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STUDIES OF MURINE B CELL DIFFERENTIATION
AFFECTED BY INTERLEUKINS PRODUCED BY T HELPER CELLS
USING CLONAL MODELS

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STUDIES OF MURINE B CELL DIFFERENTIATION AFFECTED BY INTERLEUKINS PRODUCED BY T HELPER CELLS USING CLONAL MODELS

By

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ABSTRACT

STUDIES OF MURINE B CELL DIFFERENTIATION AFFECTED BY INTERLEUKINS USING CLONAL MODELS

By

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The differentiation processes of B cells are influenced by T helper (Th) cells in the immune system, and this interaction is mediated in part by soluble factors such as Interleukins (ILs), multi-functional peptide hormones produced by T cells. Studies using inducible clonal B cell lines were carried out in order to elucidate the underlying mechanisms of the late acting factors from T cells, which are required for B cells to mature into IgM secreting plasma cells. The combined use of inducible clonal cells and recombinant ILs reduced the complexity of the cellular environment for interaction. Differentiation was assayed both at the level of the secretion of the assembled antibody molecules (IgM polymer) and transcription of antibody subunits gene (u_s and J).

The second T cell factor which induces IgM secretion by a murine B cell-type clone BCL_1-3B3,

previously shown to react to IL-2, was identified to be IL-5 in the current study. A mixture of IL-2/IL-5 and, to a lesser extent, IL-2 or IL-5 by themselves induced IgM secretion. Each factor can increase μ_s and J chain mRNA. Thus, the BCL_1-3B3 cells can be stimulated to secrete IgM by a Th-1 factor, interleukin 2 (IL-2), and/or by a Th-2 derived factor, interleukin 5 (IL-5). In addition, these interleukins act together to stimulate IgM secretion to a greater extent than can be induced by either interleukin alone. The IL-2 and IL-5, thus appear to be functionally equivalent relative to their effect on BCL_1-3B3 cell differentiation. The cell-cycle analysis experiments suggest, however, IL-2 and IL-5 appear to differ in their cell cycle dependency for signal transmision. The similarities and differences in the response of the cells to two different ILs may help to elucidate the underlying molecular mechanisms in B cell differentiation. A similar study on another cell line AKR-225 cells has been started, and the preliminary data suggest that the cell line responds to Th-2 factors, IL-4 and IL-5.

For my mother

Hatsuko Takayasu

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 5

Abbreviations

Abbreviations used in this paper: AO, Acridine Orange; AFC, antibody-forming cells; BCDFμ, B cell differentiation factor μ; BCGF II, B cell growth factor II; FBS, fetal bovine serum; IL-2, interleukin 2; IL-5, interleukin 5; 2-ME, beta mercaptoethanol; μ_s, secreted form of μ heavy chain; PC, phosphorylcholine; RIA, radioimmunoassay; SN, supernatant; SRBC, sheep erythrocytes; SSC, sodium chloride/sodium citrate solution; SSPE, SSC with 50 mM NaH_2PO_4; Th-1, type 1 T helper cell; Th-2, type 2 T helper cell.

CHAPTER 1 INTRODUCTION

Antibody production is an orchestrated effort of many types of cells to recognize and eliminate foreign antigens to protect the host. In order to recognize the specific antigens, an expanded repertoire of highly specific receptors for each antigen is necessary. It is also important to expand a selective number of clones with the specific receptor for the antigen as needed. Ehlrich was the first to propose a comprehensive theory of antibody production in 1894 to account for the cellular origin and diversity of antibody (1). It took more than sixty years before a theory of the cellular basis of humoral immune response became available. Jerne proposed in his selective theory of antibody production that the role of antigen was to selectively expand the specific antibodies preexisting in the serum (2). Further, Burnet, in his Clonal Selection Theory, restated Jerne's theory and provided the essence of the current theoretical framework (3). Any antigen (Ag) triggers two reactions in B lymphocytes which have a specific receptor for the antigen, i.e., membrane immunoglobulin (sIg or mIg). One is proliferation of the Ag-specific B cells in a clonal fashion, and the other is the initiation of differentiation of such B cell clones into

plasma cells to initiate the production and secretion of antibodies to the specific antigen.

Cellular cooperation has been recognized as an essential part of the humoral response (4,5), and T and B lymphocytes were identified as the cell types required for antibody formation with distinct functions: T cells as helpers and B cells as the antibody producers (6). T cell help can be recognized as being comprised of at least two steps. The first step is cognate T-B cell interaction through specific Ag to activate resting mature B cells. The second step is completion of the differentiation process of the activated B cells by non-specific soluble factors from T cells originally termed lymphokines (7). As discussed later in detail, a new term interleukin (IL) was introduced when such factors were later physically characterized.

Although a series of reviews on B cell growth and differentiation factors were written around the mid 1980's, there was no coherent view on how each factor contributed to the processes (8-11). There are two reasons for difficulty in attaining complete analysis. First, the factors used in most studies were heterogeneous and the existence of these factors was based only on functional assays. There was little physical characterization done since it was very difficult to obtain purified factors. Second, the cell populations studied were also heterogeneous. The

possibility of non-B cell contamination could not be excluded. There was, indeed, no guarantee that the B cell populations were homogenous.

It was generally accepted that the proliferation and stimulation of terminal differentiation (maturation) of activated B cells into antibody secreting plasma cells were regulated by soluble factors derived from T cells by the early 1980's. It was also thought that the B cell activation process could be divided into a sequential series of independent steps: induction/stimulation, proliferation, and differentiation.

In many reviews, the B cell factors were proposed to be distinguished into three functional categories: the "activation" factor of the resting B cells (BSF), the "growth" factors (BCGF) for the activated B cells, and the terminal "differentiation" factor (BCDF) of the activated B cells. The representative model can be seen in Kishimoto's review in 1988 (11). In this model the sequential progress of the resting B cells differentiating into antibody producing plasma cells is emphasized. The resting B cells are first activated by IL-4 (originally termed BSF), then IL-5 (originally termed BCGF) acts as the growth factor on the previously activated B cells, finally in this model IL-6 (BMF) is the terminal differentiation factor, which induces

the already growing activated B cells to become plasma cells.

In another review by Melchers, a model was suggested that there were three distinct signals controlling the resting B cell's growth and maturation, each acting at a restriction point in the cell cycle (12). The first signal was antigen (Ag) or anti-Ig antibodies acting in the G_0 phase to drive the cells to the G_1 phase. Then, an α factor signal from macrophages acts at the entry into the S phase. Finally, a β factor from helper T cells comes in at the late G_2 phase and the entry into the mitotic phase. Later, the same group examined different ILs using recombinant sources (13). Both interleukin-2 (IL-2) and interleukin-5 (IL-5) were found to be active as β-BCGF (β factor). In addition, both IL-2 and IL-5 were found to act also as a B-cell maturation factor (BMF), which can induce resting B cells to secrete Ig without proliferation, although IL-5 showed 1,000 times more potent BMF activity. In this model, the growth factor and differentiation factor could be separated on the basis of the point of action in the cell cycle.

Although disagreements existed on assigning specific interleukins to be designated as the activating (or stimulating) factor, growth factor or the differentiation factor; the common theme of B cell development was shared by

many groups; that is, the events are sequential, and activated B cells, expressing membrane IgM and IgD on cell surface (phenotype $sIgM^+$, $sIgD^+$, Ia^+), can receive T cell factor signals.

A. Soluble T Cell Factors: Interleukins

Interleukins (ILs) are small proteins which act as mediators of communication between lymphocytes (14-16). The interleukins are a subset of lymphokines, a collective term introduced in 1969 to encompass factors which influence blood cells (leukocytes) (15). They are hormones that allow the cells of the immune system to interact indirectly without physical contact. Subtle differences between the classical hormones, such as insulin and interleukins exist. Interleukins act at short range in the local cellular environment. A number of interleukins are produced by a variety of cell types, whereas the classical hormones are usually produced by specific cell types and tissues.

Cloning of cDNAs encoding interleukins in the 1980s was a major step in clarifying the physical nature of soluble factors previously identified in a variety of bioassays (15, 16). *In vitro* cloning of T cells, which produce a large quantity of factors, helped to isolate cDNA for such

molecules. It was also helpful to have sensitive bioassays for a specific factor, including functional assays for the translated protein product from the cDNA. Molecular biological studies of soluble factors emphasized their role in cell-to-cell communication among leukocytes; hence the term interleukins was introduced. Isolation of cDNAs for interleukins also permitted biochemical studies. The main emphasis was on physical characterization of the specific receptors and studies of signal transduction through ligand-receptor interaction (15). It has become clear that the interleukins, polypeptide hormones produced by immune cells, have a variety of physiological effects on B cells.

There are several features common to all the interleukins isolated to date (15-20). First, they are all secreted polypeptides with heavy glycosylation moiety. The sugar component may account for up to 50 % of the molecular weight of the protein. Second, their functions are pleiotropic - their effects range from growth to differentiation of many types of cells, including lymphocytes. Many ILs share overlapping activities. Thus, the long-held assumption that growth and differentiation are always regulated by different molecular entities has been challenged. The effects of a given interleukin on a given type of cell can be dependent on the developmental stage of the cell receiving the signal. Third, the interleukin

molecules act upon cells through binding specific receptors on the target cell surface. Thus, only cells with proper receptors can respond to a specific interleukin. Cloning of interleukin receptors has revealed that they have multiple subunits with distinct functions. Differential combination of such subunits can create receptors with different affinities. Moreover, some interleukin receptors are now known to share a subunit, which might partially explain their functional redundancy (21-28).

Three interleukins, IL-2, IL-4, and IL-5, are particularly important for the differentiation of "activated" B cells into IgM secreting cells (11, 12, 14). These interleukins are produced by T helper cells, and appear to be involved in the growth and differentiation of B cells.

IL-2 was first identified as a T cell mitogenic factor produced by lectin-activated mononuclear cells. It was first named T Cell Growth Factor (TCGF) for its ability to stimulate the growth of normal T cells *in vitro* (29). For many years, it was considered to be a T cell specific factor. However, it was realized in the early 1980's that IL-2 also has effects on B cells (30, 31). Taniguchi et. al. (32) successfully isolated the cDNA coding for human Interleukin-2 (IL-2) in 1983 aided by a well established bioassay and a human leukemic T cell line which produces a

high amount of IL-2 activity. The mouse cDNA for IL-2 was isolated by cross-hybridization to the human IL-2 cDNA using a cDNA library constructed from a lymphoma cell line (33).

Molecular cloning of cDNAs coding for IL-4 and IL-5 was facilitated by the establishment of a T cell line (2.19 cell) and sensitive, quantitative, and reproducible assays for BCDFγ (IgG_1 Induction Factor), BSF-1, and BCGF-II. (34,35) A well characterized murine B-cell factor, T-cell replacing factor (TRF), was previously classified as a BCDF (induction of IgM secretion), but a partially purified preparation of TRF was suggested to have BCGF II activity (stimulation of thymidine incorporation by the *in vivo* BCL_1 leukemic B-cell line.) The identity of TRF with BCGF II was proven by cloning its cDNA and the name IL-5 was proposed for this lymphokine (36). IL-4 is identical to B-cell stimulating factor-1 (BSF-1), which induces DNA synthesis when given together with anti-IgM antibodies (37-39). IL-4 also induces not only an elevated IgG_1 response in B cells activated by lipopolysaccharide but also hyper-Ia expression in B cells. Furthermore, this lymphokine reveals growth factor activities for both T and mast cells.

Receptors for IL-2, IL-4 and IL-5 (IL-2R, IL-4R, and IL-5R, respectively) belong to the recently classified Class I cytokine receptor superfamily, whose members have multiple subunits, and some share the same signal transducing subunit

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(40, 41). Both IL-2R and IL-4R share a common γ subunit along with IL-7R, IL-9R, and IL-15R. IL-5R shares a common β subunit with IL-3 and GM-CSF. Three receptors were originally identified in the human with different affinity for IL-2, with dissociation constants (kd) on the order of 10^{-11} M, 10^{-9} M, and 10^{-8} M, respectively. They are now known to be comprised of three distinct subunits. The high affinity IL-2 receptor is composed of α, β, γ subunits (IL-2R αβγ), the intermediate affinity IL-2 receptor with β and γ subunits (IL-2R βγ), and the low affinity IL-2 receptor with α subunit (IL-2R α) in human (40). In the mouse, there is no intermediate affinity IL-2 receptor complex, but only high affinity IL-2 receptor (IL-2R αβγ) and low affinity IL-2 receptor(IL-2R α) with dissociation constants (kd) on the order of 10^{-11} M and 10^{-8} M, respectively (41). The three subunits appear to have distinct functions, in the mouse, IL-2 binding appears impossible without the α subunit, and signal transduction without β or γ (41). IL-4R is a hetero-dimer consisting of IL-4Rα and IL-2Rγ (24, 27). IL-5R is also a hetero-dimer consisting of IL-5Rα and IL-5Rβ (28).

With the cloning of cDNA for more than fifteen interleukins, it has become clear that overlapping functions of different ILs is a very common phenomenon. The

multiplicity of factors, each with subtly different actions, obviously provides the opportunity for a highly regulated response. Their interaction may result in numerous consequences to a cell, and the roles of each interleukin are difficult to assign without having a homogeneous cell population, which will be discussed below. It has already emerged that the roles of B cell-active cytokines are considerably more complex than first envisaged. Consequently, the older models of B cell activation have been challenged, and newer models will be appreciated.

One of the most important findings in the pursuit of T helper cell characterization was the discovery heterogeneity in T helper cells, identified as cell surface phenotype of $CD4^+$ and $CD8^-$. In late 1980s, murine T helper (Th) cells were shown to be divided into two subsets: Th-1 and Th-2 (43, 44). This definition is based on the specific interleukins they produce. Th-1 cells produce IL-2, IFNγ, and TNF-β; and Th-2 cells produce IL-4, IL-5, IL-6, and IL-10. The difference in the cytokine profile is thought to reflect biological functions of these two subsets of $CD4^+$ Th cells: Th-1 for the classical cell mediated response and Th-2 for more efficient helper function in the humoral response i.e., B cell activation. Several diseases appear to be related to cytokine expression, such as over expression of

IL-1 and IFNγ with bacterial septic shock. Thus, the dichotomy of the immune system, first identified at the B and T lymphocyte cellular level, appears to be more complex, and each lymphocyte subset may be further divided into layers of subsets.

B. B lymphocyte Subsets and Autoimmunity

More recently, similar to the increasing subdivision of T cells, a question of heterogeneity among B lymphocytes has also arisen. Mouse B lymphocytes can be subdivided into two groups based on their cell surface characteristics: Ly-1(CD-5)$^+$ and Ly-1(CD5)$^-$ (45,46). The mouse Ly-1 molecule is a membrane glycoprotein with a molecular weight of 67,000 daltons (47). The overall homology between the mouse and its human homologue CD5 (T1 or Leu-1) is 63%, and 90% for an intracellular region (48). Ly-1/CD5 was originally considered to be expressed only on mature T cells, however recently it was also found in a limited population of B cells, so called Ly-1/CD5$^+$ B or B-1 cells. Ly-1/CD5$^-$ B cells are now termed conventional B cells. Ly-1/CD5$^+$ B cells differ from the conventional B cells in their lower expression of sIgD, lower frequency in adult animals except in the peritoneal cavity, and their capacity for self-

renewal (49). With this dichotomy in the B cell population, the possibility of different activation requirements of B lymphocyte subsets needed to be addressed.

The origin of Ly-1/CD5$^+$ B cells is a matter of controversy. Herzenberg et. al. (49) believe that there are two distinct progenitors for B lymphocytes, and that Ly-1$^+$ and Ly-1$^-$ B cells represent separate lineages. Earlier evidence supporting this view comes from adoptive transfer experiments of adult bone marrow which were able to reconstitute conventional B cells, but led to poor reconstitution of Ly-1$^+$ B cells. On the other hand, fetal omentum was shown to reconstitute B-1 cells effectively, but not conventional B cells (50). In addition, fetal liver was shown to reconstitute both conventional B cells and B-1 cells (51). Those experiments demonstrated that there are anatomical and ontological differences in B lymphocyte progenitors.

Although the separate lineage hypothesis has considerable experimental support, an alternative view that antigen contact may influence the expression of Ly-1 on B cells can not be excluded. An alternative differentiation pathway hypothesis suggests that Ly-1$^+$ B cells result from a particular type of antigen encounter during B cell development. Wortis et. al. (52) proposed that T-cell-independent type 2 (TI-2) antigen, but not T-cell-dependent

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(TD) or T cell-independent type 1 (TI-1) antigen, contact induces surface expression of Ly-1/CD5 on a newly differentiated B cells. Evidence for this view comes from experiments in which *in vitro* treatment of splenic resting B cells (Ly-1$^-$) with F(ab') fragments of anti-IgM, an analogue of TI-2 antigen, leads to a cell-surface expression of Ly-1/CD5$^+$ (52). In contrast, treatment with monovalent Fab fragment of anti-IgM and irradiated T helper cells, representing TD antigen, did not induce CD5$^+$ B cells. In addition, human CD5$^-$ cells isolated by flow cytometry were shown to become CD5$^+$ cells in the presence of EL-4 cells, suggesting the flexible phenotype for CD5 marker (53).

The role of the newly identified Ly-1/CD5$^+$ B cells is still unclear, but their involvement in autoimmune disorders has been suggested. The current view of autoimmune disease is that it represents failure of immunological tolerance to self-antigens, i.e., some self-reactive lymphocytes escape clonal deletion. Spontaneous autoimmune disease in New Zealand Black (NZB) and F_1 hybrid of NZB and New Zealand White (NZW) mice, (NZB X NZW)F_1, is associated with lymphoproliferative disorders, and the animals have been used as a spontaneous autoimmune animal model. These mice have increased numbers of Ly-1/CD5$^+$ B cells in both the spleen and the peritoneal cavity compared to normal (non-autoimmune) strains (45, 54). Ly-1/CD5$^+$ B cells appear to

be involved in production of IgM autoantibodies against a variety of self antigens including Igs, erythrocyte membranes, and denatured DNA in such animals.

An alternative hypothesis for a few B-1 cells escaping self antigen, thereby escaping clonal deletion, by residing in a privileged environment of peritoneal cavity in which self antigen was absent is supported by a more recent experiment using transgenic mice. Transgenic mice expressing anti-erythrocyte self antigen were created (55). About half of the transgenic mice exhibited an autoimmune hemolytic anemia, and selective escape from self antigen of peritoneal B cells was demonstrated in such animals. Injection of red blood cells into the peritoneal cavity of autoimmune hemolytic transgenic mice were shown to result in a rapid death of B-1(Ly-1/$CD5^+$) cells, and concurrent clearance of autoimmune symptoms of the animals. The experiment demonstrated that escape of clonal deletion by peritoneal cavity B-1(Ly-1/$CD5^+$) cells was not an inherent characteristic, but rather the result of a privileged environment in which the self antigen was absent.

With discovery of the dichotomy of B lymphocytes with different cell-surface phenotypes, i.e., Ly-1/$CD5^+$ vs. Ly-1/$CD5^-$ B cells, several classical views on B cell development have been questioned. First, the question of the origin of such B cell subsets has not been fully

answered. Second, the existence of several differentiation pathways for B lymphocytes induced by different types of antigen, i.e., TD, TI-1, and TI-2 Ag, will need to be further investigated. Finally, the association of IgM autoantibody production by Ly-1/CD5$^+$ B cells with autoimmunity suggests the importance of further investigation. In addition, animal models might be useful to continue to increase our knowledge of immuno-tolerance, as well as providing effective strategies for prevention or treatment of human autoimmune disorders. Thus, it is important to understand the origin and properties of the newly identified Ly-1/CD5$^+$ B cells.

C. Inducible B Cell Clones as In Vitro Models

It is useful to simplify a system in order to elucidate the mechanisms underlying complex biological phenomena. One way to achieve such simplicity is to limit the variables by reducing the background noise. Studies with B-cell tumor models have advanced the field of molecular immunology in the past three decades. An understanding of gene rearrangement as the underlying mechanism for the creation of the variety of receptors leading to many antigen specificities has been aided by availability of several

tumors representing different stages of normal B cell development (56). However, one of the limitations of such a frozen phenotype as in the tumor models was recognized when investigating the role of T cell factors on B cell development.

The isolation of inducible B cell lines that can mimic a normal differentiation process *in vitro* was an important step forward in studying B cell activation. Investigation of how each of the T cell soluble factors might act directly on a B cell to influence its maturation into Ig secreting cells is difficult without such cell lines. The complexity of function of interleukins and the heterogeneity of the B cell population added to the difficulty of such investigation. Inducible clonal models would have been useful, and several such *in vitro* B cell model clones were isolated.

The first such *in vitro* inducible B cell clone was the BCL_1-3B3 cell line generated in 1983 by Brooks et. al. (57) from an *in vitro* clone of the BCL_1 tumor. The BCL_1 tumor line was isolated from a spontaneous murine B-cell leukemia derived from and passaged in BALB/c mice; it was the first B cell line to arise spontaneously, unlike the earlier carcinogen or virus induced tumors (58). The cell surface markers expressed on the tumor were shown to be similar to a subset of normal B lymphocytes: IgM^+, IgD^+, Ia^+, and FcR^+

(59). *In vitro* adapted BCL_1 cells were originally shown to proliferate and differentiate to secrete IgM in response to lipopolysaccharide (LPS) (60, 61).

The *in vitro* BCL_1-3B3 cells were selected for their capacity to secrete IgM in the presence of a variety of T cell supernatant(SN) from pK7.1 and EL-4 (57). The *in vitro* BCL_1-3B3 cells were soon shown to differ from the *in vivo* tumor in their cell surface marker expression of Ly-1, and also its ability to become an IgM antibody forming cell (AFC) in the presence of IL-2 (62, 63). Both were responsive to partially purified BCDFμ, a factor presumably distinct from BCGF, IL-2, TRF, and IFNγ. BCL_1-3B3 cells were further shown to respond to EL-4 SN, which contain BCDFμ activity, in a cell cycle dependent manner (64). IgM secretion was shown to parallel the concurrent increase of μ_s mRNA intracellularly under culture conditions of lower serum concentration and no 2-ME in the growth media, in the presence of EL-4 SN (57). Under different assay conditions, in which 2-ME was present and different sources and higher concentrations of fetal calf serum was utilized in the media, J chain mRNA but not μ_s mRNA was shown to parallel IgM secretion (65).

The second inducible B cell clone was isolated independently; this was the CH12.LX cell line from an *in*

vivo CH12 tumor line isolated from a tumor from B10.H-2^a H-4^b p/Wts (2^a4^b) mice after extensive immunization with sheep red blood cells (sRBC) (66-68). The cell line was introduced as "resting cell like" as both T help and Ag, sRBC, were required to stimulate the cells to secrete IgM. The cell surface marker phenotypes were sIgM$^+$ and Ly-1$^+$, similar to BCL_1-3B3 cells (68). Later, the cells were shown to be EL-4 responsive similar to BCL_1 cells, and induction of IgM secretion paralleled the increase of μ_s mRNA (69). Although the CH12.LX line did not respond to IL-2, the cells were shown to express IL-2Rα, and binding of a monoclonal Ab (3C7) against IL-2Rα could induce IgM secretion (70). Inhibitors of proliferation, mitomycin and hydroxyuria, also induced secretion of IgM from the cells, suggesting an inverse relationship between proliferation and differentiation. CH12.LX cells were shown to secrete IgA in response to IL-5 (71).

The recently isolated AKR-225 cells are unique in the sense of the origin of the cells; this cell line is Ly-1$^-$ (Lyt-1$^-$) unlike the other inducible B cell clones. The spontaneously arising 225 lymphoma was first isolated from a male AKR/J mouse. The *in vitro* line of the AKR 225 clone was isolated by Brooks et. al. (72) after selection of *in vitro* adapted cells for both a low level of spontaneous IgM

release and an increase in IgM secretion in the presence of T cell-derived lymphokines (EL-4 SN). The cell surface phenotypes were shown to be $sIgM^+$, $sIgD^+$, IgG^-, Ia^+, and $Ly\text{-}1^-$, similar to typical B cells after activation. Characterization of the AKR-225 cell line will be part of this research, and will be discussed in a later chapter.

D. Gene Expression during B cell differentiation

We have chosen expression of two genes, Cμ and J chain, as indicators of the intracellular events that occur during the differentiation process of "activated" B cells. IgM is the initial isotype produced when B cells become Ig secreting plasma cells upon an antigen encounter. The secreted form of IgM appears mostly as pentamers of Cμ gene products bound to one molecule of the J chain (IgM_5-J) (73, 74). The Cμ and J chain genes appear to be developmentally regulated at the mRNA level (75). Immunoglobulin (Ig) genes are expressed specifically in cells of the B lymphocyte lineage, as early as the pre-B cell stage (76-78). Transcription of the J chain gene is more restricted and it appears to occur later in mature B lymphocytes (79).

Immunoglobulins are composed of light (L) and heavy (H) chains, each consisting of variable (V) and constant (C)

regions. V regions are responsible for antigen binding, and associate with different C regions that exert various effector functions such as complement fixation. The V-region repertoire of the immunoglobulin heavy chain is shared by the classes and subclasses: IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, IgE, and IgA.

Studies using B cell lineage tumor cells and normal B cells have indicated two species of the IgM (Hμ): the secreted form and membrane form (80). The membrane-associated form and the secretory form of μ heavy chain proteins are specified by mRNAs that are transcribed from a single gene which contains alternative exons for the membrane form and the secretory form at the 3' end (81). The correlation between the secretory state of the cell and the ratio of RNA for the membrane from of the Hμ chain (μ_m mRNA) to mRNA for the secretory form of the Hμ chain (μ_s mRNA) have been well established (76, 82). Indeed, in BCL_1-3B3 B lymphoma cells, which may be stimulated by a B cell differentiation factor to become an IgM secretor, the induction of μ_s mRNA was observed (62).

Very early in B cell development (pre-B cell stage), the heavy chain gene becomes transcriptionally active as soon as a productive gene rearrangement has been achieved (83). Synthesis of μ chain before light chain expression is

a normal event in the early differentiation of the B-cell line (84). The levels of heavy chain gene transcripts increase more than 30 fold in plasma cell stage from pre-B stage (83). The rates of Ig heavy chain transcription, however, differ only by a factor of less than 5 between these cell types, and most of the quantitative difference was found to be caused by post-transcriptional regulation. (82, 85)

The differential expression of mRNAs encoding membrane bound (μ_m) and secreted (μ_s) forms produced from a single transcript could be regulated at several levels. Those include (i) transcription termination, (ii) by competition of the splicing site during alternative splicing, and (iii) mRNA stability (86). When the concentration of μ mRNA in cell lines representing the pre-B, B and plasma cell stages were compared, variation in the steady state level of the mRNA during differentiation was observed, and this was attributed to differences in μ mRNA stability (87). A similar rate of transcription was observed. The transcripts were stable for at least 1 hr in B cells and more than 8 hr in various plasma cells. More accurate measurements of half-life of μ mRNA was made by Mason et. al. (88) using two cell lines each representing B cells and plasma cells. Estimates of the half-life in plasmacytomas was between 12 and 20 hr, whereas half-lives of 2 to 4 hr were observed in

the B-cell lines, and the stability of the μ mRNA was proposed to be aided by the expansion of the secretory apparatus in plasma cells.

The J chain is incorporated only in the polymeric immunoglobulins, IgM and IgA (89, 90). Unlike the heavy and light chains, the J chain does not contribute to antibody specificity. Moreover, the J chain does not belong to the Ig superfamily based on the primary sequence analysis (91, 92). Unlike the complex organization of the heavy and light chain Ig genes, sequence analysis reveals that the J chain is a simple gene with 4 exons over about 7 kb of DNA (93). Its functional importance is suggested by the high level (74%) of homology between the mouse and human genes. There appear to be multiple transcripts of the J chain in the nucleus ranging from 1.4 to 7.3 kb, however, there is only one mature mRNA of 1.6 kb in the cytoplasm (94).

In a review on the J chain, Koshland summarized the studies on the J chain from its early recognition in 1970 to the late 1980's (95). When the murine pre-B cell line, WEHI 231, and five plasmacytoma lines (including hybridomas of WEHI) were tested for J chain specific mRNA, only plasmacytomas were shown to express the J chain mRNA (75). Later, when the mouse cell lines representing progressive stages of B cell differentiation were analyzed, the correlation of the expression of both μ and J chain mRNA

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with IgM secretion was apparent (79). It was also apparent that μ chain protein proceeded the J chain in the cytoplasm. Contradictory to the mouse studies, however, expression of the J chain appears to be initiated earlier in B cell development in humans without immunoglobulin in the cytoplasm (96), and was proposed to perform a general function.

The function of the J chain is still a mystery. Throughout the 70's and the early 80's the J chain was thought to be essential for the polymerization of IgM and IgA. The transcellular transport of polymeric IgA and IgM was proposed to depend on the presence of the J chain (95). However, the role of the J chain is not clear in the intracellular movement of IgM. IgA secretion appears independent of polymerization, since both monomeric and dimeric forms of the secreted IgA exist in normal lymphoid tissues. Moreover, non-lymphoid cells can assemble and secrete polymeric IgM without J chain when co-transfected with the genes for light and μ heavy chains (97).

Two forms of IgM polymer, pentamer (IgM_5) and hexomer (IgM_6), have been identified in mouse and human as well as other species (95). There is direct evidence that J chain is not required for IgM polymerization. Neuberger et. al. (98) reported a rat glioma cell line co-transfected with a mouse μ heavy chain and a λ light chain genes which secreted

polymeric IgM without J chain. More recently, J chain was shown to be required only for pentameric IgM synthesis (99). J chain negative cell lines were shown to secrete IgM_6, and when they were transfected with J chain gene, the cells predominantly expressed IgM_5.

Our understanding of how secretion of IgM polymer may be achieved in B lymphocytes is still incomplete. Regulation of gene expression of J and μ chain genes has not been fully understood, either. IL-2 was shown to increase only μ_s mRNA, and not J chain mRNA in human leukemic B cells (100). Human precursor B cells, originally not expressing J chain nor μ_s mRNAs, were shown to express J chain mRNA without μ_s expression when transformed by Epstein-Barr virus (101). Thus, the induction of μ_s and J mRNA may not occur simultaneously in human B cells. However, in mouse, it appears that gene expression of both μ_s and J chain genes are regulated more closely during B cell development, suggested by studies utilizing B cell tumors representing progressive stages of differentiation discussed earlier. Thus, our choice of specific molecular markers to follow intracellular events were induction of both μs and J chain mRNAs. Are these genes controlled by the same factor similarly, or are they regulated separately during B cell differentiation process? This question was addressed in the following research.

E. Specific Research Goals

Our long term research goal was to study the process of differentiation of activated B cells using *in vitro* inducible clonal models. We were particularly interested in the interaction of T cells indirectly through soluble factors such as interleukins, with activated B cells causing them to obtain a terminally differentiated state, in which they secrete IgM.

With identification of T helper cell subsets, Th-1 and Th-2, with different interleukin secretion patterns, a question of how these subsets might influence B cells became important. Many investigators have reported the critical importance of the Th-2-derived lymphokines, IL-4 and IL-5, in supporting antibody responses to thymus-dependent (TD) antigens (102-105). In contrast, Th-1 cells apparently play a key role in cell-mediated immune responses (106). On the other hand, both IL-2 and IL-5 are known to stimulate B cell proliferation and immunoglobulin (Ig) production (102, 103, 107-111). These observations have raised questions concerning the physiological relevance of such redundancy.

In addition to identification of Th subsets, B cells can be divided into two subsets: B1(Ly-1/CD5$^+$) and conventional (Ly-1/CD5$^-$) cells. A question of how the Th subsets may interact with each B cell subset became important. There are two hypothesis, Separate Lineage vs.

Different Activation, regarding the origin of the newly identified Ly-1/CD5$^+$ cells. As evidence of the existence of several activation pathways for B lymphocytes by different antigens (TD, TI-1, and TI-2) accumulates, the possibility arises that the courses of each B cell subset after Ag activation might differ further. Our hypothesis was that each B subset may have different requirements for activation including interaction with different Th factors to become IgM secretors.

Our two model systems, BCL_1-3B3 cells and AKR-225 cells, were found to differ in their cell surface phenotype of Ly-1/CD5 expression and their responsiveness to different T cell SNs, and IL-2 as shown in Table 1 and Table 2 (57, 62, 72). The cell surface phenotypes of the BCL_1-3B3 cells and the AKR-225 cells are summarized in Table 1. The BCL_1-3B3 cells express high levels of surface IgM and low levels of surface IgD, and Ia antigens. The BCL_1-3B3 cells also express IL-2Rα and Ly-1/CD5. Similar to the BCL_1-3B3 cells, the AKR-225 cells express high levels of surface IgM and low levels of surface IgD, Ia antigens, and IL-2Rα. But, the AKR-225 cells differ from the BCL_1-3B3 cells in the surface phenotype in that they don't express Ly-1/CD5.

Responsiveness of BCL_1-3B3 and AKR-225 clones to T cell SNs and IL-2 are summarized in Table 2 (62, 72). The BCL_1-3B3 cells are responsive to both EL-4 SN and PK 7.1 SN, and

Table 1 Surface Phenotypes of BCL1-3B3 and AKR-225 Clones

	% Positive cells for				
	sIgM	sIgD	Ia	Ly-1	IL-2Rα
BCL_1-3B3	94	15	63	+	++
AKR-225-11	67	15	88	-	+

Table 2 Responsiveness to T cell SNs, IL-2, and IFNγ of BCL_1-3B3 and AKR-225 Clones

	EL-4	PK 7.1	IL-2	IFNγ
BCL_1-3B3	+++	+++	++	-
AKR-225	-	+++	-	n.d.

to IL-2. The AKR-225 cells are responsive to PK 7.1 SN but not to EL-4 SN, and not to IL-2. A list of lymphokine activities known to be present in the T SNs at the time when the present research was initiated is shown in Table 3 (72). These cell lines with different responsiveness to T cell factors might be useful models to further investigate and compare possible activation requirements of Ly-1/CD5$^+$ and Ly-1/CD5$^-$ B cells. Recombinant sources of ILs (IL-2, IL-4, IL-5) have been utilized in order to clarify each factor's role separately. In short, new approaches have been taken to elucidate the roles of interleukins on the differentiation of two B cell subsets.

It has been found that AKR-225 cells, representative of Ly-1$^-$ B cells, are Th-2 factor responsive; namely, IL-4 and IL-5 and that the BCL_1-3B3 cells, representative of Ly-1$^+$ B cells, are responsive to both Th-1 and Th-2 factors: IL-2 and IL-5. Molecular events during the process of differentiation of the 3B3 cells have also been further examined using cDNA probes for J chain and IgM heavy chain. Further, the BCL_1-3B3 cells and recombinant sources of IL-2 and IL-5 were used to study the similar and different roles of these interleukins on Ly-1$^+$ B cell activation and differentiation. In the present studies, the functional similarities of IL-2 and IL-5 were revealed, as well as subtle differences in their actions during the cell cycle.

Table 3 Source of T Cell-derived Lymphokines

	IL-2	TRF (IL-5)	IFNγ	BCGF (IL-4)	BCDFμ (IL-5)	BCDFγ (IL-4)
PK 7.1	-	-	-	+	+	+
EL-4	+	?	-	+	+	+

CHAPTER 2: MATERIALS AND METHODS

Reagents.

Recombinant human interleukin 2 (IL-2) and interleukin 6 (IL-6) were obtained from Amgen (Thousand Oaks, CA). Recombinant mouse interleukin 4 (IL-4) was obtained from Genzyme (Boston, MA). Recombinant human interleukin-1 (IL-1) was obtained from Cistron (Pine Brook, NJ). For all commercial lymphokines, concentrations are expressed as defined by the source. The source of recombinant interleukin 5 (IL-5) was supernatant (SN) from the transfected line X63.mIL5 described by Karasuyama et al. (1). This cell line was the kind gift of Dr. T. Honjo, Kyoto, Japan. The culture supernatant of the parent X63-Ag8-653 cell line was used as a control. For all sources of SN from cell lines, the concentrations were expressed as v/v%. Crude T cell supernatants derived from the EL-4 thymoma (EL-4) and Th-2 clone D10.G4.1 (D10) line were used for comparison with mixtures of recombinant interleukins. The phorbol ester-induced SN from EL-4 contains IL-2, IL-4, IL-5, GM-CSF, and IFNγ activities. The ^{3}H-thymidine was obtained from Amersham (Arlington Heights, IL).

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The media used in these studies was RPMI 1640 (M.A. Bioproducts) supplemented with l-glutamine, nonessential amino acids, Na pyruvate, 5 X 10^{-5} M 2-mercaptoethanol (2-ME) and antibiotics as previously described (2). Fetal calf serum (Hyclone laboratories, Logan, UT) was added to final concentrations of 3-10% v/v.

Maintenance of Cells

Maintenance of BCL_1-3B3 Cells.

The BCL_1-3B3 cells were cultured in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with l-glutamine, nonessential amino acids, sodium pyruvate, $5x10^{-5}$ M 2-mercaptoethanol and antibiotics as previously described (2). Fetal bovine serum was added to a final concentration of 3-5% v/v. For normal maintenance of the BCL_1-3B3 line, the cells were cultured in 5% FBS-RPMI 1640 media containing the above supplements at 37°C in a 6% CO_2 atmosphere. The cells were diluted 1:3 to 1:5 every 3-4 days.

Maintenance of AKR-225 Cells.

The AKR-225 cells were cultured in RPMI 1640 (M.A. Bioproducts, Walkersville, MD) supplemented with l-glutamine, nonessential amino acids, sodium pyruvate, $5x10^{-5}$

M 2-mercaptoethanol and antibiotics as previously described (2) Fetal bovine serum (Hyclone laboratories, Logan, UT) was added to a final concentration of 10% v/v. For normal maintenance of the AKR-225 cell line, the cells were cultured in 3-10% FCS-RPMI 1640 media containing the above supplements at 37°C in a 10% CO_2 atmosphere. The cells were diluted 1:5 to 1:10 every 3-4 days.

Maintenance of X63-Ag8-653 cells.

The X63-Ag8-653 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml of penicilin-steptomycin, 2 mM L-glutamine and 5 X 10^{-5} M 2-mercaptoethanol (2-ME) as described in Karasuyama et al (1).

Preparation of supernatant (SN) from EL-4 and D10.G4.1 cells

Supernatant was collected 48 hr after induction with PMA. The 85% saturated ammonium sulfate (SAS) fraction of supernatant was dissolved in 10% of its original volume and was dialyzed against RPMI-1640.

Cell-cycle Synchronization and Cytofluorometric Analysis.

Two types of cell-cycle synchronization procedures were used. Cells were cultured in excess thymidine (1 mM) for a

time period equivalent to the doubling time minus 8 hours. The cells were washed and cultured in fresh media for 8-10 hours before the thymidine was again added. Two or three cycles of thymidine blockade were used. The second method involved culturing the cells in isoleucine-deficient media for time period equivalent to about 1.5 X doubling time. At the completion of each of these procedures an aliquot of cells was prepared for analysis of the RNA and DNA content using acridine orange (AO). The cell pellet, containing up to 10^6 cells was resuspended with 0.5 ml of buffer containing 0.1% Triton-X-100, 0.2 M sucrose, 10^{-4} M EDTA and 2 x 10^2 M citrate phosphate buffer, pH 3.0. An additional 0.5 ml of buffer containing 0.1 M NaCl, 10^{-2} M citrate phosphate buffer, pH 3.8, and 1% of a 2 mg/ml solution of AO in water. The cells were then analyzed on an Ortho 50H cytofluorograph.

Differentiation Assays

Differentiation Assays on BCL_1-3B3 cells.

The BCL_1-3B3 cells were washed and resuspended at 1-2x10^5 viable cells per ml in 3% FBS-RPMI media supplemented with glutamine, nonessential amino acids, antibiotics and sodium pyruvate. Beta-mercaptoethanol (2-ME) was not added

to the media for the differentiation assays unless explicitly stated. The deletion of 2-ME from the media slows the proliferation rate of the cells resulting in reduced spontaneous IgM secretion and higher levels of IL-induced IgM secretion. Typically after 4-6 days at 37°C in 6% CO_2, the cell supernatant was harvested and the IgM concentration determined using a solid-phase radioimmunoassay (RIA). Standard curves using purified myeloma or hybridoma proteins were included in each assay. For kinetics studies, the cell supernatant was harvested as early as 12 hr post induction.

Differentiation Assays on AKR-225 cells.

The AKR-225 cells were washed and resuspended at 1-2 X 10^5 viable cells per ml in 10% FBS-RPMI media supplemented with 2-ME, Na pyruvate, nonessential amino acids, l-glutamine, and antibiotics. After 4 days at 37°C in 6% CO2, cell supernatant was harvested and assayed for IgM concentration using radioimmunoassay (RIA).

Proliferation Assays

Proliferation Assays on BCL_1-3B3 cells and AKR-225 cells..

The BCL_1-3B3 cells were washed twice and resuspended at 1-2x10^5 viable cells per ml in 3% FBS-RPMI media,

supplemented with glutamine, nonessential amino acids, antibiotics and sodium pyruvate, in 96 well microtiter plates (200 µl per well). Beta-mercaptoethanol (2-ME) was not added to the media for the proliferation assays unless explicitly stated. The deletion of 2-ME from the media slows the proliferation rate of the cells resulting higher levels of IL-induced. The cultures were pulsed with 1 µCi/well of ^{3}H-thymidine and harvested 18 hours later.

RNA Isolation.

Cytoplasmic RNA was isolated from the cultured cells using guanidine hydrochloride (guanidine-HCl) as described in White et al. (3) and Cheley and Anderson (4), and the RNA was further purified by extracting with double distilled water (ddH_2O) (5) followed by standard sodium acetate/ethanol precipitation. The concentration of the RNA was determined by spectrophotometry. The RNA was separated by electrophoresis in a 1.2% formaldehyde-agarose gel, and transferred to a nitrocellulose filter. RNA size was estimated from the ethidium bromide staining of an RNA ladder (purchased from BRL, Gaithersburg, MD), run in the same gel.

DNA labeling and Northern analysis

The DNA probes used in this study are plasmids: p-μ-12, a murine cDNA containing part of cμ2, cμ3, and cμ4) (6, a gift of Dr. R. C. Schwartz, Michigan State University), and Jc21, encoding J chain (7, a gift of Dr. M. E. Koshland, University of California, Berkeley). A 1.3 kb cloned cDNA encoding glyceraldehyde-3-phosphate-dehydrogenase (8) as well as ethidium bromide staining of ribosomal RNA was used to monitor the RNA content of each lane.

Plasmid DNAs were labeled with Â-^{32}P-dCTP (3000 Ci/mmol) by random priming to a specific activity of 4 X 10^8 cpm/μg. Hybridization was carried out in 50% formamide/5X SSPE at 43°C for 15 hr as described by Beltz et al (9). The final wash was at 60°C with 0.1X SSC for 1 hr. The film was exposed at -70°C with an intensifying screen.

IL-2 Receptor Characterization

The binding of ^{125}I-labeled IL-2 to AKR-225-11, BCL_1-3B3, and CTLL-2 cells were measured according to the methods of Robb et al. (10) with slight modifications. Cells were washed extensively with HBSS. In addition, CTLL-2 cells were incubated for 1 hr at 37°C to remove endogenous IL-2. After washing, cells were resuspended in RPMI 1640 supplemented with 1% BSA, Na azide, and 25 mM Hepes, pH 7.2.

Cells (1 X 10^6) were incubated with serial dilutions of ^{125}I-labeled IL-2 in a total volume of 150 µl at room temperature for 1 hr. Maximum binding was observed by 30 min and did not decline with up to 90 min of incubation. The cell suspension was centrifuged through a 200 µl layer of 1M sucrose-HBSS for 4 min at 12,000 X g. The tips of the tubes containing the cell pellet were cut off and their radioactivity was determined in a γ-counter. The specific binding of ^{125}I-labeled IL-2 was calculated by substracting the nonspecific binding in the presence of a 50-fold excess of unlabeled IL-2.

CHAPTER 3: BCL_1-3B3 CELLS

Introduction

Cell-cell interaction has been recognized as an essential part of antibody formation for more than two decades (1,2). Specifically, T cell help for an antibody response to most antigens is minimally required at two points in B cell differentiation. Cognate T-B interaction mediated through antigen presentation to the T cell receptor initiates B cell proliferation and T cell lymphokine production (3-5). Subsequently, these T cell-derived lymphokines augment B cell proliferation as well as control the differentiation process (6-9). Mosmann et al (10) have subdivided murine T helper cells into type 1 T helper (Th-1) cells and type 2 T helper (Th-2) cells according to the interleukins they produce. Th-1 cells uniquely produce IL-2 and IFNγ whereas Th-2 cells secrete IL-4 and IL-5. With the molecular cloning of interleukins 1 through 10 as well as other cytokines, it has become apparent that the interleukins can have overlapping or closely related functions. For example, both IL-2 and IL-5 are known to stimulate B cell proliferation and immunoglobulin (Ig) production (6-9, 11-14). These observations have raised questions concerning the physiological relevance of such

redundancy. Numerous investigators have reported the critical importance of the Th-2 derived lymphokines, IL-4 and IL-5, in supporting antibody responses to thymus-dependent antigens (9,11,14,15). In contrast, Th-1 cells clearly play a key role in supporting cell-mediated immune responses (16). In addition, there is increasing evidence of lymphokine-mediated cross-regulation between Th-1 and Th-2 cells. IFNγ can inhibit the actions of IL-4 (17-18); whereas IL-10 released by Th-2 cells and B cells can inhibit the secretion of IFNγ by the Th-1 cells (19). Relative to the B cell, it is presently unclear to what extent Th-1 derived IL-2 and Th-2 derived IL-5 perform similar functions during B cell differentiation.

The frequency of normal cells responsive to IL-2 and IL-5 depends on the nature of the primary stimulus and the anatomical derivation of the B cells. Splenic B cells bind and proliferate in response to IL-2 only after activation by stimuli such as lipopolysaccharide plus anti-immunoglobulin (6). Comparably, optimal responses by splenic B cells to IL-5 require preactivation with the mitogen dextran sulfate (20). Peritoneal B cells show a somewhat different response pattern, secreting IgM in the presence of IL-5 alone without *in vitro* costimulation (21,22).

Recent evidence suggests that the peritoneal B cells which respond to IL-5 belong to a unique B cell subset

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Recent evidence [illegible] B cells which respond [illegible]

(21,22). The complete phenotype of this subset is still being elucidated but many of its members express the Ly-1/CD5 surface marker (23-25). These data raise the question of whether all B cells are equally responsive to lymphokines such as IL-2 and IL-5, whether responsiveness depends on the nature of preceding activation signals, or whether B cells of a particular lineage are selectively responsive to Th-1 versus Th-2 derived lymphokines.

An ideal method to approach these questions would be to stimulate clonal B cells with each of the lymphokines in question. Until recently, virtually all clonal B cell lines were transformed B cells which either secreted Ig spontaneously or were refractory to lymphokine-mediated differentiation signals. The isolation of a lymphokine-responsive B cell line, BCL_1-3B3, which secretes IgM in the presence of T cell culture supernatant has been previously reported (26). BCL_1-3B3 cells can be induced to secrete IgM by a variety of T cell SN with a concurrent increase in μ_s mRNA. The factor responsible for this differentiation process was named B cell differentiation factor μ (BCDFμ). BCDFμ was originally defined by its ability to induce IgM secretion by the *in vivo* BCL_1 line (27). The lymphokine possessing BCDFμ activity was subsequently cloned by Kinashi et al. (28) and termed interleukin-5 (IL-5). During purification of BCDFμ, it was noted that some of the T cell

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culture supernatants contained an additional factor which was capable of inducing IgM secretion by the *in vitro* BCL_1-3B3 clone, but not the *in vivo* BCL_1 line. This additional lymphokine was identified as IL-2 (29).

In this chapter, the roles of IL-2 and IL-5 in stimulating IgM secretion will be evaluated using recombinant sources of the ILs. The BCL_1-3B3 cells used in this study provide a clonal B cell target for these lymphokines, thus eliminating the heterogeneous effects of the lymphokines observed when primary B cells are used. In addition, these neoplastic B cells are spontaneously proliferating which allows us to focus on the specific role of each lymphokine in the differentiation process without excessive effects from proliferative signals the lymphokines might provide. We have determined that IL-2 and IL-5 independently induced IgM secretion with concurrent increases in μ_s and J chain transcripts. However, the actions of IL-2 and IL-5 did not appear to be completely identical. Cell cycle arrest of the cells followed by a 12 hr pulse with each interleukin suggested that the reception of the signals from IL-2 and IL-5 does not occur as efficiently at all phases of the cell cycle. The receipt of the IL-5 signal appeared to be optimal in late G_1 phase, whereas IL-2 stimulation appeared to be more effective in S and G2 phase. Under the optimal differentiation condition

in the 3%FCS-RPMI-1640 media without 2-ME, IL-2 and IL-5 appeared to act synergistically.

Results

EL-4 SN induces a higher level of IgM secretion by BCL_1-3B3 cells than an optimal concentration of IL-2.

In order to confirm the presence of an additional factor(s) that can induce IgM secretion of the BCL_1-3B3 cells, we first compared the activity of EL-4 SN and recombinant IL-2 (IL-2) at various concentrations. As can be seen in Figure 1, EL-4 SN could induce more IgM secretion than the optimal concentration of IL-2 at 20 U/ml. IL-2 can induce the IgM secretion optimally at 10 to 20 U/ml, and at least 5% v/v EL-4 SN can induce the IgM secretion to a level two to three fold greater than that seen with IL-2.

This result was consistent with the fact that EL-4 SN contains other factors besides IL-2 that can act on BCL_1-3B3 cells to induce IgM secretion.

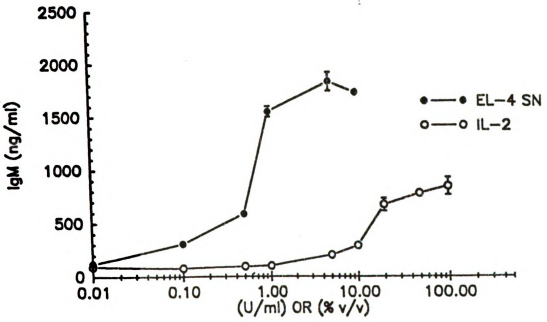
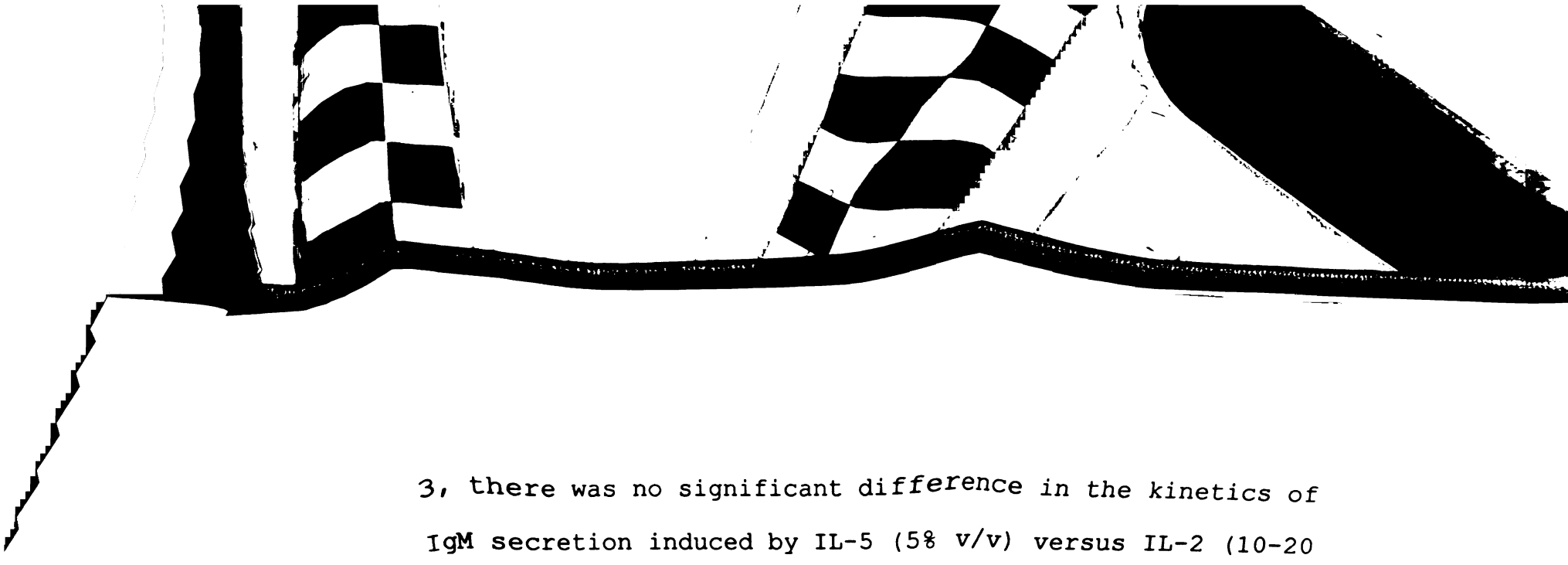


Figure 1. Differentiation response of BCL1-3B3 cells to EL-4 SN and IL-2. BCL1-3B3 cells were cultured at 2 x 10^5 cells/ml in 3%FCS-RPMI-1640 media lacking 2-mercaptoethanol (2-ME). The EL-4 SN concentration were 0.1, 0.5, 1, 5, and 10 %v/v. Recombinant IL-2 (IL-2) concentration were 0.1, 0.5, 1, 5, 10, 20, 50, and 100 U/ml. The concentration of IgM was assayed on day 5 by RIA. This data is representative of 4 additional experiments.

IL-5 can stimulate BCL_1-3B3 cells to secrete *IgM*

BCL_1-3B3 cells can be induced to differentiate into IgM antibody-forming cells by supernatant (SN) from various T cell lines (26) which contain a B cell differentiation factor, initially named BCDFμ (27). Subsequently, it was determined that IL-2 was one of the T cell-derived factors responsible for IL-induced IgM secretion by BCL_1-3B3 cells (29). In 1986, Honjo and colleagues cloned a T cell replacing factor (TRF) and demonstrated that this interleukin, termed IL-5, possesses the ability to induce both proliferation and differentiation of *in vivo*-derived BCL_1 cells (28). Thus, IL-5 was the molecule responsible for both B cell growth factor II (BCGF II) and BCDFμ activity. Thus, these studies suggested that both IL-2 and IL-5 independently induce IgM secretion by BCL_1-3B3 cells. Therefore, the response of BCL_1-3B3 cells to IL-2 and IL-5 was examined next. The optimal concentrations of IL-2 and IL-5 were determined with an assay of IgM levels on Day 6. Figure 2 shows the effect on IgM secretion of optimal IL-2 (10 U/ml), IL-5 (5% v/v), as well as the combination of IL-2 and IL-5. IL-5 can induce the IgM secretion by itself, and appears to augment differentiation induced by IL-2.

Since the IgM concentration was measured only on Day 6 in the above experiment, we next examined the kinetics of the response to the interleukins. As can be seen in Figure



3, there was no significant difference in the kinetics of IgM secretion induced by IL-5 (5% v/v) versus IL-2 (10-20 U/ml) at optimal concentrations previously determined from day 1 to day 6. In addition, the effect produced by the combination of the interleukins was always greater than IL-5 alone, and IL-5 induced more IgM secretion than IL-2 alone.

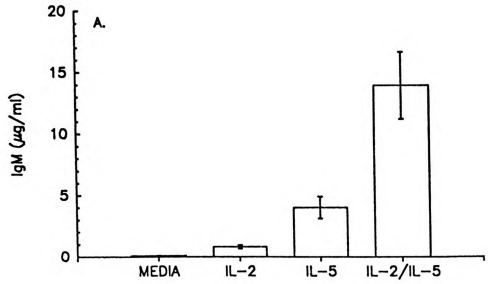


Figure 2. Differentiation response of BCL1-3B3 cells to rIL-2, rIL-5, and rIL-2/ rIL-5. BCL1-3B3 cells were cultured at 2 x 10 5 cells/ml in 3%FCS-RPMI-1640 media lacking 2-mercaptoethanol (2-ME). The concentration of rIL-2 was 10 U/ml and rIL-5 containing SN was used at 5 % v/v. The IgM concentration in the culture supernatant was assayed on day 6 by RIA. The results are representative of three additional experiments.

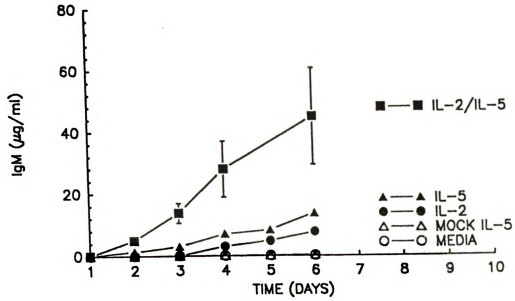


Figure 3. Kinetics of IgM secretion induced by IL-2 and IL-5. BCL_1-3B3 cells were cultured for 6 days in media only (MEDIA) or the presence of either SN from the untransfected X63 line (MOCK IL-5), IL-5 containing X63.mIL-5 SN (IL-5), IL-2 (IL-2) or both IL-2 and IL-5 (IL-2/IL-5). The concentrations of MOCK IL-5 and IL-5 were 2.5% v/v, and IL-2 at 20 U/ml. The concentration of IgM in the culture supernatant on days 1-6 was determined by RIA. This experiment is representative of the data obtained from five independent experiments.

Similar results were observed when a plaque assay, which measures the frequency of cells producing IgM, was used rather than a radioimmuno assay (RIA) on Day 3 of culture. In Table 1, the effects of IL-2 and IL-5, in media containing 3% FCS with 2-ME, were measured by both RIA and plaque assay. Results of IgM secretion measured by RIA and the frequency of cells secreting IgM measured by plaque assay paralleled.

Thus, IL-2 and IL-5 were shown to independently induce IgM secretion by BCL_1-3B3 cells, and the effect of the combination of IL-2 and IL-5 was found to be at least additive in the growth maintenance media.

a
l
p
I
b
a
c
d

Table 1. Ability of BCL_1-3B3 Cells to Become IgM-Plaque Forming Cells in the Presence of IL-2 and IL-5.

Stimulus[a,b]	PFC/10^6 cells	IgM/ml[c]
MEDIA	875	226
IL-2	6,125	1,142
MOCK-IL5	2,750	589
IL-5	5,500	1,162

a) BCL_1-3B3 cells were cultured for 3 days in 3% FCS-RPMI-1640 media with 2-mercaptoethanol (2-ME) only (MEDIA) or the presence of either SN from the untransfected X63 line (MOCK IL-5), IL-5 containing X63.mIL-5 SN (IL-5), or IL-2 (IL-2).
b) The concentrations of MOCK IL-5 and IL-5 were 2.5% v/v, and IL-2 at 20 U/ml.
c) The concentration of IgM in the culture supernatant on days 3 was determined by RIA.

IL-2 and IL-5 have similar effects on J chain and μ_s steady-state mRNA levels.

J chain gene expression appears to be related to the state of differentiation of B cells, normally occurring only during the latter stages of B cell differentiation (38). There is also clearly an increase in μ_s mRNA levels during B cell differentiation (26). We therefore examined the ability of IL-2 and IL-5 to regulate these structural genes for the IgM polymer.

Using culture conditions identical to those employed in Figures 1 through 3, the cells do not proliferate extensively except in the presence of IL-2 or IL-5 (data not shown). IL-2 and IL-5 each upregulated both J chain and μ_s cytoplasmic mRNA (Figure 4). The pattern for regulation of the μ_s and J chain mRNA appeared to parallel kinetically that for IgM secretion as shown in Figure 3. For both μ_s and J chain mRNA, levels after IL-5 stimulation were greater than that observed with IL-2 stimulation, however maximal levels were observed with the IL-2/IL-5 mixture. The drop in the RNA expression (μ_m, μ_s, and J chain) for day 4 in the presence of IL-2 and IL-5 (IL-2/IL-5) was due to low loading of the RNA. (This was confirmed by the similar drop of rRNA bands in the ethidium bromide stained gel before the RNA transfer, data not shown.) Cells treated with untransfected X63 SN (Mock IL-5) expressed predominantly μ_m mRNA for at

least the three days measured, and IgM secretion was minimal as determined by RIA (data not shown).

To determine if IL-induced changes in the proliferation rate were influencing the induced mRNA levels, the experiment was repeated in media containing 2-ME. This media supports optimal proliferation of BCL_1-3B3 cells and thus the effect of each interleukin on proliferation is minimized (data not shown). Under these culture conditions, i.e. 3% FCS media containing 2-ME, IL-2 and/or IL-5 upregulated both J chain and μ_s transcripts in the cytoplasm (Figure 5). In the absence of exogenous interleukins, the cells expressed predominantly μ_m mRNA, and the expression of the μ_s and the J chain mRNA was minimal. In contrast, interleukin-induced expression of both genes paralleled increases in IgM in the SN (data not shown).

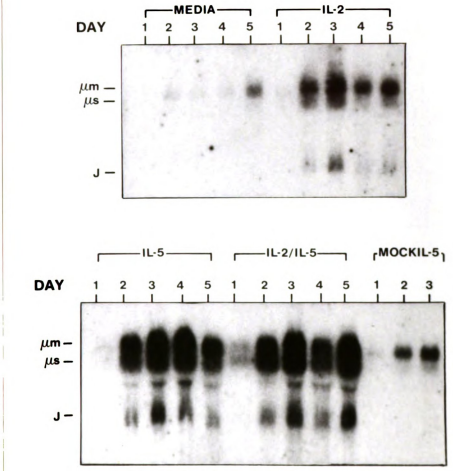


Figure 4. Northern analysis of μ_m, μ_s, and J chain in the presence of IL-2, IL-5, and IL-2/IL-5 mixture in 3% FBS-RPMI without 2-mercaptoethanol. Cytoplasmic RNA was isolated from the whole cultures 1~5 days after culture initiation with 2 x 10^5 cells/ml, except for untransfected X63 SN-treated cells for which only first 3 days are shown. The RNA was separated in 1.2% formaldehyde/agarose gel, and transferred to nitrocellulose. The filter was hybridized with p-μ-12 and pcJ21 probes.

Fig

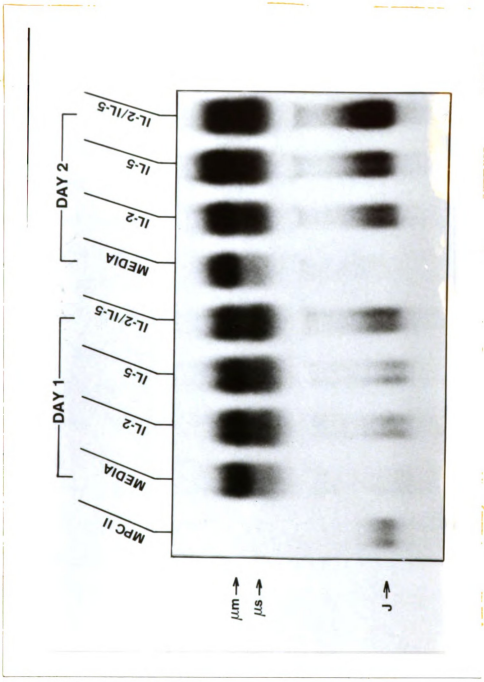


Figure 5. Northern analysis of μ_m, μ_s, and J chain in the presence of IL-2, IL-5, and IL-2/IL-5 mixture (IL-2/IL-5) in 3% FBS-RPMI containing 2-mercaptoethanol (2-ME). For days 1 and 2, 10 μg of cytoplasmic RNA from each culture was loaded on the gel. RNA from MPC 11 cells was used as a positive control for J chain mRNA. RNA was separated in 1.2% formaldehyde/agarose gel, and transferred to nitrocellulose. The filter was hybridized with p-μ-12 and pcJ21 probes.

Optimal Stimulation with IL-5 and IL-2 appears to occur at different points in the cell cycle.

Since under most circumstances B cell differentiation occurs after the cell has entered the cell cycle, we next asked if there is any cell cycle regulation of the B cell's response to IL-2 or IL-5. Two methods were used to synchronize the cells: excess thymidine and isoleucine deficient media. The efficiency of the cell cycle arrest was verified by acridine orange (AO) analysis for each experiment. Synchronized cells were pulsed for 12 hr (the minimum pulse time that consistently induced IgM secretion; data not shown) from 0-12 hr after release from the cell cycle block or 12-24 hr after the release.

First, the effect of IL-2 and IL-5 on excess thymidine synchronized cells was examined. This point in the cell cycle was chosen because previous studies had indicated that EL-4 SN containing both IL-2 and IL-5 induced maximal differentiation when provided during S and G_2 of the cell cycle (15). As can be seen in Figure 6, cells were arrested at the G_1/S border and continued through the cell cycle after the release from the block. A high percentage of the cells were in S phase at 6 hours and had returned to G_1 by 12 hr after the release.

When the effect of the ILs on IgM secretion was assessed (Figure 7), there appeared to be an increase in the

efficiency of IL-5 stimulation with the 12-24 hr pulse (i.e., from a stimulation index on day 4 of about 3-fold to 8-fold). During the 12-24 hr pulse period most of the cells were in the G_1 phase (Figure 6). In contrast, IL-2 appeared to be slightly more effective (a change in stimulation on day 4 from a 4.5-fold increase versus about a 3-fold increase), if provided at 0-12 hours when the cells were in the S and G_2 phases (Figure 6). IL-5 could also stimulate differentiation when added at 0-12 hr but the level of stimulation was about equivalent to that obtained with IL-2. Thus, the enhanced ability of IL-5 to induce IgM secretion when compared to IL-2 seemed to correlate with stimulation received during the G_1 phase of the cell cycle.

The effect of ILs on cells deprived of isoleucine, which arrests the cells in the G_1, was also examined. AO analysis revealed that most of the cells were in the G_1 phase after the treatment (Figure 8), and remained so even 36 hr after isoleucine was provided. There was, however, a gradual increase in RNA content from 12 to 24 hours after the release from the block (Figure 8, Panel B). As seen with thymidine synchronization, IL-5 provided a more effective induction signal during the 12-24 hr pulse period than IL-2 (Figure 9).

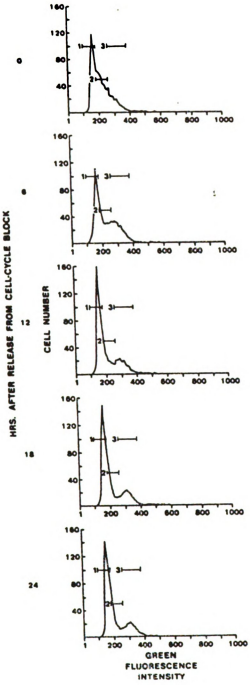


Figure 6. Cell cycle analysis of the BCL_1-3B3 cells after thymidine block. Cells were blocked at the G_1/S border by the double thymidine method. Cells were harvested at T=0, 6, 12, 18, and 24 hr after reculture in 3% FBS-RPMI-1640 media without 2-mercaptoethanol. In each histogram, regions 1, 2, 3 refer to G_1, S, and G_2/M phase of the cell cycle, respectively, as indicated by DNA content (green fluorescence).

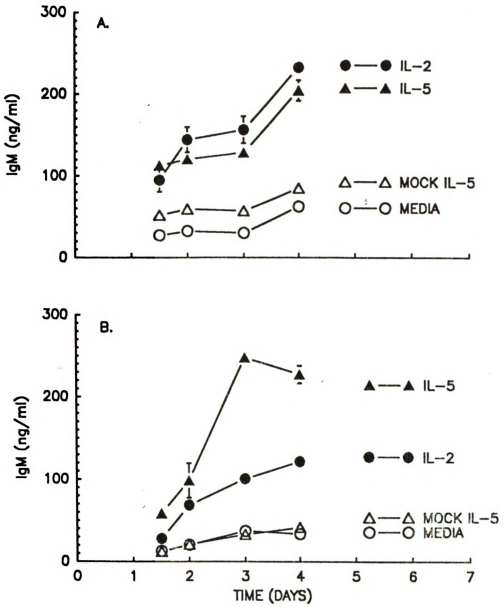


Figure 7 Effect of IL-2 or IL-5 stimulation 0-12 hr or 12-24 hr after release from a G_1/S cell cycle block on IgM secretion. In panel A, excess thymidine synchronized BCL_1-3B3 cells were pulsed from 0-12 hr of culture with IL-2 (20 U/ml) or IL-5 (2.5% v/v X63.mIL-5 SN). The controls included media only and untransfected X63 SN (Mock IL-5). In panel B, addition of IL-2 or IL-5 was delayed 12 hrs and washed out at 24 hr. The IgM concentration in the culture SN was determined by RIA over a 4 day time period. This data is representative of that obtained from four experiments.

In four experiments, the stimulation seen with IL-5 given at 12-24 hours (late G_1) was 26.4 ± 5% of that observed with continuous stimulation whereas when IL-5 was given at 0-12 hours (early G_1) the IgM concentration reached only 11.1 ± 3% of that obtained by continuously stimulated controls. On the other hand, IL-2 given at 0-12 hrs (early G_1) induced an IgM level 12.2 ± 5% of that obtained with continuous stimulation and when given at 12-24 hrs (late G_1) the level was comparable at 13.3 ± 4%. In fact, only marginal stimulation was observed using either IL when the cells were in early G_1 (Fig. 9, Panel A).

Thus, the synchronization experiments using excess thymidine and isoleucine-deficient media indicated that the IL-5 signal was most efficient when given during G_1 (Fig. 7) and in particular late G_1 (Fig. 9). In contrast, the IL-2 signal induced the highest levels of IgM secretion when given during S and G_2 of the cell cycle, although the differences observed were marginal.

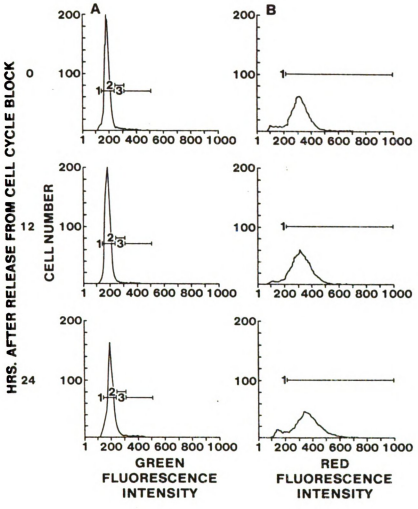


Figure 8 Cell cycle analysis of the BCL_1-3B3 cells after the isoleucine block. Cells were blocked in G_1 by culturing the cells in isoleucine deficient media for 36 hours. The cells were harvested at T=0, 12, and 24 hr after reculture in complete media. In panel A of each histogram, regions 1, 2, 3 refers to G_1, S, and G_2/M phase of the cell cycle as indicated by the DNA content (green fluorescence). In panel B, red fluorescence represents the RNA content of the cells, the mean of which were 335.8, 335.5, and 385.4 at T=0, 12, and 24 hr, respectively.

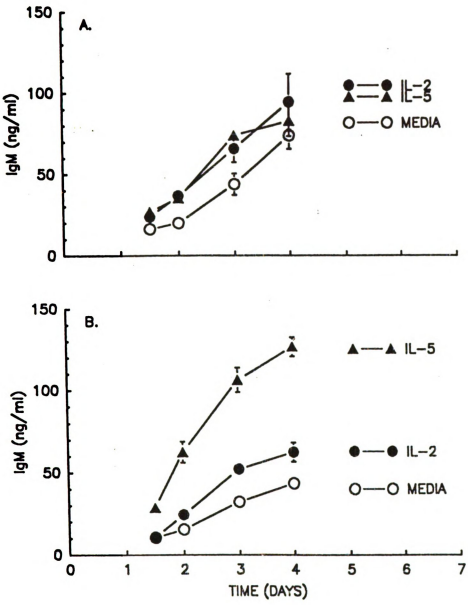


Figure 9 Effect of IL-2 or IL-5 stimulation 0-12 hr or 12-24 hr after release from an early G_1 cell cycle block. In panel A, BCL_1-3B3 cells which had been synchronized by culture in isoleucine deficient media were cultured in isoleucine-containing media only (MEDIA) or pulsed with IL-2 at 20 U/ml (IL-2) or 2.5% v/v X63.mIL-5 SN containing IL-5 (IL-5) for 12 hr. In panel B, addition of IL-2 or IL-5 was delayed 12 hr and the cells were washed and recultured 24 hr after culture initiation. IgM concentration was determined by RIA from 36 hr to 4 days after culture initiation. The data shown is representative of four experiments.

Since the enhanced responsiveness to IL-2 during S and G2 as opposed to G1 was not as dramatic as the cell-cycle-dependent differences we observed with IL-5, Scatchard analysis was used to compare high affinity IL-2R expression on unsynchronized versus cells sysnchronized at the G_1/S border with excess thymidine (Fig. 10). The unsynchronized cells had an average of 944 ± 261 receptors/cell with a K_d of 7.3 X 10^{-11} M. Whereas cells synchronized at the G_1/S border expressed 2175 ± 298 receptors/cell with a K_d of 8.0 X 10^{-11} M. Thus the number of high affinity receptors increased at least two fold at the G_1/S border, a result which is consistent with the increased IL-2 responsiveness of the BCL_1-3B3 cells during the S and G_2 phases of the cell cycle.

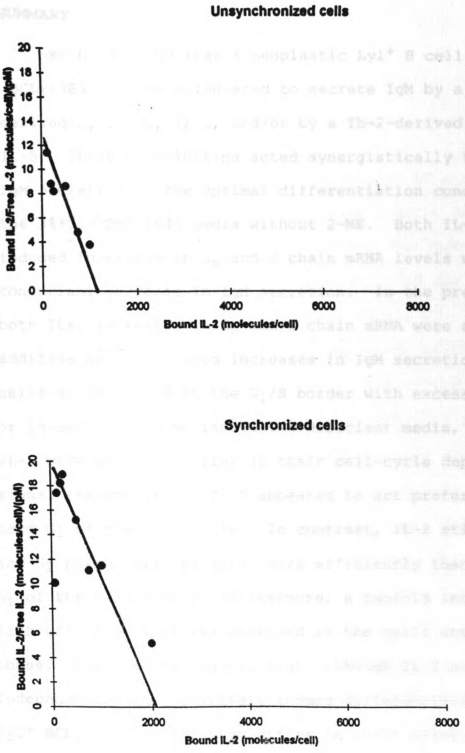


Figure 10 Scatchard analysis high affinity IL-2R expression by unsynchronized and G1/S synchronized BCL1-3B3 cells. The data shown are representative of five experiments.

SUMMARY

We have found that a neoplastic Lyl^+ B cell clone (BCL_1-3B3) can be stimulated to secrete IgM by a Th-1-derived cytokine, IL-2, and/or by a Th-2-derived cytokine, IL-5. These interleukins acted synergistically to enhance IgM secretion in the optimal differentiation condition in the 3%FCS-RPMI-1640 media without 2-ME. Both IL-2 and IL-5 induced increases in μ_S and J chain mRNA levels with concurrent increase in IgM secretion. In the presence of both ILs, increases in μ_S and J chain mRNA were at least additive and paralleled increases in IgM secretion. Using cells synchronized at the G_1/S border with excess thymidine or in early G_1 using isoleucine-deficient media, IL-2 and IL-5 were shown to differ in their cell-cycle dependency for signal transmission. IL-5 appeared to act preferentially in late G_1 of the cell cycle. In contrast, IL-2 stimulated S and G_2 phase cells slightly more efficiently than cells in G_1 of the cell cycle. Furthermore, a twofold increase in high affinity IL-2R was observed as the cells entered S phase. The results suggest that although IL-2 and IL-5 can independently and additively induce differentiation of the Lyl^+ BCL_1-3B3 cells, they differ in their point of action during the cell cycle.

CHAPTER 4. THE AKR-225 CELLS

Introduction

In this chapter, the characterization of a second inducible model system will be discussed using AKR-225 cells cells as a Ly-1$^-$ B cell representative. The AKR-225 cell-line had been adapted *in vitro*, and selected for the ability to secrete IgM in response to T cell SN (1, 2). The AKR-225 cells were derived from a spontaneous tumor which arose in the AKR strain of mice. An *in vitro* line of the AKR-225 lymphoma was generated by alternate passages *in vitro* and *in vivo* and was established after three *in vitro* cycles. Clones capable of differentiating in response to T cell-derived lymphokines were selected by limiting dilution culture with a feeder layer of 10^5 irradiated *in vitro*-adapted AKR-225 cells. This feeder layer was employed because the *in vitro*-adapted line could not be maintained at concentrations below 10^3 cells/ml and feeder layers of splenocytes or thymocytes were found to be inadequate. Selected clones were subcloned twice with a cloning efficiency of 52%. Cells were chosen for further subcloning if they demonstrated both a low level of spontaneous IgM

release and a significant increase in IgM secretion in the presence of T cell-derived lymphokines (EL-4 or PK7.1 SN).

The cell surface phenotype of AKR-225 cells is similar to that of BCL_1-3B3 cells, and can satisfy the operational definition of an activated B cell (1,2), i.e. $mIgM^+$, $mIgD^+$, and Ia^+. In contrast to BCL_1-3B3, AKR-225 cells are $Ly\text{-}1^-$, express kappa light chain instead of λ, and lack high affinity IL-2R, but are Tac^+ ($IL\text{-}2R\alpha^+$). The surface phenotype of the representative *in vitro* clone, AKR-225-11, is shown in Table 1 in Chapter 1. All the clones expressed high levels of mIgM and Ia antigens and lacked mIgG, Ly-1, and Ly-2 antigens. Four of the six clones expressed relatively low levels of mIgD while the other two expressed mIgD at easily detectable levels.

In addition to PK 7.1 SN, which lacks IL-2 activity, previous studies demonstrated that SN of an antigen-dependent Th-2 type D10 clone consistently induced IgM secretion by AKR-225 cells (2). Furthermore, a Th-1 derived lymphokine IL-2 could not induce IgM secretion by the AKR-225 (1,2). In this chapter , the roles of Th-2 derived lymphokines, IL-4 and IL-5, in stimulating IgM secretion will be evaluated. Preliminary results suggest that IL-4 and IL-5 independently induced IgM secretion. Gene expression of the uninduced cells in cell maintenance media

demonstrated that the cells express predominantly μ_m RNA, but not μ_s RNA or J chain RNA.

RESULTS

IL-4 and IL-5 can induce IgM secretion of AKR-225 cells

The AKR-225 cells secrete little IgM in the growth media. The cells were found to be induced to secrete IgM by **SN from EL-4,** PK 7.1, and D10 cells (data not shown). D10 SN consistently induced secretion of IgM in a concentration dependent manner whereas the response to EL-4 SN was less marked and quite variable between the experiments. The EL-4 thymoma produces a variety of lymphokines including both IL-2 which is produced by Th-1 cells as well as IL-4 and IL-5 which are produced by Th-2 cells (3-5). In contrast, the D10 line is an antigen-dependent Th-2 clone (6). The unresponsiveness of the AKR-225 cells to IL-2 was also confirmed (data not shown).

Since SN from the D10 line contains both IL-4 and IL-5, it was possible that only one of these lymphokines was required to stimulate IgM secretion. To determine the optimal ratio of IL-4 and IL-5, IL-4 and IL-5 were tested at various concentrations independently and in combination. In Table 1, various concentrations of rIL-4 (0,, 2.5, 5.0, 10, 20, 100, 500, 1000 U/ml) and rIL-5 (0, 0.125, 0.25, 0.5, 1.0, 2, 4, 10 %v/v) were added to the culture, and IgM secretion was measured on Day 4. When either IL-4 and IL-5 were used alone to stimulate the AKR-225-11 cells, only

modest IgM secretion was observed. Maximum stimulation of IgM secretion was noted with a concentration of 100 U IL-4/ml and 4% v/v X63.mIL-5 SN (IL-5). In addition, when both IL-4 and IL-5 are present and concentrations of IL-4 below 100 U/ml were used, no consistent enhancement of IgM secretion was observed. However, at a concentration of 100 U/ml of IL-4 and concentrations of 1-4 %v/v of IL-5, the level of IgM secreted was greater than that observed with either IL alone.

In order to confirm the release of IgM was not due to cell death, the frequency of the IgM secreting cells was examined by plaque assay. As can be seen in Table 2, a synergistic response to IL-4 and IL-5 was observed at concentrations of 100 U/ml and 5-10% v/v respectively.

Table 1 Concentration Optimums for IL-4 and IL-5 on AKR-225 Cells Differentiation.[a]

IL-4[b] / IL-5[b]	0	2.5	5.0	10	20	100
0	4.9	17.5	10.7	18.0	41.0	52.4
0.125	3.7	9.4	11.9	15.2	36.4	52.0
0.25	5.8	14.7	14.4	8.9	40.0	73.3
0.5	8.7	12.2	14.9	19.1	36.4	72.9
1.0	25.2	24.4	17.3	32.1	57.0	122.7
2.0	22.5	33.0	34.9	38.3	80.0	123.3
4.0	33.6	43.7	35.9	60.5	73.1	134.2

IL-4[b] / mock IL5[b]	0	2.5	5.0	10	20	100
0	4.9					52.4
1.0	0.0					54.8
2.0	0.0					41.3
4.0	2.4					63.2

a) The AKR-225 cells were cultured at 10^5 cells/ml and IgM concentration (ng/ml) was measured on Day 4 with RIA.
b) IL-4 concentration is expressed as Unit/ml and IL-5 v/v and mock IL-5 concentrations are expressed as % v/v.

TABLE 2. Ability of AKR-225 to become IgM Plaque-Forming Cells in the presence of both IL-4 and IL-5.

	PFC/10^6 cells	
Stimulus[a]	EXP 1.	EXP 2.
Media	5	0
Mock IL-5 [b]	3	0
IL-5 [b]	95	175
IL-4 [c]	nd[d]	525
IL-4[c]/IL-5 [b]	1,450	5,275

a) AKR-225 cells were cultured for 3 days in 10%FCS-RPMI-1640 media with 2-mercaptoethanol (2-ME) only (Media) or the presence of either SN from the untransfected X63 line (Mock IL-5), IL-5 containing X63.mIL-5 SN (IL-5), IL-4 (IL-4), or combination of IL-4 and IL-5 containing X63.mIL-5 SN (IL-4/IL-5).
b) The concentrations of Mock IL-5 and IL-5 were 4 % v/v.
c) The concentration of IL-4 was 100 U/ml.
d) Not determined.

RNA status of the uninduced AKR-225 cells along with other B related cell lines.

The cytoplasmic RNA from the cells, Kalc, 3B3, D133, AKR-225 in low (3%) serum media, and AKR-225 in high (10%) serum media, were analyzed for the specific gene expression of the structural component of IgM genes: μ (Figure 1) and J (Figure 2) chains. A house keeping gene (GAPDH) expression was also measured to ensure equal loading (Figure 1). Kalc and D133 cells represent the pre-B and spontaneous secretor stage, respectively. The expression of both μ and J chain mRNAs seen with these cell lines was consistent with those previously reported results. That is, Kalc cells only express μ_m, and do not express μ_s or J chain mRNA; whereas, the D133 cells express all three mRNAs. The amount of μ mRNA appears to correlate with the concentration of the IgM secreted from the cells.

The result from a parallel RIA assay showed that the D133 secretes more IgM than 3B3 cells. 3B3 cells and D133 cells express both μ_m and μ_s; but the ratio of the membrane to secreted form differs in these cells. It appears that the spontaneous secretor D133 cells express both forms equally, and 3B3 cells express more membrane form (μ_m) mRNA than secreted form (μ_s) mRNA.

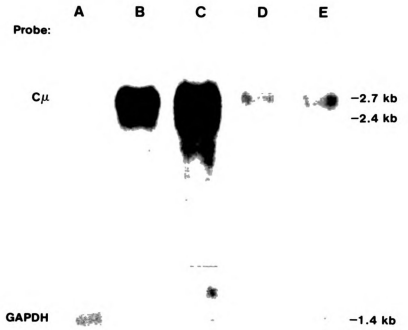


Figure 1. Expression of μ and GAPDH mRNA by AKR-225 cells. 5 μg each of cytoplasmic RNA from A) Kalc, B) D133, C) BCL_1-3B3, D) AKR-225 in low serum media, and E) AKR-225 in high serum media, respectively. The RNAs were separated in 1% agarose Formaldehyde-MOPS gel, and transferred to nitrocellulose filter. The filter was first hybridized with p-μ-12 probe and later with pGAPDH.

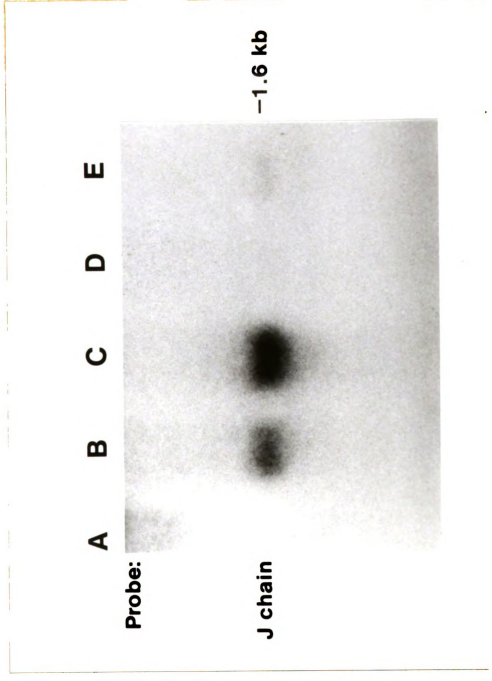


Figure 2 Expression of J Chain mRNA by AKR-225 cells. 20µg each of cytoplasmic RNA from A) Kalc, B) D133, C) BCL_1-3B3, D) AKR-225 in low serum media, and E) AKR-225 in high serum media, respectively. The RNAs were separated in 1.2% agarose Formaldehyde-MOPS gel, and transferred to nitrocellulose filter. The filter was and hybridized with pc21 probe.

As shown in Figure 1 and Figure 2, the AKR-225 cells appear not to express much of μ nor J chain transcripts compared to other cell lines. The AKR-225 cells express predominantly μ_m mRNA at both low and high concentration of serum. Only a slight expression of J chain gene was observed at higher concentration of serum (10%), but not at low serum concentration (3%). These observations are consistent with the lack of spontaneous IgM secretion from this cell line.

Summary

In the present chapter, the second neoplastic murine cell line, AKR-225 cells, was introduced as a Ly-1$^-$ representative. AKR-225 cells was found to be selectively responsive to Th-2 specific factors for differentiation into IgM secreting plasma cells. Specifically, an IL-4/IL-5 mixture and, to a lesser extent IL-4 or IL-5 by themselves, can induce the differentiation. Although an additive or slightly synergistic response was observed when IgM secretion was measured, a clearly synergistic effect was observed in terms of frequency of the cells which produces IgM as determined by a plaque assay.

Although the molecular studies were not as comprehensive as those using BCL_1-3B3 cells, some preliminary data were obtained. Northern analysis of cytoplasmic RNA from the uninduced cells demonstrated that the cells predominantly express mRNA encoding the membrane form of μ. Expression of mRNA for the secreted form of μ and J chain was not observed in these cells at low serum concentration, and a slight expression of J chain was observed at high serum concentration. Thus, the unstimulated AKR-225 cells expressed predominantly μ_m mRNA; there was little detectable μ_s or J chain mRNA.

CHAPTER 5 SUMMARY AND DISCUSSION

In the current research, two B cell clones, BCL_1-3B3 and AKR-225, have been utilized to elucidate the responsiveness of phenotypically distinct B cell subsets to specific T helper cell factors. With identification of T helper cell subsets, Th-1 and Th-2, with different interleukin secretion patterns, the question of how these subsets might influence B cells became important. The difference in cytokine profile was thought to reflect biological functions of these two subsets of $CD4^+$ Th cells: Th-1 cells for classical cell mediated response and Th-2 cells for more efficient helper function in the humoral response. In addition, with discovery of the dichotomy of B lymphocytes with cell-surface phenotype, i.e., $Ly\text{-}1/CD5^+$ vs. $Ly\text{-}1/CD5^-$ B cells, the possibility of existence of several differentiation pathways for B lymphocytes has arisen. The major difference between $Ly\text{-}1^+$ BCL_1-3B3 and $Ly\text{-}1^-$ AKR-225 clones was that the BCL_1-3B3 cells were responsive to a Th-2 derived lymphokine IL-2 and the AKR-225 cells were not. The question of whether $Ly\text{-}1^+$ B cells preferentially respond to Th-1 lymphokines and $Ly\text{-}1^-$ B cells preferentially respond to Th-2 lymphokines was asked using the representative clones.

It was found that BCL_1-3B3 cells and AKR-225 cells have different requirements for interleukins during their

differentiation. BCL_1-3B3 cells respond to both Th-1 and Th-2 factors, specifically IL-2 and IL-5. The effects of IL-2 and IL-5 appear to be very similar in that they both stimulate proliferation and IgM secretion. In addition, when simultaneously present, these effects are at least additive. With both ILs, stimulation of IgM secretion was much greater than stimulation of proliferation. The response to IL-2 and IL-5 was similar with increases of IgM μ_s and J chain genes mRNAs which paralleled increases in IgM secretion. However, cell cycle analysis suggested that IL-2 and IL-5 may act at slightly different points during the cell cycle.

On the other hand, AKR-225 cells responded only to Th-2 factors, namely IL-4 and IL-5, and not to IL-2, a Th-1 factor, to become IgM secreting cells. Both IL-4 and IL-5 were required to induce IgM secretion by AKR-225 cells. This IL-2Rα positive line does not express detectable high affinity IL-2 receptors (1). BCL_1-3B3 does express high affinity IL-2 receptors at a density of approximately 950 receptors per cell and is capable of differentiating into an IgM secreting cell after stimulation with either IL-2 or IL-5. Besides the expression of the p75 IL-2 receptor β chain (IL-2Rβ) and the IL-2Rγ and the use of λ light chains by BCL_1-3B3, the only other known phenotypic difference between

the AKR-225 clone and BCL_1-3B3 is the expression of Ly-1 by BCL_1-3B3. Whether there is linkage between the expression of the IL-2Rβ and the IL-2Rγ and Ly-1 and, in addition, whether some B cell subsets are restricted in their capacity for interaction with the two types of T helper cells can only be determined when additional clones become available for study.

The BCL_1-3B3 model for IL-mediated IgM secretion is comparable to the CH12 B cell lymphoma isolated by Haughton and colleagues (2). This Ly-1/CD5$^+$ lymphoma was induced by hyperimmunization with sheep erythrocytes (sRBC) and expresses IgM capable of binding phosphatidylcholine (3). The original reports on the differentiation requirements of CH12 cells indicate a need for both antigen and cell contact with an appropriate T helper cell (4,5). More recently, Swain et al. (6) have shown that highly purified CH12 cells can be induced to secrete IgM in the presence of lysed sRBC and IL-5. CH12 cells appear unresponsive to IL-2, although monoclonal antibody to the IL-2R p55 subunit (IL-2Rα) can stimulate the cells to release IgM (7). Thus, the primary differences between the activation requirements of these two clonal B cell lines are a) a requirement by the CH12 cells for a membrane Ig-transmitted signal in addition to IL-5 and

b) the ability of the BCL_1-3B3 cells to differentiate in the presence of IL-2.

Relative to a requirement for mIg crosslinking, the study by Webb et al. (8) is informative. These investigators transfected phosphorylcholine (PC)-specific μ and kappa chain genomic sequences into the parent *in vitro* BCL_1 line from which BCL_1-3B3 cells were cloned. After selecting a clone which did not spontaneously secrete IgM at a high rate, it was found that IL-5 alone increased both the proliferation rate and quantity of anti-PC IgM released into the culture supernatant. When steady-state mRNA levels were determined for both the transfected and endogenous μ chains, a 3-4 fold increase occurred for both mRNAs in the presence of both IL-5 and antigen (PC-KLH); however, no increases were seen when the cells were stimulated with IL-5, IL-2 or antigen alone. This indicates that the BCL_1 cells are capable of responding to a differentiation signal mediated by antigen-mIg interaction. Thus, when compared to the BCL_1 cells, the antigen requirement for CH12 differentiation may be more a quantitative rather than a qualitative difference.

Relative to the IL-2 and IL-5 mediated differentiation of the BCL_1-3B3 cells, another *in vitro* clone of BCL_1 (BCL_1-CL-3) was isolated by Nakanishi et. al.(9). BCL_1-CL-3 cells are also induced to secrete IgM in the presence of both IL-2

and IL-5 (9, 10). Although IL-5 alone could induce some IgM secretion the additional presence of IL-2 increased the percent of cytoplasmic IgM+ cells approximately 6-fold (10). In the absence of IL-5, IL-2 did not induce differentiation and IgM secretion by the BCL_1-CL-3 cells. Sequential addition of the ILs indicated that maximal differentiation was observed when IL-5 preceded IL-2 and that an 8-9-fold increase in low-affinity IL-2R (IL-2Rα subunit) preceded the peak percentage of cytoplasmic IgM^+ cells (9). Thus, this BCL_1 clone is unresponsive to IL-2 alone even though it expressed low levels of both low and high-affinity IL-2R.

Consistent with the present research observations on the BCL_1-3B3 cells, Matsui et al. (11) found that IL-5 was capable of inducing increases in both μ_s and J chain mRNA in BCL_1-CL-3 cells and that maximal J chain steady-state mRNA levels were observed in the presence of both IL-2 and IL-5. Since BCL_1-CL-3 cells do not respond to IL-2 without costimulation with IL-5, no induction of μ_s or J chain mRNA in the presence of IL-2 was observed. Using the BCL_1-3B3 cells in the current research, it was found that IL-2 also stimulated increases in both μ_s and J chain mRNA levels.

Blackman et. al. (12) have also shown that the BCL_1-3B3 cells can be induced to secrete IgM in the presence of IL-2 and that IL-2 induces marked increases in J chain mRNA.

However, their results differ from the current study in that they did not observe a significant increase in μ_S mRNA levels (12). This difference is most likely due to slight differences in the maintenance and preparation of the cells for analysis. Similar to studies reported on CH12 cells by Bishop and Haughton (7), culture conditions which inhibit cell proliferation, i.e., 2-ME deprivation, were observed to enhance IgM secretion of BCL_1-3B3 cells. It was found that the optimal conditions for IL-mediated differentiation include a preculture in 3% FBS-RPMI media containing 2-ME for 2-4 days followed by IL stimulation in 3% FBS-RPMI 1640 media without 2-ME. Long-term maintenance of BCL_1-3B3 requires the presence of 2-ME, but the reduced proliferation rate which ensues shortly after 2-ME removal seems to facilitate the differentiation process.

Using BCL_1-3B3 cells, Tiggs et. al. (13) reported that IL-4 inhibited both proliferation and J chain gene expression induced by IL-2 and IL-5, but IL-4 did not decrease the number of high affinity IL-2R. The authors concluded that there was no cross talk between IL-4 and IL-2 at the receptor level, and suggested antagonistic action of IL-4 therefore may be exerted intracellularly at signal transduction pathways. In contrast, Fernandez-Botran et. al. (14) reported that preincubation of BCL_1-3B3 cells with IL-4 resulted in a partial decrease in the number of high

affinity IL-2R. Inhibitory effects of IL-4 on high affinity IL-2R expression on BCL_1-3B3 cells observed before these receptors were shown to share a common γ subunit, may be explained partially by competition for the γ subunit.

The interaction of the three ILs (IL-2, IL-4, and IL-5) on IL-2R up-regulation was carried out with recombinant sources on the BCL_1-CL-3 cell subline (15). Both low and high affinity IL-2R were up-regulated (9 fold) with co-incubation with IL-2 and IL-5, whereas IL-5 by itself could only affect the high affinity IL-2R slightly (3 fold). IL-5 appears to induce the expression of IL-2R subunits, although the induction is much lower than the IL-2/IL-5 mixture. The dose dependent down-regulation of IL-2Rα was clearly observed when the BCL_1-CL-3 cells were first incubated with IL-4 and IL-5 for 12 hours, and then incubated with IL-2 for the next 12 hours. The result is similar to study on the BCL_1-3B3 cells by Tiggs et. al. (13) in that IL-2 and IL-5 act as positive factors, and IL-4 a negative factor for the cells to differentiate, but differs in the mode of interaction of IL-2 and IL-4. A study of the effect of IL-5 on IL-2R expression was not carried out with the current research on BCL_1-3B3 clone. However, under suboptimal IL concentration in media without 2-ME, a synergistic interaction was observed. Thus, it might be interesting to

see if the synergistic interaction was partially caused by induction of high affinity IL-2R expression on BCL_1-3B3 cells as in the case of BCL_1-CL-3 cells.

Cell cycle analysis of BCL_1-3B3 cells revealed that regulation of signal transduction by the IL-2R and IL-5R may differ somewhat in the extent to which the phase of the cell cycle modulates responsiveness. To date, most investigations of lymphokine responses have focused on the requirement for B cells to enter the cell-cycle prior to receipt of the lymphokine-mediated signal (16-19) and/or the ability of B cells to differentiate without progressing through S phase and cell division (20, 21). Previous studies of proliferative activity of IL-2 and IL-5 have indicated that both factors are ineffective until the cell is triggered to enter the cell cycle (i.e. G_1) via stimulation through mIg or a B cell mitogen such as dextran sulfate (22, 18, 19). It has previously been shown that the spontaneously proliferating clone BCL_1-3B3 possesses an increased capacity to absorb BCDFμ (i.e. IL-5) during the S and G_2 phases of the cell cycle (23). In the present study it was found that the BCL_1-3B3 cells also express a high density of high-affinity IL-2 receptors at the G_1/S border . Consistent with this pattern of receptor expression it was found that the BCL_1-3B3 cells were responsive to an IL-2

pulse given during the S and G_2 periods of the cell cycle. In contrast, BCL_1-3B3 cells appeared most responsive to IL-5 when exposed to this lymphokine during late G_1. The earlier study utilized EL-4 SN which contains both IL-2 and IL-5 and, consistent with the present results, maximal responses to EL-4 SN during the S/G_2 phases of the cell cycle was observed (23).

Further clarification of the regulation of the IL-5R during the cell cycle would provide additional support for our tentative conclusions. Antibodies to the IL-5R subunits are now available for flow cytometry analysis. Expression of high affinity IL-5R can be carried out with purified IL-5. In addition, alternative approaches to cell cycle synchronization may prove helpful. The separation of cells might be achieved by physical means such as elutration. Density of cells change as the cells are in different stages, and this might help separate the cells in different phases of the cell cycle or stages of differentiation.

The role of IL-5 *in vivo* was examined in both IL-5Rα-deficient mice and IL-5 deficient mice. Analysis of IL-5Rα-deficient mice ($IL\text{-}5R\alpha^{-}/^{-}$) demonstrated a significant decrease in peritoneal cavity $Ly\text{-}1/CD5^{+}$ B cells, without altering the conventional B cell population in the spleen at six to eight week in age (24). The study suggests that IL-5

Rα contributes at least in part to the early development of B-1 cells. Serum levels of IgM and IgG_3 were lower in IL-$5R\alpha^{-/-}$ mice as compared to the wild type. Thus, IL-5Rα appears to be important in IgM and IgG_3 isotype production. In addition, IL-$5R\alpha^{-/-}$ mice were shown not to respond to a TI-2 antigen, TNP-Ficoll, whereas the wildtype produced an anti-TNP IgM response which was stimulated by the addition of IL-5.

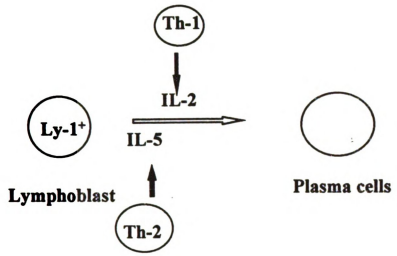
Studies on IL-5-deficient mice (IL-$5^{-/-}$) revealed a reduction of Ly-1/$CD5^{+}$ B cells at 2 weeks of age, but these levels returned to normal in adult mice of 6 to 8 weeks of age (25). Thus, IL-5 appears to aid the development of $Ly\text{-}1^{+}$/B-1 B cells, but is not essential to the process. Interestingly, there was no difference in the Ig levels in IL-$5^{-/-}$ mice as compared to wildtype mice. The responses of IL-$5^{-/-}$ mice to the TI-2 antigen TNP-Ficoll were shown to be comparable to wild type. Thus, other factors with an overlapping activity seem to compensate for the IL-5 activity reported in the *in vitro* studies.

As discussed in Chapter 1, the prevalent model for B cell differentiation can be seen in Figure 1. In this model, the sequential progress of the resting B cells differentiating into antibody producing plasma cells is emphasized. The resting B cells are first activated by IL-4, then IL-5 acts as the growth factor on the previously

Figure 1 Model for B cell Differentiation

activated B cells, finally in this model IL-6 was the terminal differentiation factor, which induces the already growing activated B cells to become plasma cells. The data obtained from the Ly-1$^-$ B clone AKR 225 is reasonably consistent with Kishimoto's model in that these cells require both IL-4 and IL-5 to differentiate into IgM secreting plasma cells. However since AKR-225 cells are already proliferating these factors seem to be contributing directly to the differentiation process rather than clonal expansion as suggested by Kishimoto's model. Likewise, the Ly-1$^+$ BCL$_1$-3B3 cells differentiate in response to IL-5, but in contrast to the AKR-225 cells, do not need stimulation from IL-4. Rather, they can respond to IL-2 and IL-2 and IL-5 can synergize during the differentiation. Thus, while Kishimoto's model might require only slight modification to explain the responses of AKR-225 cells, it can not be applied to the responses of the Ly-1$^+$ BCL$_1$-3B3 cells.

In conclusion, a model for two separate pathways for the later stage of differentiation of Ly-1$^+$ B and Ly-1$^-$ B cells is presented (Fig. 2). In both pathways IL-5 can mediate IgM secretion. However, Ly-1$^-$ B cells require the presence of IL-4 for optimal IgM secretion, whereas Ly-1$^+$ B cells can respond to either IL-2 or IL-5, but these two interleukins can synergize to give optimal IgM secretion.



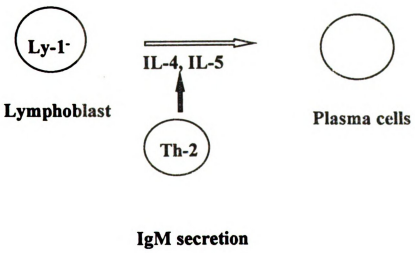


Figure 2 Model for Separate pathways for Ly-1$^+$ B and Ly-1$^-$ B cell differentiation

As our data for Ly-1$^-$ AKR-225 cells are preliminary, this will not be discussed further.

In the Ly-1$^+$ model, IL-5 would act prior to IL-2 for the already proliferating cells to secrete IgM, and a synergistic response would be observed partly due to IL-5's ability to induce expression of high affinity IL-2R. This is consistent with all three Ly-1$^+$ B *in vitro* inducible models (BCL_1-3B3, CH-12, and BCL_1-CL-3) in that they are capable of responding to IL-5 alone (note that CH-12 requires antigen, however). In addition, peritoneal Ly-1$^+$ B cells have been shown to express IL-5R without *in vitro* stimulation (26). The recent transgenic mice study demonstrated that IL-5Rα, a IL-5 specific subunit of IL-5R complex, is important for IgM production and Ly-1$^+$ B cell development *in vivo* (24) although signals other than IL-5 may also be involved as suggested by the IL-5 knockout mice which do not show any reduction in IgM (25). Furthermore, the recent study with the BCL_1-CL-3 cells demonstrated that the responsiveness to IL-2 in the presence of IL-5 was partly due to higher expression of high affinity IL-2R (15). In addition, the present study suggests IL-5 might act preferentially in G_1 phase and IL-2 at S or G_2 phase.

The effect of IL-2 and IL-5 on Ly-1$^+$ B cells are similar in that IL-2 and IL-5 independently induces IgM

secretion by increasing both μ_s and J chain mRNA. This result differs somewhat from a previous studies (12, 13) where gene expression of μ_s appears to precede that of J chain and was not upregulated by IL-2. However, the present study on BCL_1-3B3 cells confirms the studies using BCL_1-CL-3 cells and suggest that both μ_s and J mRNAs can be regulated by IL-5 and IL-2 (11).

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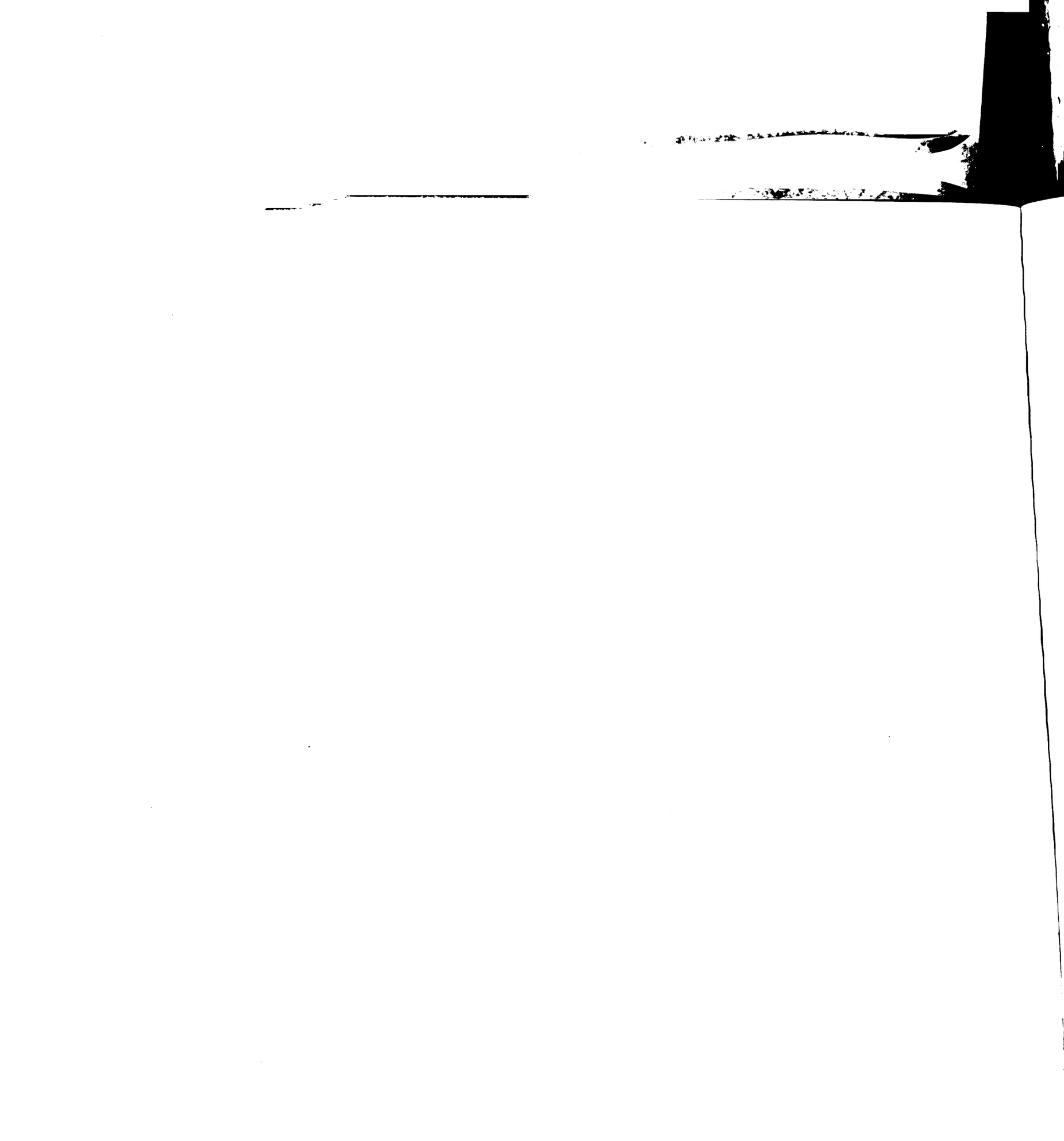

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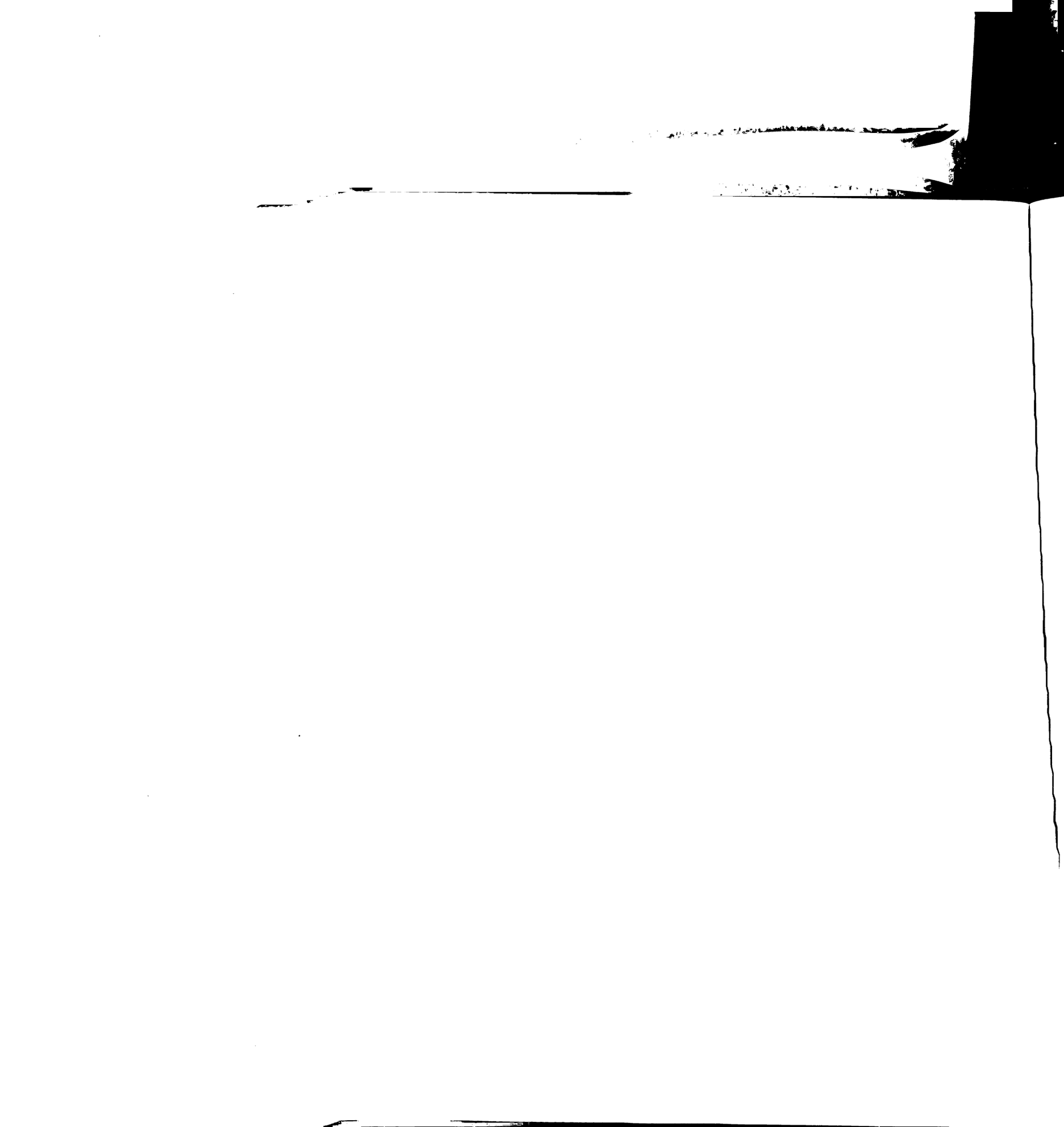
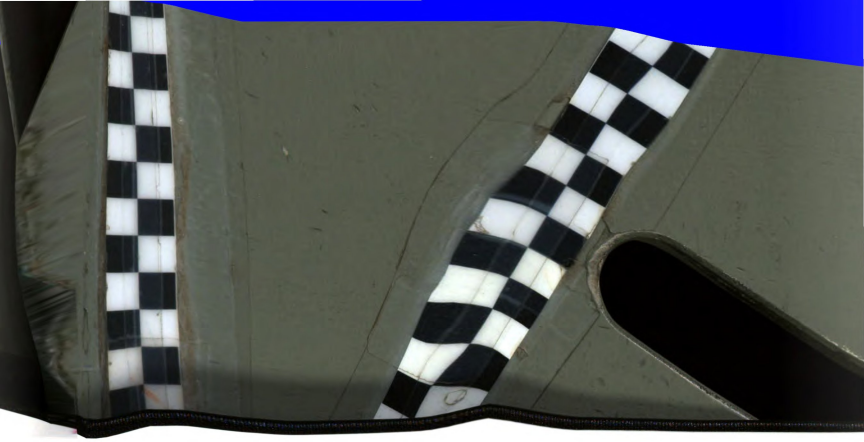
APPENDIX



APPENDIX A

Cloning and characterization of mammalian homologs of the Drospphila dunce' gene

Ronald L. Davis, Hiroko Takayasu, Mary Eberwine, and James Myers



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Biochemistry

Cloning and characterization of mammalian homologs of the *Drosophila* dunce$^+$ gene

(cAMP phosphodiesterase)

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ABSTRACT **A probe representing the *Drosophila* dunce$^+$ (*dnc*$^+$) gene, the structural gene for a cAMP phosphodiesterase (PDEase), detects homologous sequences in many different organisms, including mouse, rat, and human. Genomic and cDNA clones representing a homolog of the *Drosophila dnc*$^+$ gene were isolated from rat libraries and characterized. This gene has been named ratdnc-1. One cDNA clone defines a large open reading frame of ≈1.8 kilobases (kb), predicting a protein sequence of 610 amino acids with significant homology to a conserved domain of ≈275 residues found in most other PDEases. The amino acid identity value to the *Drosophila* cAMP PDEase within this domain is a striking 75%. Other cDNA clones show blocks of sequence divergence from this cDNA clone close to the predicted N terminus, indicating the potential existence of a family of related enzymes encoded by alternatively spliced messenger RNAs from ratdnc-1. Genomic blotting experiments suggest the existence of at least one other rat gene with homology to ratdnc-1. RNAs homologous to ratdnc-1 are heterogeneous in size between tissues, with heart containing a major transcript of 4.4 kb and brain one of 4.0 kb. The potential identity of the product of the ratdnc-1 gene with known PDEases is discussed.**

The acceptance of *Drosophila melanogaster* as a model organism for studying learning and memory processes has increased dramatically in the last decade. Several mutants defective in these processes have been isolated and studied by behavioral, biochemical, and molecular biological approaches (1). The mutant dunce, which was the first to be isolated (2), has been studied most extensively. These mutants demonstrate appreciable learning but have an abbreviated memory of the learned information (3–5). Molecular analyses of the *dnc*$^+$ gene have revealed an unusual complexity to its organization. The gene extends over >100 kilobases (kb) of the genome, encodes a remarkable array of RNAs, and contains several other genes within an enormous intron of almost 80 kb (6, 7). In addition, the sequence analysis of *dnc*$^+$ cDNA clones (8) has confirmed biochemical studies which suggested that the gene encodes the enzyme cAMP phosphodiesterase (PDEase) (9).

This enzyme is a member of the complex family of cyclic nucleotide PDEases, whose function is to hydrolyze cyclic 3′,5′-nucleoside monophosphates into 5′-nucleoside monophosphates. This function places the enzymes in a central position in the regulation of information flow from extracellular hormones, neurotransmitters, or other signals that use the cyclic nucleotides as intracellular messengers. The complexity of the family is evident from the many different forms that have been reported as well as apparent differences in their modes of regulation. In a recent review, Beavo (10) classified the mammalian PDEases into several different subfamilies, primarily based on substrate affinity, substrate specificity, and selective sensitivity to cofactors or drugs. Five types we recognized as being reasonably distinct include the following: (*i*) cyclic nucleotide (hydrolyzes both cAMP and cGMP) PDEases, which require Ca^{2+} and calmodulin for maximal activity; (*ii*) cGMP PDEases, which are generally specific for cGMP as substrate; (*iii*) cGMP-stimulated cyclic nucleotide PDEases; (*iv*) cGMP-inhibited cyclic nucleotide PDEases; and (*v*) cAMP PDEases, which are specific for cAMP as substrate. A genetic basis for at least some of this complexity was recently established. Sequence comparisons between a bovine Ca^{2+}/calmodulin-dependent PDEase, a bovine cGMP-stimulated PDEase, and yeast and *Drosophila* cAMP PDEases revealed a conserved domain of ≈275 residues between the enzymes (11). The existence of a homologous but nonidentical domain between the bovine enzymes indicates that at least some different subfamilies of PDEases are encoded by different members of a gene family.

Since it is clear that the normal function of *Drosophila dnc*$^+$ locus and its product cAMP PDEase is required for the normal memory of learned information in flies, it is critical to know whether the locus and its enzyme product are found in mammalian organisms, and, if so, whether they also serve the neurophysiological processes underlying mammalian behavior. We report here the detection, isolation, and characterization of mammalian homologs of the *Drosophila dnc*$^+$ gene.‡ The sequence of a rat PDEase predicted from the sequence of cDNA clones is strikingly homologous to the *Drosophila dnc*$^+$-encoded PDEase, confirming a high degree of structural conservation. The isolation of a rat homolog of the *Drosophila dnc*$^+$ gene will potentially allow us to determine whether their biological roles are also similar.

MATERIALS AND METHODS

Sprague–Dawley rats were used for the isolation of DNA or RNA. The genomic library used was constructed and provided by T. Sargeant (National Institutes of Health). It is a partial *Eco*RI library of Sprague–Dawley rat liver DNA. The rat brain cDNA library was constructed by B. Popko (California Institute of Technology) in the vector λgt10, using brain poly(A)$^+$ RNA isolated from 18-day-old rats as substrate, and random-priming to initiate first-strand synthesis.

Standard procedures for molecular biological research were used throughout. Probes were prepared from isolated DNA by either nick-translation or random priming. RNA was isolated from fresh or frozen tissue after homogenization in guanidinium hydrochloride as described (12). Genomic DNA was isolated from either rat brain or liver nuclei as described (29) and purified by CsCl density-gradient centrifugation. RNA blots were prepared following formaldehyde agarose

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Abbreviation: PDEase, phosphodiesterase.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04554).

gel electrophoresis (6). DNA sequencing utilized bacteriophage M13 single-stranded subclones or double-stranded plasmid DNA with dideoxynucleotide sequencing primed by universal primers or synthetic oligonucleotides.

Standard hybridizations for DNA blots, RNA blots, or library screening were at 42°C in 0.1 M Pipes/0.8 M NaCl/0.1% sarkosyl/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/~1 × 10^6 dpm of probe per ml/10% dextran sulfate/0.1 mg of alkali-sheared salmon sperm DNA per ml/50% formamide. After hybridization, filters were washed at room temperature in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.05% sarkosyl/0.02% sodium pyrophosphate. Final washes were performed at 50°C in 0.1× SSC/0.05% sarkosyl/0.05% sodium pyrophosphate. Low-stringency hybridizations were performed by adjusting the formamide concentration in the hybridization mixtures and the salt concentration in the final wash solutions. For example, the blots in Fig. 1 were hybridized at about 23, 33, and 43°C below the theoretical melting temperature (t_m) (13) of duplex DNA in solution by using 50%, 36%, or 20% formamide in the hybridizations. The final washes were performed at t_m −21, −31, or −41°C by using 0.13, 0.54, or 2.2× SSC in the final wash solutions.

RESULTS

A *Drosophila dnc*⁺ cDNA Clone Detects Homologous Sequences in Other Organisms. In a preliminary survey to determine whether the *Drosophila dnc*⁺ gene is conserved in sequence in other eukaryotes, portions of a cDNA clone containing most of the open reading frame for cAMP PDEase were used to probe genomic blots of a variety of species at several different stringencies. Fig. 1 illustrates some of the results. Each genomic DNA sample contains one to several hybridizing fragments at one or more of the three different

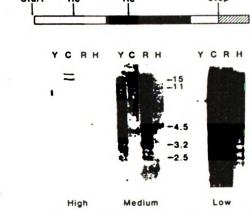

FIG. 1. Genomic blots probed with a *Drosophila dnc*⁺ cDNA clone. Schematic diagram of the *Drosophila dnc*⁺ cDNA clone ADC1 (8) showing the location of the PDEase conserved domain (11) within the open reading frame and *Hin*cII (Hc) sites. [We previously reported the open reading frame to be completely encompassed within ADC1 (8). Recent results (unpublished) have uncovered a single base omission, which, when corrected, extends the previous open reading frame to an ATG codon residing 13 nucleotides to the left of the first nucleotide of ADC1 in an overlapping cDNA clone (7). The revised conceptual amino acid sequence is shown in Fig. 3.] *Eco*RI digests of genomic DNA samples from yeast (lanes Y, 2.7 μg), chicken (lanes C, 15 μg), rat (lanes R, 20 μg), and human (lanes H, 20 μg) were probed at high, medium, and low stringencies with the three *Hin*cII fragments. Results obtained with the rightmost *Hin*cII fragment are shown. Sizes (kb) of the hybridizing fragments in the rat sample are indicated.

hybridization and wash conditions using the right half of the cDNA clone as probe. Of particular interest here are the results of hybridization to rat DNA, which shows five hybridizing *Eco*RI fragments at medium stringency. The left and middle *Hin*cII fragments also hybridized to some of the bands observed in the middle panel of Fig. 1, but much more faintly (data not shown). The bands detected may represent authentic homologs of the *Drosophila dnc*⁺ gene or artifactual hybridization, which is common in reduced stringency hybridizations. In addition to the species indicated in Fig. 1, we have also detected hybridizing fragments to genomic DNAs from the mouse, cockroach, and electric ray (data not shown).

To determine whether the hybridizing genomic fragments were authentic homologs of the *Drosophila dnc*⁺ gene, we screened a rat genomic library and isolated and characterized the positive clones. Using the rightmost *Hin*cII fragment of the *Drosophila dnc*⁺ cDNA clone as probe (Fig. 1), we recovered more than 30 positives from ~10^6 phage screened. Restriction mapping revealed that all but one of these were identical. The majority class contained an *Eco*RI fragment of ~15 kb (Fig. 2), which corresponds to the largest *Eco*RI fragment visualized in Fig. 1. We name the locus represented by this clone ratdnc-1, and any of its representative genomic clones as ratdnc-1G. The unique clone exhibited a completely different restriction pattern than ratdnc-1G and contained a 2.5-kb *Eco*RI fragment with homology to ADC1. We believe that this clone contains the 2.5-kb *Eco*RI genomic fragment visualized in Fig. 1.

The region of ratdnc-1G homologous to ADC1 was delimited to 1.15-kb *Hin*dIII/*Taq* I and 0.47-kb *Taq* I fragments by Southern blotting (Fig. 2). The 0.47-kb *Taq* I fragment was sequenced, revealing an exon (see below) of 183 residues with a high degree of homology to the *Drosophila dnc*⁺ sequence.

The Predicted Sequences of Rat PDEases Are Strikingly Similar to That of the *Drosophila* cAMP PDEase. The 470-base-pair (bp) *Taq* I fragment of ratdnc-1G was used to probe 5 × 10^5 phage from a rat brain cDNA library at high stringency. Four cDNA clones were isolated. Two of these (RD1 and RD2) were sequenced in their entirety (Fig. 3). The clone RD3 was extensively analyzed with restriction enzymes and found to map identically to RD1 except for near the left end. Both ends of this clone were also sequenced. The fourth cDNA clone isolated, although very large (4.5 kb), has proven to be unstable upon propagation under a variety of conditions and has not been analyzed further.

The cDNA clones are identical in sequence except for near their 5′ ends (Figs. 3 and 4). Clones RD1 and RD3 contain a 99-bp sequence within their open reading frames that is missing from the open frame in RD2. This predicts an additional 33 amino acids within the putative protein products of RD1 and RD3. In addition, the sequences diverge from one another in a modular fashion near their 5′ ends. The consequence of this divergence is that each clone has a different

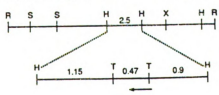

FIG. 2. Partial restriction map of the genomic clone, ratdnc-1G. R, *Eco*RI; H, *Hin*dIII; X, *Xho* I; S, *Sma* I. An expanded view of the 2.5-kb *Hin*dIII fragment shows the location of the 0.47-kb *Taq* I (T) fragment, which hybridizes strongly to the right half of the *Drosophila dnc*⁺ cDNA clone (Fig. 1).

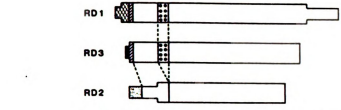

FIG. 3. Schematic diagram of ratdnc cDNA clones. The wide portions of each bar diagram represent the longest open reading frame and the narrow portions represent the predicted 5′ and 3′ untranslated regions. Regions of sequence in common are unshaded, while those that differ are marked.

ATG triplet as the first potential translation start codon (Fig. 4). Although some of the sequence differences could be due to cDNA cloning artifacts, the sequence identity of RD1 and RD2 on both sides of the 99-bp insert in RD1 suggests that at least this difference is authentic, most likely produced by alternative splicing of transcripts from the ratdnc-1 gene.

The clone RD1 has a complete open reading frame, with RD2 and RD3 being truncated at their 3′ ends. The open reading frame of RD1 predicts a protein molecule of 610 amino acids with a molecular mass of ≈68 kDa. An alignment of the conceptual translation sequence of RD1 with other known PDEases is shown in Fig. 5. The homology with the *Drosophila* cAMP PDEase is striking, with close to 75% of the residues within the conserved domain being identical. In contrast, the RD1 product shares between 20% and 40% identity with residues in a Ca^{2+}/calmodulin-dependent PDEase, a cGMP-stimulated PDEase, and the α-subunit of a retinal cGMP PDEase.

At Least One Other Gene Exists in the Rat with Homology to ratdnc-1. Genomic blots were probed with ratdnc-1 probes to estimate the number of homologous genes within the rat genome. Two hybridizing fragments (Fig. 6*A*) were observed after probing several different digests with the 0.47-kb *Taq* I genomic fragment at high stringency (Fig. 2). Each lane contains one fragment that hybridizes strongly and one that hybridizes weakly. The fragments that hybridize strongly correspond to those contained within ratdnc-1G. The fragments observed retain their relative hybridization intensity on blots prepared with genomic DNA isolated from several different animals or single animals, indicating that the weakly hybridizing extra bands are not due to polymorphism of the ratdnc-1 gene. This suggests that two genes exist in the rat genome that share a high degree of homology with the 0.47-kb *Taq* I fragment. A similar but more complex pattern was obtained upon probing genomic blots with a restriction fragment from RD1 representing a portion of the protein coding sequence (Fig. 6*B*). The additional bands observed probably represent additional genomic fragments of the two genes identified with the 0.47-kb *Taq* I fragment or additional genes

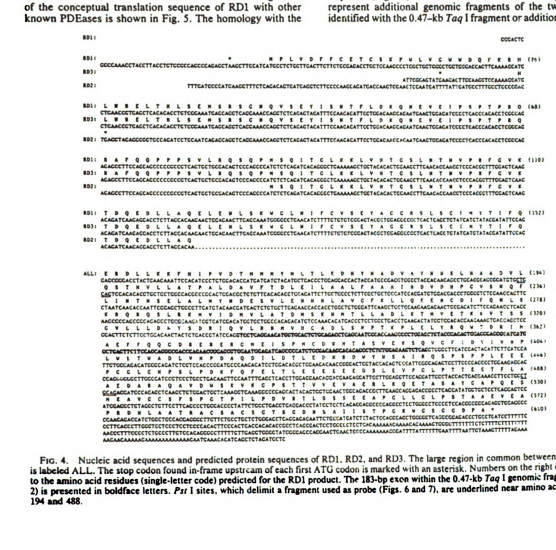

FIG. 4. Nucleic acid sequences and predicted protein sequences of RD1, RD2, and RD3. The large region in common between the clones is labeled ALL. The stop codon found in-frame upstream of each first ATG codon is marked with an asterisk. Numbers on the right correspond to the amino acid residues (single-letter code) predicted for the RD1 product. The 183-bp exon within the 0.47-kb *Taq* I genomic fragment (Fig. 2) is presented in boldface letters. *Pst* I sites, which delimit a fragment used as probe (Figs. 6 and 7), are underlined near amino acid residues 194 and 488.

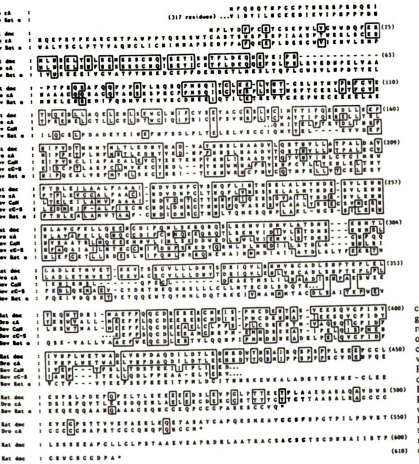

FIG. 5. Alignment of the conceptual amino acid sequence (single-letter code) of RD1 with representatives of several subfamilies of PDEases. Dro cA, *Drosophila* cAMP PDEase (8); Bov CaM, bovine Ca^{2+} calmodulin-stimulated PDEase (11); Bov cG-S, bovine cGMP-stimulated PDEase (11); Bov Ret α, bovine retinal cGMP PDEase α-subunit (14). Identities with the conceptual sequence of RD1 are boxed. The PDEase conserved domain begins approximately at RD1 residue 148 and ends at ≈425.

homologous to regions of RD1 outside of the sequence shared with the 0.47-kb *Taq* I fragment. The intensity of hybridization of RD1 (Fig. 6*B*) to the 15-kb *Eco*RI and 2.5-kb *Hin*dIII genomic fragments contained in ratdnc-1, as well as the sequence identity between the exon within the 0.47-kb *Taq* I fragment (Fig. 2) and a portion of RD1, indicates that the isolated cDNA clones are products of ratdnc-1 and not the other highly related gene.

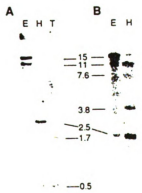

FIG. 6. Blots of rat genomic DNA digested with *Eco*RI (lanes E), *Hin*dIII (lanes H), or *Taq* I (lane T) probed at high stringency with the 0.47-kb *Taq* I genomic fragment (see Fig. 2) (*A*) or an internal *Pst* I fragment of RD1 (see Fig. 4) (*B*).

Tissue Expression Pattern of Transcripts Homologous to ratdnc-1. RNA blots of poly(A)$^+$ RNA were probed with RD1 to size the homologous RNAs and to examine their pattern of expression among several tissues. These transcripts were detected in all tissues examined, which includes brain, cerebellum, heart, lung, and testes; with minor differences in the level of expression (Fig. 7). However, there do exist qualitative differences in the RNAs among tissues, which have been observed in several different blots with two separate RNA preparations. The major hybridizing RNA measures 4.0 kb in brain and cerebellum, whereas in heart, lung, and testes, it is ≈4.4 kb. In addition, most of the tissues contain a second less abundant and/or more weakly hybridizing RNA. In neural tissue, for example, an RNA of ≈4.4 kb can be detected (Fig. 7). These qualitative differences may reflect tissue differences in transcription initiation sites or processing of ratdnc-1 transcripts or transcripts from a related gene.

DISCUSSION

The *Drosophila dnc*$^+$ gene has become an important model gene for studying the memory of conditioned behavior (1), oogenesis and development (15, 16), gene structure (7), and cyclic nucleotide metabolism (8, 9). Given this importance, we became interested in learning whether the gene is structurally and functionally conserved in mammals.

FIG. 7. Tissue expression pattern of RD1 homologous transcripts. (*A*) Hybridization of an internal *Pst* I fragment of RD1 (see Fig. 4) with 10 μg of poly(A)$^+$ RNA isolated from rat brain (lanes B), cerebellum (lane C), heart (lanes H), lung (lane L), and testes (lane T). (*B*) Hybridization of heart and brain poly(A)$^+$ RNA with the 0.47-kb *Taq* I fragment (Fig. 2), which shows the doublet character of hybridizing RNAs better than *A*. The second lane of *B* contains 50 μg of poly(A)$^+$ RNA.

As demonstrated here, the gene is sufficiently conserved to detect apparent homologs in all mammalian species examined. The isolated rat homolog is extremely conserved with the *Drosophila dnc*$^+$ gene, at least between the predicted protein coding regions. An amino acid sequence identity value of 75% between the predicted *Drosophila* and rat products over the conserved domain is very striking. For comparison, the conserved domains of the putative sodium channel of *Drosophila* are ≈50% homologous with the rat sodium channel protein (17). Approximately 65% of the amino acids are identical between a probable mouse potassium channel protein and that from the fly (18), while 40% identity exists between the nicotinic acetylcholine receptors of *Drosophila* and rat (19, 20). This strong conservation of cAMP PDEase, at least equivalent to and exceeding some of the very important proteins of ion channels, indicates that the conserved domain of the PDEases is under extreme selective pressure. This pressure has been maintained for at least 600 million years, since the separation of vertebrate and invertebrate phyla.

Colicelli *et al.* (21) have isolated a rat brain cDNA clone very similar to RD1 by its ability to suppress the phenotypes associated with a constitutive *ras2* mutation in yeast. The predicted amino acid sequence is >90% identical to that of RD1 within the conserved domain but diverges dramatically on both sides. However, close to 50% of the identical amino acid residues within the conserved domain utilize alternative codons. These comparisons indicate that the two cDNA clones represent mRNAs from two distinct but related genes. In addition, the cDNA clone isolated by Colicelli *et al.* (21) expresses in yeast into a low K_m cAMP PDEase very similar in properties to the one encoded by the *Drosophila dnc*$^+$ locus.

The homology of the predicted product of RD1 with other PDEases leaves little doubt that RD1 represents a mRNA that encodes a member of the PDEase enzyme family. The specific type of PDEase has not been demonstrated, but the high homology with the *Drosophila* cAMP PDEase and the related rat brain cAMP PDEase (21) predicts that it may encode an enzyme with very similar properties. Many different mammalian tissues, including rat brain, have been shown to contain "low K_m," cAMP-specific PDEases, similar to the *Drosophila* cAMP PDEase (10). One interesting mammalian enzyme of this PDEase subclass has an apparent molecular mass between 45 and 62 kDa (10, 22, 23) and a K_m for cAMP of ≈2 μM. This type of enzyme binds to, and is inhibited (10, 23–27) by, the new antidepressant drug rolipram (28). It is possible that the rolipram-inhibited cAMP PDEase is encoded by ratdnc-1 or by a related member of the ratdnc-1 subfamily of PDEase genes.

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