleted splenic B cells were cultured in LPS for three days, then washed and recultured in medium alone to collect putative inhibitor produced by the proliferating cells. This B cell-conditioned medium was tested in conventional lymphocyte mitogen assays.

Table 2. B cell-derived inhibitor of B cell proliferation

Assay Condition	Cell Type Assayed* B Cell	T Cell
Basal	5.1	1.8
Mitogen stimulated	11.2	9.4
+ B-CM (10%)	3.2	9.9
+ T-CM (10%)	13.6	14.8

B cell mitogen: Fc fragment of human IgG (Weigle and Berman, 1979)
T cell mitogen: Concanavalin A
B-CM: Conditioned medium from LPS-induced B cell lymphoblasts
T-CM: Conditioned medium from Con A-induced T cell lymphoblasts
*Thymidine incorporation, cpm x 10^{-3}

In Table 2, B cell-conditioned medium produced a striking inhibition of B cell proliferation, but had no effect on a similar level of T cell proliferation. In contrast, conditioned medium derived from T lymphoblasts (produced by B cell-depleted splenic T cells cultured by the same protocol (using Conconavalin A) had

no inhibitory effect; the slight enhancement may be due to the effect of interleukin-2 produced under these conditions.

Other experiments have further defined parameters of this regulatory pathway (Kiely, 1984): 1) in positive cell fractionation studies, the inhibitor producer was membrane IgG positive; T cells and adherent cells were inactive. 2) Production of the inhibitor could be induced by several B cell mitogens, including LPS, the Fc fragment of human gammaglobulin, and mycoplasma bovorrhinis. 3) The inhibitor effectively suppressed proliferation to the latter panel of B cell mitogens; it was ineffective in Concanavalin A- or interleukin-2-induced T cell growth. 4) The inhibitor was produced at a characteristic point during cell activation, typically two to three days after addition of mitogen. 5) Interferon, interleukin-1, and interleukin-2 activities were absent in B cell-conditioned medium.

These observations indicate that B lymphocytes produce a growth-regulatory autokine at a specific point in their activation cycle. In the past, the regulatory influence of B cells was primarily recognized with respect to the role of antibody and idiotype networks (Jerne, 1974) and Fc-mediated functions (Weigle, 1979). Recently, interferon and polyclonal stimulatory activities have been obtained from B cells in certain myeloma lines (Okumura, 1982; Walker, 1982; Vesole, 1979) as well as inhibitory factors which block primary and secondary humoral immune responses (Gilbert and Hoffman, 1983). The characterization of the differentiative activity by chemical identity of the growth inhibitor in B cell-conditioned medium is necessary for comparison to these previous observations. In any event, the existence of a B lymphocyte self-regulatory pathway is clearly relevant to the propagation of B cell clonal expansion and may clarify features of B cell tolerance, and certain autoimmune and neoplastic states.

REFERENCES

Braun J (1983) Spontaneous in vitro occurrence and long-term culture of murine B lymphoblast cell lines. J Immunol 130:2113-2117

Gilbert KM, Hoffmann NK (1983) Supressor of B lymphocytes. Immunol Today 4:253-255

Hayakawa K, Hardy RR, Parks DR, Herzenberg LA (1983) The "Ly-1 B" cell population in normal, immunodefective, and autoimmune mice. J Exp Med 157:202-218

Howard M, Kessler S, Chused T, Paul WE (1981) Long-term culture of normal mouse B lymphocytes. Proc Natl Acad Sci USA 78:5788

Jerne N (1974) Towards a network theory of the immune system. Ann Immunol (Inst. Pasteur) 125c:373-389

Kiely J-N, Braun J, Unanue ER (1984) Autoregulation of B cell growth by a B cell product. Manuscript submitted for publication

Lanier LL, Warner NL, Ledbetter JA, Herzenberg LA (1981) Quantitative immunofluorescent analysis of surface phenotypes of murine B lymphomas and plasmacytomas with monoclonal antibodies. J Immunol 127:1691-1697

Manohar V, Brown E, Leiserson WM, Chused TM (1982) Expression of Lyt-1 by a subset of B lymphocytes. J Immunol 129:532-540

Okumura K, Hayakawa K, Tada T (1982) Cell-to-cell interaction controlled by immunglobulin (Ig) genes: role of Thy-1(-), Ly-1(+), Ig(+) (B') cell in allotype-restricted antibody formation. J Exp Med 156:443-452

Sredni B, Kumaga S, House F, Green I, Paul WE (1981) Long-term culture and cloning of non-transformed human B cells. J Exp Med 154:1500-1513

Vesole TH, Joust JM, Fett JW, Fudenberg HH (1979) Stimulators and inhibitors of lymphocyte DNA synthesis in supernatants from human lymphoid cell lines. J Immunol 123:1322-1328

Walker EB, Lanier LL, Warner NL (1982) Characterization and functional properties of tumor cell lines in accessory cell replacement assays. J Immunol 128:852-859

Wang CY, Good RA, Ammirato P, Dymbert G, Evans RL (1980) Identification of a p69/70 complex expressed on human T cells sharing determinants with B-type chronic lymphocyte leukemia cells. J Exp Med 151:1539-1546

Weigle WO, Berman MA (1979) Role of the Fc portion of antibody in immune regulation. In: Pernis, B, Vogel HJ (eds), Cells of Immunoglobulin Synthesis. Academic Press, New York, pp. 223-252
Whitlock CA, Witte ON (1982) Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci USA 79:3608-3613

Identification of a Transforming Virus from a Lymphoma of a Mouse Infected with a Wild Mouse Retrovirus

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A. Introduction

Cas-Br-M is a biologically-cloned ecotropic murine leukemia virus (MuLV) isolated from wild mice of the Lake Casitas region of California (1). This virus induces both hind limb paralysis and hematopoietic neoplasms when inoculated into newborn mice (2,3). The tumors of mice inoculated with Cas-Br-M are first evident at 18-30 weeks and include T- and B-cell lymphomas, lymphoblastic lymphomas, erythroleukemias, myelogenous leukemias and megakaryocytic leukemias (4). Cas-Br-M therefore appears to be unique among murine retroviruses in its ability to cause a wide spectrum of hematopoietic tumors. The mechanisms involved in generating this range of tumors are not known, however mink lung cell focus-inducing (MCF) MuLVs may play a role since these viruses appear rapidly in the tissues of Cas-Br-M infected mice (5).

Recently we isolated a defective spleen focus-forming virus (SFFV) from erythroleukemic mice infected with Cas-Br-M (6). The SFFV was originally identified in the erythroleukemic spleens of mice inoculated at birth with an extract from a Cas-Br-M-induced lymphoma. In the course of studying whether SFFVs may be generated at high frequency from Cas-Br-M, newborn mice were inoculated with a number of extracts from lymphomatous and erythroleukemic spleens. One of these extracts, from a Cas-Br-M-induced pre-B-cell lymphoma, was found to consistently cause lymphomas with the same histologic and cell-surface antigenic phenotype as the primary tumor. This result was in contrast to earlier results where Cas-Br-M was found to induce a broad spectrum of hematopoietic tumors (4). On further examination, the extract was found to contain a virus capable of transforming mouse and rat fibroblasts, raising the possibility that the transforming virus may have recombined with host DNA sequences involved in the generation of pre-B-cell lymphomas. Here we report the biological and preliminary biochemical properties of this virus.

B. Materials and Methods

Mice - Inbred NFS/N mice were obtained from colonies of the National Institutes of Health. Litters were inoculated ip or ic within 3 days of birth and observed weekly for the development of tumors.

Viruses and virus assays - The origin and characteristics of Cas-Br-M have been previously described (1). Extracts were prepared from lymphomatous spleens by homogenization in an appropriate volume of minimal essential medium (MEM) to make a 10% weight/volume suspension.

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The suspension was clarified twice by centrifugation at 3,000 rpm for 15 minutes and stored in 1 ml aliquots at -70°C. Ecotropic virus in the extracts was assayed on SC-1 cells using the XC plaque assay, and the titer expressed as the log of plaque-forming units (PFU)/ml (7). MCF virus was assayed by focus-induction directly in mink lung cells or in SC-1 cells by the UV-mink procedure and the titer expressed as the log of focus-forming units/ml (8). The presence of transforming virus in the extract was assayed on subconfluent monolayers of SC-1 and mink cells. The cells were observed for the development of foci and the titer expressed as the log of FFU/ml. Harvests from infected tissue culture cells were prepared by pooling 24 hr supernatant fluid with cells disrupted by freezing, thawing, and passing through a 22 gauge needle, and clarifying at 2500 rpm for 20 min.

Pathology - All diagnoses were made on the basis of gross observations and microscopic examinations of tissues fixed in Tellyesneczky's solution, sectioned, and stained with hematoxylin and eosin.

Flow microfluorometry (FMF) - Single cell suspensions from spleens of tumor-bearing and normal mice were prepared and stained with fluoresceinated antibodies as previously described (9). The specificities and sources of the monoclonal antibodies used in this study have been detailed previously (4). Briefly, the specificities of the monoclonal antibodies utilized in this study are anti-Thy-1.2 (all T-cells), anti-Lv-1.2 (all T-cells, some B-cells), anti-IgM (B cells), anti-Ly-5(clone F-11, all T cells, B-cells, and macrophages), anti-Ly-5(B220) (clone 6B2, pre-B-cells and B-cells), anti-ThB (thymocytes and B-cells), anti-Mac-1 (macrophages and immature granulocytes), anti-Ly-b-2 (pre-B-cells and B-cells) and Ly-17 (60% of bone marrow cells, pre-B-cells and B-cells). FMF assays were performed on a fluorescence-activated cell sorter (FACS 11; Becton and Dickinson & Co., Sunnyvale, Calif.) fitted with a logarithmic amplifier. Tumor cells were readily distinguished from residual normal cells on the basis of increased forward-angle light scatter.

Isolation of total cell RNA - Total cellular RNA was isolated from tumor cells by a modification of the method of Strohman et al. (10) and has previously been described in detail (6).

Formaldehyde gels - RNA samples (4μg) in 10mM sodium phosphate, pH7.4/50% (vol/vol) formamide/2·2 M formaldehyde/0.5mM EDTA, were heated for at 68°C 5 minutes and added to submerged gel slabs consisting of 1% agarose in 10mM sodium phosphate, pH 7.4/2.2 M formaldehyde. The samples were electrophoresed at 25V for 22-24 hours in a recirculating buffer consisting of 10mM sodium phosphate, pH 7.4/2.2 M formaldehyde. The RNA was transferred directly to nitrocellulose membranes using 20XSSC buffer and procedures outlined by Thomas (11).

Preparation of retroviral and oncogene probes - Retroviral probes included the long terminal repeat (LTR) prepared by isolating the 640-base-pair Kpn1 fragment from cloned Harvey sarcoma proviral DNA (12); a 500-base-pair xenotropic/MCF envelope probe, pXenv, constructed from a cloned NFS xenotropic proviral fragment (13); a 500-base-pair BamH1/Bg111 ecotropic-envelope specific probe, pEc-B4, constructed from cloned ecotropic AKR-MuLV (14); and a 800-base-pair Bg111 envelope probe, pT3, constructed from cloned ecotropic AKR-MuLV. This probe consists of sequences that code for the carboxy terminus of gp70 and amino terminus of p15(E) and recognizes ecotropic and endogenous xenotropic/MCF related sequences (C. Buckler and M. Martin, unpublished results). Oncogene probes included a v-abl probe prepared from a 1.2-kbp Bg111 fragment isolated from cloned A-MuLV (15); a

c-myc probe constructed from a 850-base-pair Cla 1/EcoR1 fragment derived from the second exon of chicken c-myc (16); a v-myb probe constructed from a 1.0 kbp Hae11/Xba1 fragment isolated from cloned avian myeloblastosis virus (17); a rat c-Harvey-ras probe constructed from a 2.3 kbp EcoR1/Xba1 fragment (18); and a v-mos probe constructed from a 1.2 kbp Bgl1/Hind111 fragment of cloned Moloney murine sarcoma virus (19).

Nucleic acid hybridization - Recombinant plasmid DNAs were labeled with ^{32}P by nick-translation (20) and had specific activities of $6\text{-}13x10^7$ cpm/μg DNA. Blot hybridizations were carried out at 42°C for 16-20 hours using the dextran sulphate procedure of Wahl et al. (21), as modified by Thomas (11). Membranes were washed in 0.1X SSC at 50°C and exposed to Kodak AR film at -70°C with a Dupont Lighting plus intensifier screen.

C. Results

Phenotypic and histological characterization of pre-B-cell lymphomas induced by viral extract NS-1

Biologically cloned Cas-Br-M virus was inoculated intracerebrally (ic) into newborn NFS/N mice and a range of hematopoietic neoplasms developed between 18-30 weeks (4). One of these tumors was diagnosed as a pre-B-cell lymphoma by FMF. The tumor cell population was found to be positive for the pre-B and B-cell surface antigen recognized by the monoclonal antibody 6B2 (anti-Ly5-B220) and negative for Thy-1.2, sIg and Mac-1. Consistent with the FMF results the tumor was diagnosed as a lymphoblastic lymphoma by histological examination.

A 10% (w/v) cell-free extract was prepared from the lymphomatous spleen and inoculated into newborn NFS/N mice. This extract, termed NS-1, induced 14 tumors between 12-18 weeks after inoculation. Twelve of the tumors were diagnosed as pre-B-cell lymphomas by FMF and as lymphoblastic lymphomas by histology. The two exceptions were diagnosed as an erythroleukemia and a myelogenous leukemia. One mouse, NS 35, also had an early transitional cell carcinoma in one kidney. The details of the 14 tumors are shown in Table 1.

Virus assays of pre-B-cell lymphomas

The extract prepared from the primary pre-B-cell lymphoma (NS-1) was examined for MCF and transforming viruses. As shown in Table 2 the extract contained both virus types. Transformed foci could be detected at 10-12 days post infection of SC-1 cells and supernatant harvested from these cells was able to transform NIH-3T3 and NRK cells but no transforming activity for mink cells was detected. The viruses present in the spleen of an NFS/N mouse with an NS-1-induced pre-B-cell lymphoma (NS 176) were also tested. Table 2 shows the spleen contained a mixture of ecotropic, MCF and transforming viruses.

Characterization of tumors induced by supernatants from NS-1 transformed cells

Newborn NFS/N and AKR/J mice were inoculated ip with tissue culture supernatant from SC-1 cells transformed with NS-1 spleen extract. To date 7 NFS/N and 2 AKR mice have been examined for tumors that arose 10-16 weeks after inoculation. Four of the 10 tumors (3 NFS

Table 1. FMF and histological analyses of NS-1 tumors

Mouse	Histology[a]	Thy-1.2	sIg	Ly-5 (B220)	ThB	Ly-5	Mac-1	Lyb-2	Ly-17	Ly-1.2	FMF Diagnosis
34	LL	-	-	+	+	+		+	+		Pre-B
35	LL	-	-	+	-	+		+	+		Pre-B
36	LL	-	-	+	-	+		+	+		Pre-B
37	LL	-	-	+	+	+		+	+		Pre-B
38	LL	-	-	+	+	+		+	+		Pre-B
48	E	-	-	-	-	+		-	+	-	Null
49	LL	-	-	+	-	+		+	+	-	Pre-B
64	ML	-	-	-	-	+	+	-/+	+	-	Macrophage
117	LL	-	-	+	-	+	-	+	+	+	Pre-B
118	LL	-	-	+	-	+	-	+	+	+	Pre-B
127	LL	-	-	+	+/-	+	-	+	+	+	Pre-B
128	LL	-	-	+	-	+	-	+	+	-	Pre-B
176	LL	-	-	+	-	+	-	+	+	+	Pre-B
178	LL	-	-	+	-	+	-	+	+		Pre-B

[a]LL = lymphoblastic lymphoma; E = erythroleukemia; ML = myelogenous leukemia.

Table 2. Retroviral assays of extracts from pre-B-cell lymphomas

Spleen Extract	Transformed Foci[a]	MCF MuLV[a]	Ecotropic MuLV[b]
NS-1	+	0.9	ND
NS-176	4.5	3.9	4.6

[a]Number of transformed foci and mink cell foci expressed as log FFU/ml.

[b]Number of XC plaques expressed as log PFU/ml.

N.D. Not Determined.

and 1 AKR) were diagnosed as pre-B-cell lymphomas by FMF (Ly-5(B220)$^+$, sIg$^-$, Thy-1$^-$ and Mac-1$^-$). Three NFS tumors were found to be both Ly-5(B220)$^+$ and Mac-1$^+$ and have been termed pro-B cell lymphomas. Finally, one NFS and one AKR mouse were found to contain two distinct tumor populations - one population of pre-B-cells and the other of macrophage/granulocytes. The macrophage/granulocyte tumor cells were clearly distinguishable from the pre-B-cells by their larger size as determined by forward angle light scatter. This tumor population was positive for both Mac-1 (macrophages and immature granulocytes) and 8C5, a monoclonal antibody that recognizes mature granulocytes. The tissue culture supernatant used to inoculate two mice that developed pre-B-cell lymphomas and two mice that developed both pre-B-cell and granulocyte/macrophage lymphomas had no detectable MCF virus by mink or SC-1 UV-mink assay. However more extensive testing would be needed to rule out trace amounts of virus, or a few MCF-producing cells.

Retroviral and oncogene analysis of lymphoma RNAs

Total cell RNA was prepared from an NFS/N mouse that developed a pre-B-cell lymphoma following inoculation with tissue culture supernatant from NS-1-transformed SC-1 cells. RNA was also prepared from two T-cell lymphomas of Cas-Br-M-infected mice. As shown in Fig. 1 the lymphomas contained the 8.8kb and 3.0kb retroviral RNA species, consistent with replication competent MuLVs. These retroviral messages hybridized to all four MuLV probes, including the pXenv and Ec-B4 probes, suggesting the lymphomas contained both MCF and ecotropic viruses. However, RNA from the pre-B-cell lymphoma (lane 1) contained two additional MuLV messages that were not present in the T cell lymphomas (lanes 2,3). These messages were slightly smaller in size than the 8.8kb genomic message and hybridized to the LTR, pT3 and pXenv probes, but not the pEc-B4 probe. The envelope message from the pre-B-cell tumor RNA also appeared to be slightly smaller in size.

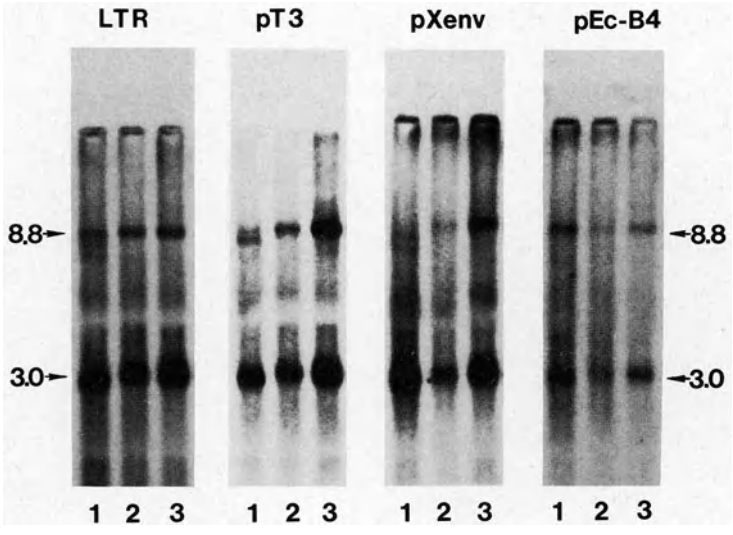

Fig. 1. Total cell RNA from an NS-1-induced pre-B-cell lymphoma (lane 1) and two Cas-Br-M-induced T cell lymphomas (lanes 2 and 3) was electrophoresed, transfered to nitrocellulose and hybridized to the probes shown above.

The same RNAs were also hybridized with the oncogene probes v-abl, c-myc, v-myb, c-mos and c-ras. As shown in Fig. 2 none of the probes hybridized to RNA species of the same size as those recognized by the retroviral probes. However the lymphoma RNAs did contain 6.7kb and 5.4kb v-abl messages, a 2.4kb c-myc message and a 3.5kb v-myb message (Fig. 2). None of the RNAs contained c-mos or c-ras messages that could be detected following one week exposures (data not shown).

D. Discussion

In the course of studying tumors of mice inoculated with the wild mouse ecotropic virus, Cas-Br-M, it was observed that a spleen extract from one such mouse was capable of inducing a high frequency of tumors of the same phenotype as the primary tumor. The primary tumor had been diagnosed as a pre-B-cell lymphoma (4) and of 12 tumors that developed following inoculation of the extract 10 were pre-B-cell lymphomas. This finding was in contrast to the results of a preceding study in which we found mice inoculated with biologically-cloned Cas-Br-M developed a broad spectrum of hematopoietic tumors (4). Further examination of the primary pre-B-cell tumor extract resulted in the detection of a virus that transformed mouse and rat fibroblasts. Inoculation of tissue culture supernatant from the transformed cells into newborn mice also resulted in the development of pre-B-cell lymphomas. Interestingly two of the mice also contained a second tumor population consisting of cells from the macrophage/granulocyte lineage. These results suggest the generation of a transforming retrovirus with a specificity to induce lymphomas of the pre-B-cell (and occasionally macrophage) lineage.

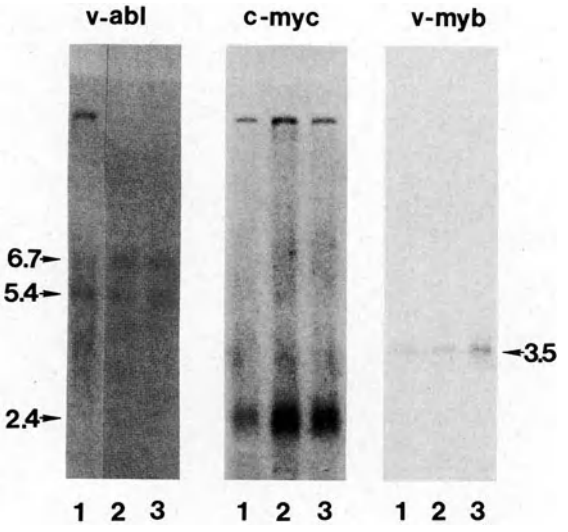

Fig. 2. Total cell RNA from an NS-1-induced pre-B-cell lymphoma (lane 1) and two Cas-Br-M-induced T cell lymphomas (lanes 2 and 3) was electrophoresed, transfered to nitrocellulose and hybridized to the probes shown above.

The majority of transforming retroviruses isolated to date have defective genomes that can be identified by RNA blot hybridization procedures using retroviral DNA probes. RNA prepared from a pre-B-cell lymphoma was hybridized with four MuLV probes in an attempt to detect a retrovirus with a genome of unique size. Hybridization with an LTR probe and two envelope probes (a generalized gp70/p15E probe and an MCF/xenotropic envelope-specific probe) detected two additional RNA species not present in two T-cell lymphomas associated with Cas-Br-M. These messages were only slightly smaller in size than the 8.8kbp genomic message of replication-competent MuLVs. In addition these probes hybridized to envelope messages that appeared slightly smaller in size than the normal 3.0kbp env message. The ecotropic envelope-specific probe did not however hybridize to the smaller genomic messages present in the pre-B-cell lymphoma RNA. Assuming the genome of the transforming virus is represented by one of the smaller messages it is possible that the virus was generated from an MCF virus and not directly from Cas-Br-M.

A number of oncogene probes were also utilized to examine whether the transforming virus had recombined with host sequences related to oncogenes associated with tumors of the B-cell lineage. For this reason the v-abl, c-myc and v-myb probes were selected but none hybridized to RNAs of equivalent sizes as detected by the MuLV probes. The v-mos and c-ras oncogenes were also examined because of their murine origin and fibroblast transformation properties but they did not detect message in the lymphoma RNA.

Work is presently in progress to biologically and molecularly clone this transforming virus. Studies with the cloned virus should enable characterization of its B-cell specificity and its oncogenic sequence.

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References

1. Hartley, J.W., Rowe, W.P.: Naturally occurring murine leukemia viruses in wild mice: characterization of a new "amphotropic" class. J. Virol. 19, 19-25 (1976).
2. Gardner, M.B.: Type C viruses of wild mice: characterization and natural history of amphotropic, ecotropic, and xenotropic MuLV. Curr. Top. Microbiol. Immunol. 79, 215-259 (1978).
3. Gardner, M.B., Klement, V., Henderson, B.E., Casagrande, J., Bryant, M.L., Daugherty, M.F., Estes, J.D.: Lymphoma, paralysis, and oncornaviruses of wild mice. In: Tumors of Early Life in Man and Animals. Severi, L. (ed.), Perugia Quadrennial International Conferences on Cancer, Montelvce, Italy, 1978, pp. 343-356.
4. Fredrickson, T.N., Langdon, W.Y., Hoffman, P.M., Hartley, J.W., Morse, H.C. III: Histologic and cell surface antigen studies of hematopoietic tumors induced by Cas-Br-M murine leukemia virus. J. Natl. Cancer Inst. 72, 447-454 (1984).
5. Hoffman, P.M., Davidson, W.F., Ruscetti, S.K., Chused, T.M., Morse, H.C. III: Wild mouse ecotropic murine leukemia virus infection of inbred mice: Dual-tropic virus expression precedes the onset of paralysis and lymphoma. J. Virol. 39, 597-602 (1981).
6. Langdon, W.Y., Hoffman, P.M., Silver, J.E., Buckler, C.E., Hartley, J.W., Ruscetti, S.K., Morse, H.C. III: Identification of a spleen focus-forming virus in erythroleukemic mice infected with a wild mouse ecotropic MuLV. J. Virol. 46, 230-238 (1983).
7. Rowe, W.P., Pugh, W.E., Hartley, J.W.: Plaque assay techniques for murine leukemia viruses. Virology 42, 1136-1139 (1970).
8. Hartley, J.W., Wolford, N.K., Old, L.J., Rowe, W.P.: A new class of murine leukemia virus associated with the development of spontaneous lymphomas. Proc. Natl. Acad. Sci., U.S.A. 74, 789-792 (1977).
9. Morse, H.C. III, Chused, T.M., Boehm-Truitt, M., Mathieson, B.J., Sharrow, S.O., Hartley, J.W.: XenCSA: Cell surface antigens related to the major glycoproteins (gp70) of xenotropic murine leukemia virus. J. Immunol. 122, 433-454 (1979).
10. Stroham, R.C., Moss, P.S., Micou-Eastwood, J., Spector, D., Przybyla, A., Paterson, B.: Messenger RNA from myosin polypeptides: Isolation from single myogenic cultures. Cell 10, 265-273 (1977).
11. Thomas, P.S.: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77, 5201-5205 (1980).
12. Hager, G.L., Chang, E.H., Chan, H.W., Garon, C.F., Israel, M.A., Scolnick, E.M., Lowy, D.R.: Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: Initial structural and biological characterization. J. Virol. 31, 795-809 (1979).

13. Buckler, C.E., Hoggan, M.D., Chan, H.W., Sears, J.F., Khan, A.S., Moore, J.L., Hartley, J.W., Rowe, W.P., Martin, M.A.: Cloning and characterization of an envelope-specific probe from xenotropic murine leukemia proviral DNA. J. Virol. 41, 228-236 (1982).
14. Chan, H.W., Bryan, T., Moore, J.L., Staal, S.P., Rowe, W.P., Martin, M.A.: Identification of ecotropic proviral sequences in inbred mouse strains with a subgenomic DNA fragment. Proc. Natl. Acad. Sci., U.S.A. 77, 5779-5783 (1980).
15. Srinivasan, A., Reddy, E.P., Aaronson, S.A.: Abelson murine leukemia virus: Molecular cloning of infectious integrated proviral DNA. Proc. Natl. Acad. Sci., U.S.A. 78, 2077-2081 (1981).
16. Robins, T., Bister, K., Garon, C., Papas, T., Deusberg, P.: Structural relationship between a normal chicken DNA locus and the transforming gene of the avian acute leukemia virus MC29. J. Virol. 41, 635-642 (1982).
17. Perbal, B., Baluda, M.A.: Avian myeloblastosis virus transforming gene is related to unique chicken DNA regions separated by at least one intervening sequence. J. Virol. 41, 250-257 (1982).
18. Chang, E.H., Gonda, M.A., Ellis, R.W., Scolnick, E.M., Lowy, D. R.: Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. Proc. Natl. Acad. Sci., U.S.A. 79, 4848-4852 (1982).
19. Reddy, E.P., Smith, M.J., and Aaronson, S.A.: Complete nucleotide sequence and organization of the Moloney murine sarcoma virus genome. Science 214, 445-450 (1981).
20. Maniatis, T., Jeffrey, A., Kleid, D.G.: Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci., U.S.A. 72, 1184-1188 (1975).
21. Wahl, G.M., Stern, M., Stark, G.R.: Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci., U.S.A. 76, 3683-3687 (1979).

Transformation Capacities of a Murine Retrovirus Encoding an Avian myc Oncogene

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In trying to establish a system for the study of myc-induced in vitro transformation of mammalian cells, we have constructed a mammalian retrovirus encoding an avian myc oncogene. Its LTRs are derived from the Moloney Murine Leukemia Virus (MoMuLV) and the myc oncogene from the avian retrovirus OK10. A virus, denoted murine myc virus (MMCV), was recovered after cotransfecting NIH3T3 cells with MMCV and MoMuLV helper virus DNAs.

MMCV efficiently transforms fibroblast cells of established lines (such as mouse NIH3T3 and rat 208F) to anchorage independent growth. Molecular analysis of several clones of transformed cells shows that the structure of the viral genome is unperturbed upon replication and that the virus expresses a 57,000 dalton myc protein similar to that found in OK10 transformed avian cells.

Most clones of fibroblast cells transformed by MMCV have a rather flat morphology, particularly when 208F cells are used, and the virus only poorly induces foci of transformed cells in monolayer cultures. Indeed, only cells from clones with a very transformed morphology are capable of forming foci when seeded into cultures of normal 208F cells. This finding might explain the difficulties of other investigators when trying to transform cultured cells with myc. MMCV also transforms primary mouse embryo macrophages in vitro. The transformed cells grow in semisolid and liquid media, and can easily be expanded into mass-cultures. They have been identified as macrophages as based on: i) requirement for the macrophage growth factor CSF-1 for growth; ii) presence of the macrophage-neutrophil specific mac-1 antigen on the cell-surface; and iii) ability to phagocytize bacteria. Transformation of primary mouse embryo fibroblasts has, however, not yet been detected.

In conclusion, MMCV transforms both fibroblasts and macrophages in culture, and thus has in vitro-transformation properties similar to those of the avian myc retroviruses, the only difference so far detected being the inability of MMCV to transform primary fibroblasts. Experiments to transform cells of other hemopoietic lineages with MMCV and to assess the tumorigenicity of the MMCV-transformed fibroblasts are in progress.

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The CH Series of Murine B Cell Lymphomas: Identification of Cross-reactive Idiotypes and Restricted Antigen Specificities

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INTRODUCTION

Mice of the double congenic strain, Bl0.H-2^aH-4^bp/Wts (2^a4^b), display a high incidence of lymphoma following intense adoptive immunization with sheep erythrocytes (SRBC). The tumors which arise comprise a heterogenous group, including the UNC series of T cell lymphomas (Corley 1983), the CH series of B cell lymphomas (Lanier 1982), and a set of lymphomas which are not readily characterized as belonging to either the B cell or T cell lineage (unpublished data). Presently available data are insufficient to define the etiology of this disease, but we previously had found that heterologous anti-idiotype sera raised against the surface IgM of CH1 and of CH2 each reacted with one other of the first 11 B cell lymphomas described (CH9 and CH5, respectively) (Arnold 1982). This suggested that a limited subset of the total normal idiotype repertoire might be expressed by CH lymphomas. We also had found that the surface immunoglobulin of CH12 was specifically reactive with an epitope present on SRBC, suggesting that adoptive immunization had led to the expansion of a subset of B cells with neoplastic potential (Arnold 1983).

MATERIALS AND METHODS

Mice and Tumors

Bl0.H-2^aH-4^b mice were bred and maintained in our pathogen free mouse colony. This strain was derived from selected F2 progeny of Bl0.A x Bl0.129 (2lM) mice as described (Wettstein 1974).

The CH series of B cell lymphomas was induced in 2^a4^b mice by adoptive hyperimmunization with SRBC, except for CH19 which arose in an old (487d), unmanipulated 2^a4^b mouse. Details of the induction protocols have been reported elsewhere (Lanier 1982). The tumors were maintained by serial transplantation in syngeneic animals. MOPC104E and TEPC183 plasmacytomas (Potter 1972) were maintained by transplantation in pristane primed BALB/c mice.

Characterization of cell surface antigens and idiotypes

Cell surface antigens were detected by standard methods of indirect immunofluorescence (Arnold 1983). Details of the cell surface antigens expressed by CH1-CH12 have been reported (Lanier 1982) and the characteristics of CH15-CH39 will be presented elsewhere. Briefly, all bear surface IgM (sIgM) as well as I-A, I-E, and T200/Ly5 antigens. Five lymphomas (CH5, CH6, CH12, CH25 and CH35) also express

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the Lyt-1 antigen while none of the tumors tested bear Thy1, Lyt-2, or Lyt-3. CH15 bears sIgD in addition to sIgM.

Bromelain treatment of autologous erythrocytes

A 5% suspension of washed MRBC was incubated with bromelain (100 μg/ml) for 45 min at 37°C. The erythrocytes (BrMRBC) were washed 3 times in HBSS and stored in Alsever's solution for up to 1 week.

Rosette assays

The erythrocyte rosette assays were performed as described (Arnold 1983). The *E. coli* rosette assay was similar except that the mixture of tumor and *E. coli* cells was centrifuged at 43 x G for 10 min.

Preparation of anti-idiotype sera

Rabbit antisera were raised against the detergent solubilized, affinity-purified surface immunoglobulin (sIg) of 9 CH lymphomas as described (Haughton 1978). Each serum was absorbed extensively with either immobilized myeloma proteins or other CH tumor cells followed by normal 2^a4^b spleen cells. Antisera raised against the IgM, κ bearing tumors (with the exception of CH5 and CH12) were absorbed with TEPC183 (μ,κ) while MOPC104E (μ,λ) was used to absorb antisera raised to the IgM, λ tumors. Since both CH5 and CH12 expressed idiotopes present on TEPC183, the anti-CH5 and anti-CH12 idiotype sera (anti-id) were absorbed with CH15 cells. An anti-id was considered specific when it reacted with >95% of the appropriate tumor cells but not detectably with normal spleen cells (indirect immunofluorescence assay).

RESULTS

The specificity of the anti-ids was established both by immunofluorescence and by antigen binding inhibition studies. In these experiments, tumor cells were incubated with either a specific or an irrelevant anti-id under capping conditions (in RPMI1640 for 60 min at 37°C) before the addition of antigen (erythrocytes or *E. coli*; see Table 2). In all cases ability to inhibit rosette formation corresponded precisely with the presence of anti-idiotope antibody demonstrable by immunoflorescence.

Analysis of cross-reactive idiotypes

We tested the panel of anti-ids against the entire series of CH lymphomas. This revealed that 21 of the 27 CH lymphomas bore cross-reactive idiotopes (IdX), as defined by 7 of the anti-ids. We accordingly classified these tumors into 5 IdX groups. Four of the groups (CH1IdX, CH2IdX, CH12IdX and CH27IdX) were connected, one to another, by tumors which reacted with more than one anti-id, thereby linking 16 of the CH lymphomas in a single network (the other 5 tumors only bear one IdX, the CH10IdX). In order to determine the minimum number of idiotopes expressed by the lymphomas in each IdX group, we absorbed each anti-id with each of the tumors with which it reacted and retested on all the tumors in the IdX group defined by that serum. This analysis revealed that a minimum of 16 idiotopes are expressed by 23 of the 27 CH lymphomas (see Table 1). Only 4 idiotopes (b,n,o,p) appear to be private (IdI); the rest are IdX. At least 7 (a,d,e,f,i,j,k) of the 12 IdX are located on the heavy chain variable (V_H) region, as evidenced by their expression on lymphomas

bearing either light chain class. Four of these idiotopes (e,f,i,k) are also expressed by the BALB/c myeloma protein, TEPC183.

Table 1. IDIOTOPES EXPRESSED BY THE CH LYMPHOMAS

		Idiotopes															
Tumor	sIg	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p
CH1	μ,λ	+[1]	+	-	+	-	-	-	-	ND[2]	-	ND	ND	-	-	-	-
CH9	μ,κ	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
CH15	μ,δ,κ	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
CH39	μ,λ	+	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-
CH34	μ,κ	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CH26	μ,κ	+	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	-	-
CH2	μ,λ	-	-	-	-	+	+	-	-	ND	-	ND	ND	-	-	-	-
CH5	μ,κ	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
CH27	μ,κ	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-
CH28	μ,κ	-	ND	-	-	-	-	+	+	-	-	-	-	-	-	-	-
CH35	μ,κ	-	ND	-	-	-	-	+	+	-	-	-	-	-	-	-	-
CH32	μ,κ	ND	-	ND	ND	-	-	+	+	+	-	-	-	-	-	-	-
CH25	μ,κ	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	-
CH23	μ,κ	-	-	-	-	-	-	ND	ND	+	-	+	-	-	-	-	-
CH12	μ,κ	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
T183	μ,κ	-	ND	-	-	+	+	-	-	+	-	+	+	-	-	-	-
CH38	μ,κ	-	-	-	-	-	-	-	-	+	ND	ND	ND	-	-	ND	-
CH10	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
CH29	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
CH31	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
CH33	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
CH36	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
CH6	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CH7	μ[3],κ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
CH4	μ	-	-	-	-	-	-	-	-	ND	-	ND	ND	-	-	-	-
CH19	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CH21	μ,κ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CH30	μ,κ	ND	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	-	-

[1]cells positive in an indirect immunofluorescence assay.

[2]Not done.

[3]sIgM only detected by flow cytometry analysis.

Antigen specificities of CH lymphomas

Thirteen of the 27 CH lymphomas bind determinants on the surface of erythrocytes or *E. coli* or both (see Table 2). We first discovered that CH12 bore sIg specific for an epitope on SRBC when we tested it for Fc and C3 receptors using the EA and EAC rosette assays (Arnold 1983). Subsequently, we found that CH27, 28, 32, 34, and 35 also formed rosettes with ChRBC and SRBC and that these tumors (including CH12), in addition to CH9, 15 and 23, bore sIg reactive with determinants on BrMRBC.

Table 2. ANTIGEN SPECIFICITIES OF CH LYMPHOMAS

Lymphoma	SRBC	BrMRBC	ChRBC	E. coli
CH1	-	-	ND[1]	ND
CH2	-	-	-	-
CH4	ND	ND	ND	ND
CH5	-	ND	ND	-
CH6	-	-	-	-
CH7	-	-[2]	-	-
CH9	-	+[2]	-	-
CH10	-	-	-	+
CH12	+	+	+	-
CH15	-	+	-	ND
CH19	-	-	-	-
CH21	-	-[3]	-	-[3]
CH23	-	+[3]	-	+[4]
CH25	-	-	-	+
CH26	ND	ND	-	ND
CH27	+	+	+	-
CH28	+	+	+	-
CH29	-	-	-	-
CH30	-	-	-	-
CH31	-	-	-	+
CH32	+	+	+	-
CH33	-[3]	ND	-[3]	-
CH34	+[3]	+	+[3]	+
CH35	+	+	+	-
CH36	-	-	-	-
CH38	-	-	-	+
CH39	-	-	-	-

[1] Not done.

[2] >80% unless otherwise noted.

[3] 10-15%

[4] 50%

Two tumors, CH23 and CH34, formed rosettes not only with BrMRBC but also with *E. coli* (the percent of CH23 rosette forming cells was low, 13%, with both antigens). CH10, 25, 31, and 38 all displayed high percentages of rosette forming cells with *E. coli*.

For every tumor tested, the binding of erythrocytes or *E. coli* was attributable to the sIg. Evidence for this came from the capping experiments mentioned above, plus the observation that supernatants from the CH12, 27, 28 and CH10 hybridomas agglutinated the appropriate erythrocytes or *E. coli*, respectively (the preparation of a representative CH hybridoma was described by Arnold 1983).

DISCUSSION

Idiotypes are sets of serologically defined antigenic determinants (idiotopes) associated with immunoglobulin-variable regions (Oudin 1963). The CH lymphomas display a restricted repertoire of idiotopes defined by heterologous anti-idiotype sera. Twenty-one of the 27 tumors examined bore IdX and were classified into 5 IdX groups, 4 of which overlapped (accounting for 16 tumors). Two tumors (CH6

and CH7) bore idiotypes (IdI) only detectable with the homologous anti-id sera, and 4 tumors (CH4, 19, 21, and 30) did not react with anti-id sera, when tested (homologous anti-id sera were not available for these tumors). It seems likely that all 27 CH lymphomas are idiotypically related but that some common idiotopes have yet to be detected. Approximately half (7/16) of the CH-idiotopes are certainly located in the V_H domain, as evidenced by their presence on lymphomas expressing either kappa or lambda light chains; presently available data are uninformative regarding the involvement of V_H and V_L in expression of the other 9 idiotopes. Interestingly, the tumors (CH25, 27, 32, 39, and TEPC183) that link the 4 IdX groups together each bears at least one proven V_H-associated idiotope. On the basis of these observations, we hypothesize that the CH lymphomas derive from a subset of B cells, defined by the expression of a restricted number of V_H sequences.

The humoral response of certain inbred strains of animals to particular antigens is dominated by characteristic, idiotypically restricted sets of immunoglobulins (murine systems are reviewed by Makela 1977). Murine strain-specific, recurrent idiotypes are associated predominately with V_H regions and, in those cases examined, are encoded by a paucity of V_H genes (Bothwell 1981; Siekevitz 1983; Crews 1981). Although we do not know yet if the CH lymphomas utilize a restricted number of V_H genes (these experiments are underway) we do know that their sIg are related not only by V_H region encoded idiotopes but also by their antigen-binding specificities.

Nine tumors (CH9, 12, 15, 23, 27, 28, 32, 34, and 35), including members of 4 of the 5 IdX groups, bear sIg specifically reactive with a determinant on bromelain-treated autologous erythrocytes (BrMRBC) but not on untreated MRBC. Six of these tumors (CH12, 27, 28, 32, 34, and 35) also bind SRBC and ChRBC (the ChRBC determinants are not MHC or major blood group related; data not shown). To our knowledge, murine immunoglobulins specific for determinants on both SRBC and ChRBC have not been reported previously (Tim Mosmann, personal communication). SRBC were used in the induction protocols for each CH lymphoma (except CH19), but these tumors do not represent a population of B cells with typical anti-SRBC specificities.

The treatment of erythrocytes with bromelain reveals otherwise cryptic determinants which are expressed on MRBC modified (perhaps due to aging) in vivo (Linder 1972). Endogenous antigenic determinants such as these may provide a constant source of stimulation to the immune system, as would common antigens on environmentally encountered pathogens. Immunoglobulins specific for antigens on pathogens often express dominant idiotypes characteristic of strain specific antigenic systems (Marion 1984). Six of the CH lymphomas (CH10, 23, 25, 31, 34, and 38) bear sIg reactive with a determinant(s) on *E. coli* and interestingly, CH23 and CH34 also bind BrMRBC, suggesting that the *E. coli* and BrMRBC associated epitopes are structurally similar. This limited spectrum of antigenic reactivities further suggests that the CH lymphomas represent a subset of B cells. By analogy, Potter (1977) and Hood (1977) suggested that the BALB/c and NZB myeloma proteins represent restricted spectra of B cell products, suggestions based partially on the myelomas' antigen binding characteristics.

Bona (1981) first proposed the existence of regulatory idiotopes, idiotopes that could serve as recognition units in mini-regulatory networks. Cells expressing sIg which bear a given regulatory idiotope, regardless of the antigen-binding specificity of the sIg, presumably are controlled

by the same set of regulatory cells (Gleason 1982). The CH lymphomas may be members of such a mini-network defined by a V_H encoded regulatory idiotope. The specificity of some of the cells in this network for endogenous antigens would provide the selective pressure necessary for the maintenance of the network and for the conservation in the genome of the necessary V_H genes. Briles (1982), for example, has shown that anti-PC antibodies bearing the dominant T15id confer protection to mice infected with Streptococcus pneumoniae. The specificity of some of the normal B cell counterparts of the CH lymphomas may be physiologically relevant, being directed against determinants on E. coli and modified autologous erythrocytes. Somatic mutation and different V_H-D-J_H combinations would result in cells producing sIg with different antigen specificities but if they still expressed the same regulatory idiotope, then they would still be regulated by the same set of cells. The CH lymphomas that express IdX but do not bind BrMRBC or E. coli would be in this category.

Perturbation of the putative CH-idiotype network by hyperimmunization with SRBC, an antigen for which some of the members are specific, in some way increases the risk of the members for neoplasia. A possible mechanism involves dysfunctioning regulatory cells which normally would recognize a CH-associated regulatory idiotope. This implies a T cell function and the involvement of H-2 products and idiotopes. There are several systems in which idiotype expression is correlated with MHC haplotypes (Bekoff 1982; Yamamoto 1983). To investigate the genetics of susceptibility to antigen-driven lymphomagenesis, we induced tumors in 2^a4^b congenic strains and found a correlation between H-2 haplotype and the incidence of lymphomas (manuscript in preparation) Thus, the CH series of B cell lymphomas bear immunoglobulins related by idiotype and by antigen specificity whose regulation seems to be under the influence of H-2.

REFERENCES

Arnold LW, Pennell CA, LoCascio NJ, Haughton G (1982) Cross-reactive idiotypes of murine B cell lymphomas: implications for etiology and immunotherapy. In: Vitetta ES, Fox CF (eds) B and T Cell Tumors. Academic Press, New York, p 499.

Arnold LW, LoCascio NJ, Lutz PM, Pennell CA, Klapper DG, Haughton G, (1983) Antigen-induced lymphomagenesis: identification of a murine B cell lymphoma with known antigen specificity. J Immunol 131:2064-2068.

Bekoff MC, Levine H, Schlossman SF (1982) T cell and Ir gene regulation of expression of a cross-reactive idiotype. J Immunol 129: 1173-1180.

Bona CA, Heber-Katz E, Paul WE (1981) Idiotype-anti-idiotype regulation. I. Immunization with a levan-binding myeloma protein leads to the appearance of auto-anti-(anti-idiotype) antibodies and to the activation of silent clones. J Exp Med 153:951-967.

Bothwell ALM, Paskind M, Reth M, Imanishi-Kari T, Rajewsky K, Baltimore D (1981) Heavy chain variable region contribution to the NP^b family of antibodies: somatic mutation evident in a γ2a variable region. Cell 24:625-637.

Briles DE, Forman C, Hudak S, Claflin JL (1982) Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J Exp Med 156:1177-1185.

Corley RB, Arnold LW, Lutz PM, Kuhara T, White DA, Staniszewski C, Haughton G (1983) T cell lymphomas with helper T cell function. In: Parker JW, O'Brien RL (eds) Intercellular Communication in Leucocyte Function. John Wiley and Sons Ltd., Sussex, p 521.

Crews S, Griffin J, Huang H, Calame K, Hood L (1981) A single V_H gene segment is correlated with the class of the antibody. Cell 25:59-66.

Gleason K, Kohler H (1982) Regulatory idiotypes. T helper cells recognize a shared V_H idiotope on phosphorylcholine-specific antibodies. J Exp Med 156:539-549.

Haughton G, Lanier LL, Babcock GF, Lynes MA (1978) Antigen-induced murine B cell lymphomas. II. Exploitation of the surface idiotype as tumor specific antigen. J Immunol 121:2358-2362.

Hood L, Loh E, Hubert J, Barstad P, Eaton B, Early P, Fuhrman J, Johnson N, Kronenberg M, Schilling J (1977) The structure and genetics of mouse immunoglobulins: an analysis of NZB myeloma proteins and sets of BALB/c myeloma proteins binding particular haptens. CSH Symp Quant Biol 41:817-836.

Lanier LL, Arnold LW, Raybourne RB, Russell S, Lynes MA, Warner NL, Haughton G (1982) Transplantable B-cell lymphomas in B10.H-2^aH-4^b p/Wts mice. Immunogen 16:367-371.

Linder E, Edgington TS (1972) Antigenic specificity of anti-erythrocyte autoantibody responses by NZB mice: identification and partial characterization of two erythrocyte surface antigens. J Immunol 108:1615-1623.

Makela O, Karjalainen K (1977) Inherited immunoglobulin idiotypes of the mouse. Immunol Rev 34:119-138.

Marion TN, Dzierzak EA, Lee HS, Adams RL, Janeway CA (1984) Non-dinitrophenyl-binding immunoglobulin that bears a dominant idiotype (Id460) associated with antidinitrophenyl antibody is specific for an antigen on Pasteurella pneumotropica. J Exp Med 159:221-233.

Oudin J, Michel M (1963) A new allotype form of rabbit serum-globulins, apparently associated with antibody function and specificity. CR Seances Acad Agr 257:805.

Potter M (1972) Immunoglobulin-producing tumors and myeloma proteins of mice. Physiol Rev 52:631-719.

Potter M (1977) Antigen-binding myeloma proteins of mice. Adv Immunol 25:141-211.

Siekevitz M, Huang SY, Gefter ML (1981) The genetic basis of antibody production: a single heavy chain variable region gene encodes all molecules bearing the dominant anti-arsonate idiotype in the strain A mouse. Eur J Immunol 13:123-132.

Wettstein PJ, Haughton G (1974) Production, testing, and utility of double congenic strains of mice. Transplantation 17:513-517.

Yamamoto H, Bitoh S, Torii M, Fujimoto S (1983) Idiotype-specific T lymphocytes. I. Regulation of antibody production by idiotype-specific H-2 restricted T lymphocytes. J Immunol 130:1038-1042.

Interaction of Acute Transforming Retroviruses with Murine Hematopoietic Cells

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INTRODUCTION

There is emerging evidence that several retroviral onc gene products possess functional, structural, and amino acid sequence homology. Rous sarcoma virus (RSV), Abelson-murine leukemia virus (Abelson-MuLV), and Snyder-Theilen feline sarcoma virus (ST-FeSV) code for related phosphoproteins that have associated tyrosine-specific protein kinase activity (Barbacid et al., 1980; Witte et al., 1980; Reddy et al., 1983). BALB-, Harvey-, and Kirsten-murine sarcoma viruses (MSVs) are members of another family whose 21,000 dalton transforming proteins possess GDP binding and autophosphorylation activity (Shih et al., 1979; Andersen et al., 1981). Investigation of the diversity of hematopoietic target cells susceptible to neoplastic transformation by specific retroviruses might be expected to provide insights into the relationship of the differentiated state of a cell and its susceptibility to the action of a family of onc genes. As an approach to this question, we took advantage of the availability of hematopoietic cell transformation assay systems to analyze the array of cellular targets whose growth and differentiation could be altered by different retroviral onc genes.

BALB- AND HARVEY-MSVs TRANSFORM NOVEL LYMPHOID PROGENITOR CELLS AND PROMOTE GROWTH AND DIFFERENTIATION OF MYELOID PRECURSOR CELLS

Ras-containing retroviruses, in addition to their ability to cause sarcomas, induce proliferation of erythroid cells both in vivo (Scher et al., 1975) and in tissue culture (Hankins and Scolnick, 1981). To investigate the effects of BALB- and Harvey-MSVs on other murine hematopoietic cell lineages, we utilized a bone marrow colony-forming assay developed by Rosenberg and Baltimore (1976) that detects lymphoid cell transformation by Abelson-MuLV. BALB- and Harvey-MSVs induced two types of colonies in the bone marrow assay. One colony type was compact in morphology, and cells from these colonies exhibited an undifferentiated blast cell morphology (Pierce and Aaronson, 1982) (Figure 1a, b). Compact colony formation was sarcoma virus-dependent, followed single-hit kinetics (Table 1), and required the presence of mercaptoethanol. Similar compact colonies were obtained when bone marrow from leukemic mice infected with BALB- or Harvey-MSV was utilized in the colony-forming assay. Cells from BALB- and Harvey-MSV-induced compact colonies could be established into continuous lines. The success rate for establishment of cell lines could be greatly improved with the initial use of adherent normal bone marrow feeder layers. After a short adaptation period, the transformed lines could be propagated independently. Cells from these lines expressed high levels of the p21 transforming protein and released sarcoma virus. They formed colonies in soft agar at a high efficiency and very large hematopoietic tumors when inoculated subcutaneously into syngeneic adult mice (Table 2). These transformants had a blast cell morphology and lacked markers of mature cells within myeloid and erythroid

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series. They did not synthesize cytoplasmic immunoglobulin μ chain and lacked surface Thy-1 antigen. However, the transformants contained very high levels of terminal deoxynucleotidyl transferase (TdT), an enzyme believed to be specific to early stages within the lymphoid differentiation pathway (Bollum, 1979) (Table 2). This phenotype distinguishes these BALB- and Harvey-MSV transformants from any previously reported hematopoietic targets of transforming retroviruses, including the pre-B lymphoid cells transformed by Abelson-MuLV under identical assay conditions (Siden et al., 1979).

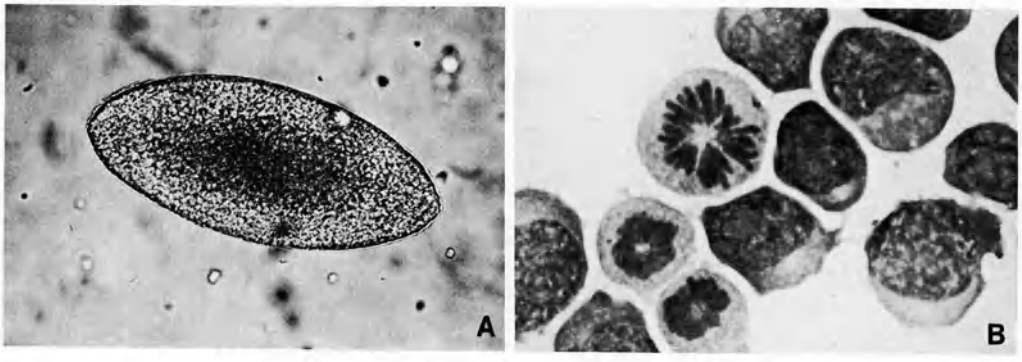

Fig. 1 (a). Morphology of a compact colony; (b) Wright-Giemsa stained cells from a compact colony.

A second colony type which was induced by in vitro or in vivo infection of bone marrow with BALB- or Harvey-MSV was diffuse in morphology and was shown to be comprised of cells from the myeloid lineage (Pierce, unpublished observations) (Figure 2a, b). Unlike compact colony induction, diffuse colony formation did not require the presence of mercaptoethanol. No diffuse colonies were observed after infection of bone marrow with Abelson-MuLV or ST-FeSV. The presence of granulocyte-macrophage colony stimulating factors in virus stocks was excluded as being the cause of diffuse colony formation. The single-hit titration pattern (Table 1) and release of virus from diffuse colony cells provided evidence that sarcoma virus was required for the induction of diffuse colonies. Moreover, the presence of p21 could be detected in the cells from these colonies by radioimmunoprecipitation. Cells from diffuse colonies exhibited morphological characteristics of mature macrophages. Phenotypic analysis revealed that the majority expressed high levels of lysozyme, nonspecific esterase, mac-1 cell surface antigen, Fc receptors, and the ability to phagocytize latex beads (Table 2). The doubling time, saturation density, and replating efficiency of diffuse colony cells was greatly increased in comparison to normal mouse macrophages. Cells from these colonies could be propagated for several passages, but only one permanently growing line could be established. Cells from the BALB-MSV-induced continuous line grew in soft agar and were tumorigenic. They expressed high levels of the p21 transforming protein and displayed phenotypic markers similar to those of diffuse colony cells, although at relatively low levels. These cells could be induced to differentiate to a more mature macrophage phenotype with the phorbol ester, TPA.

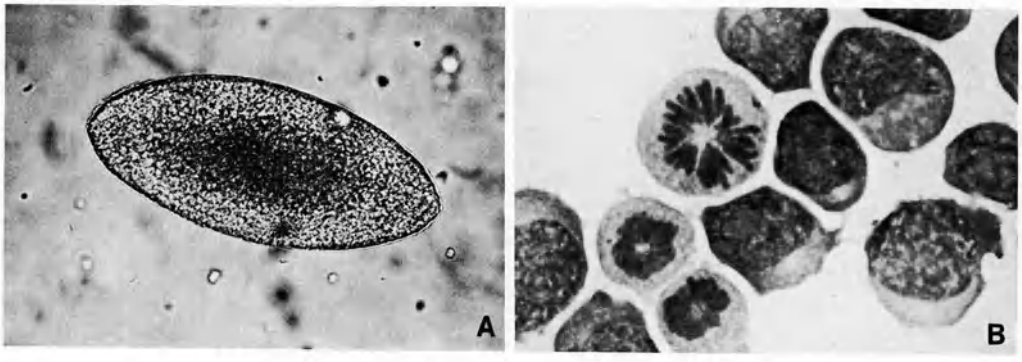

Fig. 2 (a). Morphology of a diffuse colony; (b) Wright-Giemsa stain of cells from a diffuse colony.

TRANSFORMATION OF MURINE PRE-B LYMPHOID CELLS BY ST-FeSV

ST-FeSV codes for a protein with many in vitro functional properties similar to the Abelson-MuLV protein (Barbacid et al., 1980; Witte et al., 1980). Although ST-FeSV is capable of transforming fibroblasts in vitro, there had been no studies performed on its ability to transform hematopoietic cells in culture. ST-FeSV was demonstrated to induce compact colony formation at a low efficiency (Pierce and Aaronson, 1983)(Table 1). Cell lines established from ST-FeSV-induced compact colonies were shown to produce the putative ST-FeSV transforming protein, P85, by radioimmunoprecipitation. ST-FeSV-transformed cell lines formed colonies in soft agar at a high efficiency and were tumorigenic (Table 2). Phenotypic analysis of ST-FeSV transformants revealed that they possessed low levels of Fc receptors, were mercaptoethanol-dependent for growth, and were positive for the pre-B cell surface antigen, Lyb-2. One half of the ST-FeSV transformants were shown to synthesize readily detectable levels of μ as determined by radioimmunoassay (Table 2). No ST-FeSV transformants synthesized κ or λ light chains. These results indicate that ST-FeSV is capable of transforming pre-B cells similar to those that are preferentially obtained after Abelson-MuLV infection of murine bone marrow (Siden et al., 1979).

HEMATOPOIETIC CELL TRANSFORMATION BY A MURINE RECOMBINANT RETROVIRUS CONTAINING THE *src* GENE OF RSV

Efforts to demonstrate transformation of avian hematopoietic cells by RSV have not been successful. For example, although RSV replicates efficiently in avian macrophages, it is unable to transform them in culture (Durban and Boettiger, 1977). Recently, the RSV *src* gene was introduced into an amphotropic murine helper virus, yielding a recombinant virus, designated murine Rous sarcoma virus (MRSV) (Anderson and Scolnick, 1983). Since this virus was capable of inducing erythroleukemia and sarcomas in newborn mice, we examined its ability to alter the growth properties of mouse hemato-

poietic cells in culture. MRSV induced compact colonies that followed single-hit kinetics (Table 1) and required mercaptoethanol in the agar medium (Pierce et al., in press). Cells from the colonies induced by MRSV could be established as continuous cell lines. The initial use of normal adherent bone marrow feeder layers was required to establish permanent lines. Once MRSV-induced cell lines became independent of the feeder layer requirements, they demonstrated unrestricted self-renewal and were tumorigenic (Table 2). The seven transformants isolated each expressed high levels of pp60^{src} that was active in the protein kinase assay. Cells from these lines lacked Fc receptors and detectable immunoglobulin μ chain synthesis. The majority contained high levels of TdT. One cell line expressed Thy-1 antigen but none expressed Lyt-1 or Lyt-2 antigens (Table 2). These findings provide the first evidence that the src gene of RSV is capable of inducing malignant transformation of lymphoid progenitor cells.

Table I. In Vitro Hematopoietic Colony Formation Induced by Acute Transforming Retroviruses

	Colony forming units/plate* induced by:						
FFU added/ plate	BALB-MSV Compact	BALB-MSV Diffuse	Harvey-MSV Compact	Harvey-MSV Diffuse	ST-FeSV Compact	MRSV Compact	Abelson-MuLV Compact
10^5	82, 93	18, 16	75, 88	34, 42	8, 5	25, 19	>100
10^4	10, 8	1, 0	12, 6	2, 3	1, 1	3, 0	9, 18
10^3	1, 2	0, 0	1, 1	0, 0	0, 0	0, 0	1, 2
10^2	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0
FFU/CFU	6.7×10^2	2×10^4	1×10^3	4×10^3	1×10^4	6.7×10^3	6.7×10^2

*Bone marrow cell suspensions from NFS/N weanlings were infected with varying virus dilutions and plated at a concentration of 2×10^6 nucleated cells per plate into soft agar medium containing RPMI-1640 with 20% fetal calf serum and 5×10^{-5}M 2-mercapto-ethanol. Hematopoietic colony formation as scored at day 14.

SUMMARY

Retroviruses containing abl, src and fes genes were each shown to transform murine lymphoid cells in culture but did not always arrest lymphoid cell differentiation at the same stage. Whereas abl and fes preferentially transformed pre-B lymphoid cells (Siden et al., 1977; Pierce and Aaronson, 1983), the targets of src were lymphoid progenitors which did not appear to be committed to the B-cell pathway (Pierce et al., in press). The presence of Thy-1 antigen on the cell surface of one of the transformants indicated that these cells may be T-cell progenitors. Comparison of their predicted amino acid sequences has not revealed any closer homology of abl and fes gene products to each other than to pp60^{src} (Reddy et al., 1983). However, the transforming proteins encoded by fes and abl have been shown to be gag-onc fusion proteins, while the src gene product is not. It is possible that these structural differences play a role in determining the hematopoietic cell specificity for transformation by otherwise closely related onc gene products.

The ras-containing retroviruses, BALB- and Harvey-MSVs, were also shown to transform cells within the lymphoid lineage (Pierce and Aaronson, 1982). These viruses specifically transformed lymphoid progenitor cells that lacked detectable immunoglobulin or thy-1 antigen expression but demonstrated high levels of TdT. Such transformants appear to be at an earlier stage of lymphoid cell differentiation than those induced by Abelson-MuLV or ST-FeSV. The analysis of immunoglobulin gene rearrangement patterns in the BALB- and Harvey-MSV-induced transformants, as well as the use of temperature-sensitive mutants for transformation, could be useful in determining whether these cells have the capacity to differentiate along either the T- or B-cell pathways and may help to determine the functional role of TdT in normal lymphocyte development.

It has been reported that cells from many Abelson-MuLV-induced lymphoid colonies are initially poorly oncogenic and that they become progressively tumorigenic only after propagation on normal adherent bone marrow feeder layers (Whitlock and Witte, 1981). The establishment of permanent cell lines from blast cell colonies induced by other retroviruses was greatly enhanced by the initial use of feeder layers. These findings suggest that a variety of replication-defective retroviruses can initiate lymphoid cell transformation but additional events may be required for the expression of the fully transformed state. Nonetheless, the rapid induction of highly malignant cells by these viruses argues that the interaction of their onc genes with primary hematopoietic cells must play a major role in the development of the transformed phenotype.

The growth-promoting action of BALB- and Harvey-MSVs for hematopoietic cells in culture is not limited to their transforming effects on early lymphoid cells. These viruses also induce erythroid burst formation in vitro (Hankins and Scolnick, 1981). The growth stimulating and differentiating actions of BALB- and Harvey-MSVs on myeloid cells appear to resemble effects of these viruses on cells from the erythroid lineage, since the majority of these cells are also induced to proliferate and proceed through a normal program of differentiation to a mature macrophage phenotype. Through the use of different assay systems it has been possible to demonstrate that ras-containing viruses alter the growth properties of three separate targets within different hematopoietic lineages. In the present report, Abelson-MuLV, ST-FeSV, and MRSV failed to transform myeloid cells under the same conditions in which BALB- and Harvey-MSVs readily induced formation of macrophage colonies. These findings distinguish the effects of these two retrovirus groups on hematopoietic cells. Continued investigation of the targets for transformation by different families of retroviral onc genes may provide additional insights into the mechanisms by which these genes alter growth and differentiation and could help to dissect the pathways of normal hematopoietic cell development.

Table II. Characterization of Retrovirus-Transformed Hematopoietic Cell Lines

	BALB-MSV and Harvey-MSV		MRSV	ST-FeSV	Abelson-MuLV
	Compact	Diffuse*	Compact	Compact	Compact
Growth Properties					
Tumor incidence	12/12	NT	6/6	6/6	6/6
Growth in soft agar	12/12	NT	6/6	6/6	6/6
Continuous growth in culture	12/12	1/12	6/6	6/6	6/6
Hematopoietic Markers					
Mercaptoethanol dependence	11/12	0/12	6/6	6/6	6/6
Cytoplasmic μ	0/12	0/12	0/6	3/6	2/6
Fc receptor	7/12	12/12	0/6	6/6	4/6
TdT	11/12	NT	5/6	0/6	0/6
Thy-1 antigen	0/12	0/12	1/6	0/6	0/6
Mac-1 antigen	0/12	12/12	0/6	0/6	0/6
Nonspecific esterase	0/12	12/12	0/6	0/6	0/6
Lysozyme	0/12	12/12	0/6	0/6	0/6
Hemoglobin synthesis	0/12	0/12	0/6	0/6	0/6

*Results were obtained directly from diffuse colony cells since a significant number of cell lines were not available.

REFERENCES

Andersen PR, Devare DG, Tronick SR, Ellis, RW, Aaronson, SA, Scolnick EM (1981) Generation of BALB-MuSV and Ha-MuSV by type C virus transduction of homologous transforming genes from different species. Cell 26:129-134

Anderson SM, Scolnick EM (1983) Construction and isolation of a transforming murine retrovirus containing the src gene of Rous sarcoma virus. J Virol 46:594-605

Barbacid M, Beemon K, Devare SG (1980) Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus. Proc Natl Acad Sci USA 77:5158-5163

Bollum FT (1979) Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 54:1203-1215

Durban EM, Boettiger D (1981) Differential effects of transforming avian RNA tumor viruses on avian macrophages. Proc Natl Acad Sci 78:3600-3604

Hankins DW, Scolnick EM (1981) Harvey and Kirsten sarcoma viruses promote the growth and differentiation of erythroid precursor cells in vitro. Cell 26:91-97

Pierce JH, Aaronson SA (1982) BALB- and Harvey-murine sarcoma virus transformation of a novel lymphoid progenitor cell. J Exp Med 156:873-887

Pierce JH, Aaronson SA (1983) In vitro transformation of murine pre-B lymphoid cells by Snyder-Theilen feline sarcoma virus. J Virol 46:993-1002

Pierce JH, Aaronson SA, Anderson SM (1984) Hematopoietic cell transformation by a murine recombinant retrovirus containing the src gene of Rous sarcoma virus. Proc Natl Acad Sci USA (in press)

Reddy E, Smith MJ, Srinivasan A (1983) Nucleotide sequence of Abelson murine leukemia virus genome: Structural similarity of its transforming gene product to other onc gene products with tyrosine-specific kinase activity. Proc Natl Acad Sci 80: 3623-3627

Rosenberg N, Baltimore D (1976) A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. J Exp Med 143:1453-1463

Scher CD, Scolnick EM, Siegler R (1975) Induction of erythroid leukemia by Harvey and Kirsten sarcoma viruses. Nature 256:225

Shih TY, Weeks MO, Young HA, Scolnick EM (1979) p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature-sensitive for the maintenance of transformation. J Virol 31:546-560

Siden E, Baltimore D, Clark P, Rosenberg N (1979) Immunoglobulin synthesis by lymphoid cells transformed in vitro by Abelson murine leukemia virus. Cell 16:389-396

Whitlock CA, Witte ON (1981) Abelson virus infected cells can exhibit restricted in vitro growth and low oncogenic potential. J Virol 40:577-584

Witte OM, Dasgupta A, Baltimore D (1980) Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature 283:826-831

Monoclonal Antibody to Abelson Lymphoma Cells

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INTRODUCTION

The Abelson murine leukemia virus (A-MuLV) is a defective transforming retrovirus that rapidly induces a variety of hematopoietic neoplasms including those of B-cells when injected into mice (reviewed in Risser 1982; Rosenberg and Baltimore 1980). A-MuLV is a genetic recombinant between Moloney MuLV (M-MuLV) and a normal cellular proto-oncogene, c-abl (Goff et al., 1980). The strain of A-MuLV used here encodes a novel gag-abl fusion protein of 160 kd (Goff et al 1981; Grunwald et al 1982). This v-abl product is a phosphoprotein that will phosphorylate itself and other proteins on tyrosine residues in immunoprecipitates (Van de Ven et al 1980; Witte et al 1980; Sefton et al 1981). Using tumor regressor antiserum Witte et al (1979) identified a cellular protein of 150 kd (NCP150) in uninfected cells that is serologically related to the v-abl product; thus NCP150 is a candidate for the c-abl product. Ponticelli et al (1982) demonstrated that NCP150 shares 5 phosphopeptides with v-abl p160. Wang and Baltimore (1983) demonstrated that c-abl mRNA was found in all normal cells and cell lines tested, though in highest quantities in mouse thymocytes and NIH 3T3 cells. We report here the isolation of a monoclonal antibody, 442, which reacts with normal protein(s) that have molecular properties reminiscent of those of the c-abl product or some receptors for growth factors (Cohen et al 1982).

RESULTS

Isolation of Monoclonal Antibody 442

Spleen cells from C57BL/6 mice immune to a syngeneic A-MuLV lymphoma line (Risser et al 1978) were used as normal donor cells in fusions with the NS-1 plasmacytoma line. Hybridomas were screened for antibodies that immunoprecipitate a 160 kd protein from A-MuLV nonproducer cells in the presence of exogenous M-MuLV, i.e. abl-specific antibodies (Witte et al 1979). One antibody, 442, having those properties was recovered, and the hybridoma cells recloned twice by terminal dilution.

Reactivity of Antibody 442 with A-MuLV and Other Cell Lines

To investigate the nature of the epitope recognized by 442 antibody we metabolically labeled p160 v-abl nonproducer cells, p160 v-abl tumor cells or cells from an A-MuLV$^-$ tumor line. Cell lysates were immunoprecipitated with 442 antibody and analyzed by gel electrophoresis (Fig. 1). The results of such experiments, summarized in Table 1, indicated that all cell lines expressed three 442 reactive molecules, one of 160 kd, one of 150 kd, and one of 140 kd. The same three molecules were found in NIH3T3 cells and A-MuLV nonproducer cells expressing variant forms of v-abl. When cell lysates of p160 v-abl containing cells were repeatedly pre-cleared with 442 antibody plus Staph A protein, and then analyzed for the presence of v-abl p160, no decrease in the v-abl p160 was observed, although all 442 reactive proteins had been removed.

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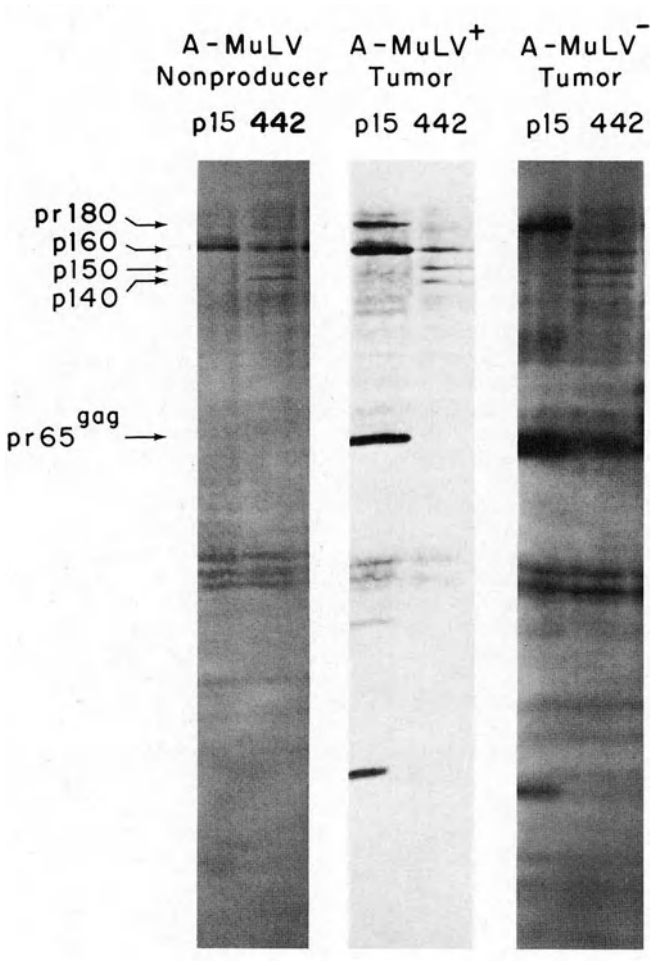

Fig. 1. Immunoprecipitation of 442 molecules from cell lines. The A-MuLV v-abl p160 nonproducer N54, the A-MuLV v-abl p160 lymphoid tumor E2 or the A-MuLV$^-$ lymphoid line 2A was metabolically labeled with ^{35}S-methionine. Cell lysates were precleared with normal mouse serum and S. aureus A protein, and then immunoprecipitated with anti-Moloney MuLV p15 antiserum or 442 monoclonal antibody. Immunoprecipitates were separated electrophoretically and detected autoradiographically.

Therefore, it seems unlikely that 442 antibody recognizes an epitope carried on v-abl p160 or any other v-abl protein.

We also investigated the expression of 442 proteins in thymus, spleen and bone marrow cells of normal mice. We found that 442 proteins p160, p150, and p140 were present in all three cells, and clearly were more readily detected in thymus than in the other two tissues (Table 1). This last observation is reminiscent of NCP 150, which is also more readily detected in thymus than spleen or bone marrow (Witte et al., 1979).

Table 1: Expression of 442 Proteins p160, p150, p140 in Normal Cells and Cell Lines

Positive cell lines and tumors:

NIH 3T3
NIH 3T3 A-MuLV nonproducer expressing v-abl p160, p120, p90 or p105
A-MuLV tumor lines expressing v-abl p160 or p120
A-MuLV$^-$ tumor line

Positive normal cells:

Thymus > spleen, bone marrow

In vivo and In vitro Phosphorylation of 442 Proteins

To determine if 442 proteins were phosphorylated in vivo, thymus cells were incubated with ^{32}P-orthrophosphate, and lysates were examined for expression of 442 proteins. 442 proteins p160, p150 and p140 were each found to be phosphorylated in vivo in mouse thymocytes.

Although no enzymatic activity has been associated with NCP150, it is clear that v-abl p160 will phosphorylate itself in immunoprecipitates (Witte et al., 1980). To determine if any 442 reactive protein could be labeled in vitro, lysates of unlabeled thymus or A-MuLV nonproducer cells were immunoprecipitated with 442 antibody and incubated in Mn^{2+} containing buffer in the presence of γ-^{32}P-ATP. Immunoprecipitates were then analyzed electrophoretically; a prominent band at 140 kd was found in each case. From this we conclude that p140 can be phosphorylated in vitro. It remains to be determined which, if any, of the 442 proteins is the in vitro kinase.

DISCUSSION

The properties described for 442 reactive molecules are reminiscent of those described for receptors associated with some growth factors (Cohen et al., 1982) or cellular or viral oncogenes (Bishop, 1983), particularly c-abl. Further biochemical experiments currently in progress should clarify the nature of 442 proteins and their relation to A-MuLV transformation.

REFERENCES

Bishop JM (1983) Cellular oncogenes and retroviruses. Ann Rev Biochem 52:301-354

Cohen S, Ushiro H, Stoscheck C, Chinkers M (1982) J Biol Chem 257:1523-1531

Goff SP, Gilboa E, Witte ON, Baltimore D (1980) Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell 22:777-785

Goff S, Witte O, Gilboa E, Rosenberg N, Baltimore D (1981) Genome structure of Abelson murine leukemia virus variants: proviruses in fibroblasts and lymphoid cells. J Virol 38:460-468

Grunwald DJ, Dale B, Dudley J, Lamph W, Sugden B, Ozanne B, Risser R (1982) Loss of viral gene expression and retention of tumorigenicity by Abelson lymphoma cells. J Virol 43:92-103

Ponticelli AS, Whitlock CA, Rosenberg N, Witte ON (1982) In vivo tyrosine phosphorylations of the Abelson virus transforming protein are absent in its normal cellular homologue. Cell 29:953-960
Risser R (1982) The pathogenesis of Abelson virus lymphomas of the mouse. Biochim Biophys Acta 651:213-244
Risser R, Stockert E, Old LJ (1978) Abelson antigen: a viral tumor antigen that is also a differentition antigen of BALB/c mice. Proc Natl Acad Sci USA 75:3918-3922
Rosenberg N, Baltimore D (1980) Abelson virus. In: Klein G (ed) Viral oncology. Raven Press, New York, p 187-203
Sefton B, Hunter T, Raschke WC (1981) Evidence that the Abelson virus protein functions in vivo as a protein kinase that phosphorylates tyrosine. Proc Natl Acad Sci USA 78:1552-1556
VandeVen WJ, Reynolds FH, Stephenson JR (1980) The nonstructural component of polyproteins encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associate protein kinase activity. Virology 101:185-187
Wang JYJ, Baltimore D (1983) Cellular RNA homologous to the Abelson murine leukemia virus transforming gene: expression and relationship to the viral sequence. Mol Cell Biol 3:773-779
Witte ON, Dasgupta A, Baltimore D (1980) Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature 283:826-831
Witte ON, Rosenberg NE, Baltimore D (1979) Identification of a normal cellular protein cross-reactive to the major Abelson murine leukemia virus gene product. Nature 281:396-398

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