CELL SURFACE ANTIGENS IN NORMAL AND NEOPLASTIC HUMAN B LYMPHOCYTE DIFFERENTIATION: CELLULAR DISTRIBUTION AND FUNCTIONAL IMPLICATIONS

by

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ABSTRACT

Differentiation within the lymphoid system produces effector cells which are involved in a variety of immune functions. For T cells these include the provision of help, suppression, cytolytic activity and the regulation of cooperative cellular interactions. The primary function of B lineage cells is the production of specific antibody. Understanding the regulation of normal lymphocyte proliferation and differentiation may lead to a better appreciation of those factors which result in the development of malignancy. The non-Hodgkin's lymphomas are neoplasms of the immune system, the majority of which are B cell in origin. Despite advances in immunology and molecular biology, little is known about the mechanisms involved in B cell activation, proliferation and differentiation or about those events leading to their malignant transformation.

The advent of monoclonal antibody technology a decade ago has revolutionized our ability to identify and characterize cell surface antigens. Because the activation and control of proliferation of B cells was already known to involve structures at the cell surface, it was logical to utilize monoclonal antibodies to identify additional cell surface molecules that might be important in the function of normal B lymphocytes and that might allow normal and various types of neoplastic B cells to be distinguished.

To achieve this goal, we developed monoclonal antibodies that showed differential reactivity between large actively dividing lymphoma cells and small inactive (quiescent) lymphocytes. These were tested for their ability to inhibit various T and B lymphocyte functions (i.e. responses to anti-μ, lipopolysaccharide, phytohemagglutinin and the mixed lymphocyte response) as

well as for their reactivity with cell suspensions from a variety of malignant and nonmalignant hematopoietic tissues.

From these studies emerged the following: 1) Cell surface molecules other than immunoglobulin are involved in regulating the activation of normal B cells. This was shown by the discovery that monoclonal antibodies to both lymphocyte function associated antigen (LFA-1) and certain HLA class II determinants were able to inhibit the activation of peripheral blood mononuclear cells by the B cell mitogens anti-μ and LPS. This inhibition was shown to be mediated via effects of these antibodies on T cells and/or monocytes. 2) B lymphoma cells appear to express unique cell surface antigens (defined by monoclonal antibodies LM-26 and LM-155) not detectable on cells of other lineages, and absent from normal resting or activated B lymphocytes.

Future investigations will attempt to define the mechanisms by which the indirect involvement of LFA-1 and HLA class II molecules in B cell activation in vitro suggests new regulatory interactions not previously identified. Further studies will be required to define the mechanisms underlying these interactions and their significance in vivo. Similarly, the structure and function of the antigens detected by LM-26 and LM-155 remains to be determined. Nevertheless, the expression of apparently unique molecules on B lymphoma cells holds new promise for the diagnosis, classification and treatment of this group of diseases.

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"There is a tide in the affairs of men
which, taken at the flood, leads on to fortune;
omitted, all the voyage of their life
is bound in shallows and in miseries.
On such a full sea are we now afloat,
and we must take the current when it serves,
or lose our ventures."

W. Shakespeare (Julius Caesar)

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C H A P T E R I

THE LYMPHOID SYSTEM

"There are two kinds of confidence which a reader may have in his author...there is a confidence in facts and a confidence in vision...The former requires simple faith. The latter calls upon you to judge for yourself and form your own conclusions."

Anthony Trollope

1) GENERAL CONCEPTS OF LYMPHOCYTE DIFFERENTIATION

(A) The Cell Surface

Many important physiological functions are mediated at the surfaces of cells. These include: selective transport of small molecules and ions, cell-cell interactions, cell adhesion, cell activation by hormones, growth factors and mitogens, phagocytosis, exocytosis and endocytosis and metabolic regulation (1). The lipid bilayer determines the basic structure of biological membranes. However, proteins are responsible for most membrane functions and serve as specific receptors, enzymes or transporters. The matrix of cell membranes is a bilayer composed predominantly of phospholipids, glycolipids and cholesterol. These molecules are amphipathic and associate with their more hydrophobic portions oriented internally and their hydrophilic ends protruding externally. Embedded in the bilayer are the integral proteins of the membrane. Many of these are transmembrane proteins; most are glycoproteins. At physiological temperatures, the membrane exists as a two-dimensional fluid, in which protein and lipid components unless specifically restricted, may move freely. The cell

membrane does not exist in isolation. In contrast to integral proteins, peripheral proteins are not associated with the lipid bilayer but exist within the aqueous phase of the cell membrane, non-covalently attached to protruding regions of integral proteins. The concept of peripheral proteins may help to explain how cell membranes relate to their external (exoskeleton) and internal (cytoskeleton) environments (e.g. microfilaments, intermediate filaments and microtubules in the cytoskeleton; fibronectin and collagen in the exoskeleton (1, 2).

Recent evidence has implicated a quantitatively minor group of membrane phospholipids (the polyphosphoinositides) in signal transmission for a wide variety of growth factors, neurotransmitters and hormones. Activation of the polyphosphoinositide system results in the release of products which act as "second messengers" in evoking the cell's responses. Two products released from polyphosphoinositide as a consequence of receptor activation are inositol triphosphate and diacylglycerol. Inositol triphosphate causes an increase in intracellular calcium ions which modulates further reactions within the cell. Diacylglycerol appears to act independently by stimulating a protein phosphokinase (3).

The possible involvement of this system in lymphocyte activation is just beginning to be explored. However, recent evidence suggests that perturbations of the T3-antigen receptor complex by monoclonal antibodies results in the release of inositol triphosphate. This in turn causes the release of calcium ions from intracellular stores; an effect which is thought to be important in the activation of the human T cell line, Jurkat, to produce interleukin 2 (4). Furthermore, some evidence supports the hypothesis that cross-linking of B cell surface immunoglobulin leads to subsequent activation through a series of events including phosphatidyl

inositol hydrolysis, followed by the generation of diacylglycerol and protein kinase C activation (5, 6).

(B) Monoclonal Antibodies

Recently, technology has become available which has revolutionized the ability to study cell surface molecules. First developed by Kohler and Milstein in 1975, monoclonal antibodies promise to be of major value in furthering the understanding of cell interactions and function (7). By combining an antibody producing cell with a neoplastic plasma cell, a hybrid (or hybridoma) can be generated with the desired characteristics of each. Usually, a mouse of the same genetic background as the myeloma cells is immunized with an antigen. The spleen of the mouse is removed and teased apart to yield a cell suspension. Myeloma cells and splenic B cells are then fused together to form a single cell or hybrid. Fusion is facilitated using polyethyleneglycol, an electrical pulse or Sendai virus. Hybrids are then selected, screened for antibody production, cloned, rescreened, recloned and finally the antibodies are characterized. Inherited from the parent myeloma cell is the property of immortality in culture; from the immune lymphocyte the production of specific antibody. Monoclonal antibodies, because of their exquisite specificity can be used to detect distinct cell surface molecules or epitopes. Furthermore, monoclonal antibodies may be used to purify antigens or to assess biologic functions in in vitro assays (8). To a great degree, monoclonal antibodies have been responsible for the characterization and increased understanding of lymphocyte differentiation that has occurred in the last decade.

(C) Lymphocyte Ontogeny, Subpopulations and Differentiation Antigens

It is now clear that there are two classes of lymphocytes each mediating distinct functions (Figure 1). B cells secrete antibody; T cells subserve

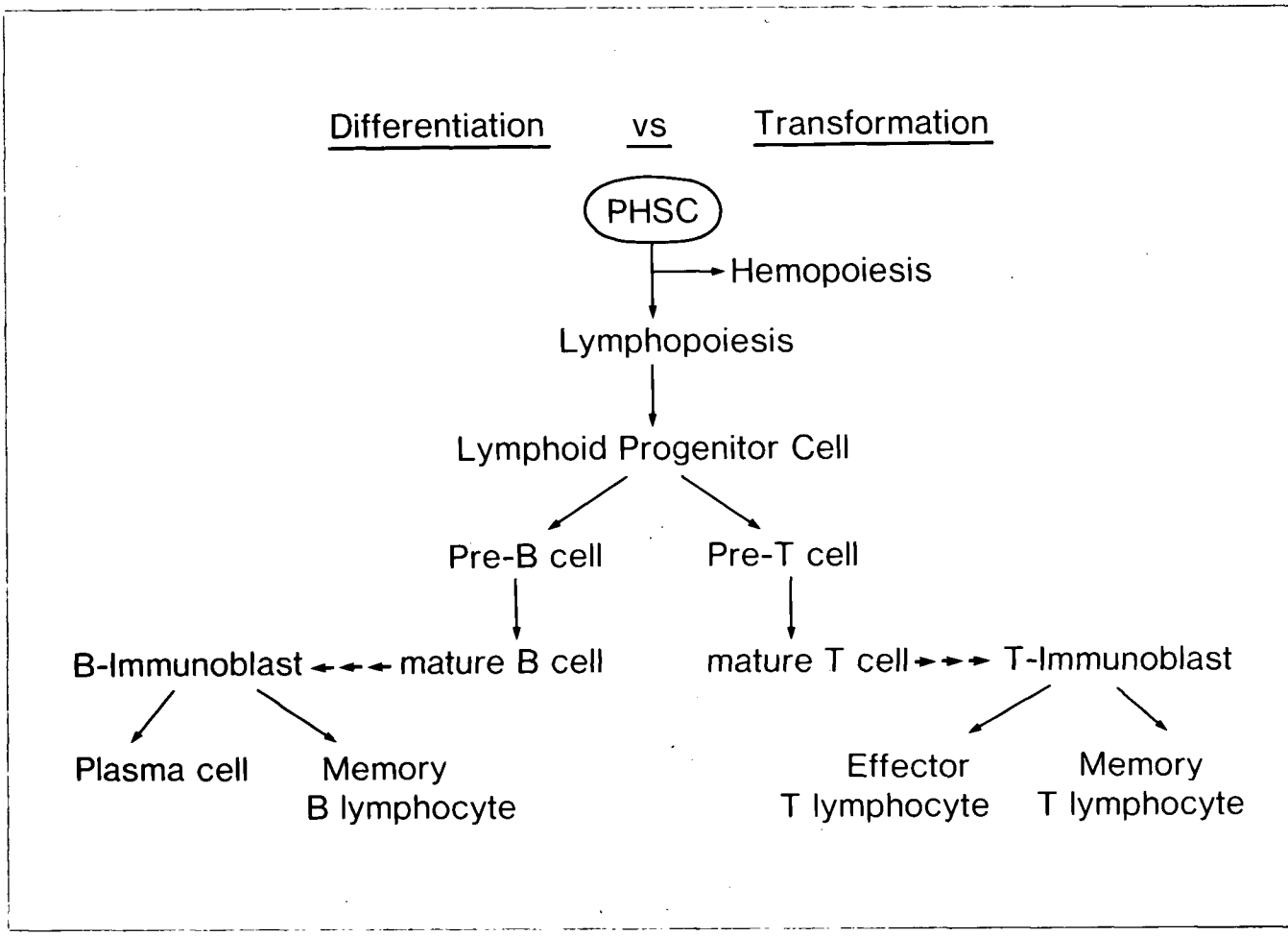

FIGURE 1 Schematic diagram of lymphocyte differentiation. Beginning with the pluripotent hematopoietic stem cell (PHSC) lymphocytes pass through a series of stages ending with functionally mature cells of B or T lineage (vertical arrows). For contrast, the transformation of mature B and T lymphocytes to large actively dividing immunoblasts is represented by the horizontal arrows. Immunoblasts may differentiate further to effector cells of T or B lineage (e.g. cytotoxic T cells, plasma cells). The mechanism of transformation is illustrated in more detail in Figures 2 and 3.

regulatory and effector functions. In birds, B cells are generated in the lymphoid organ called the bursa of Fabricius. In mammals, hematopoietic stem cells migrate from the yolk sac to the fetal liver where they differentiate into erythroid, myeloid and B cells. Stem cells then populate the bone marrow which becomes the major organ of hematopoiesis. Thereafter, B cells and other cells are continuously produced in marrow throughout life (9).

B lymphocytes are categorized into functional subpopulations on the basis of the different classes of immunoglobulin they synthesize. B lymphocytes and their terminally differentiated progeny plasma cells synthesize and/or secrete all classes of immunoglobulin molecules (IgM, IgG, IgA, IgD and IgE). Evidence now supports the hypothesis that the earliest progenitors of antigen-specific B cells possess receptors of the IgM class. More mature B cells express both cell surface IgM and IgD. Further maturation may result in the expression of IgG with or without IgD. So-called "memory" B lymphocytes may express surface IgG, while "virgin" B lymphocytes are thought to have IgM on their cell surfaces. Memory B cells are functionally important for development of rapid secondary (anamnestic) antibody responses upon subsequent antigenic exposure (10).

It is clear that B cells undergo a process of immunoglobulin heavy chain switching during differentiation. Isotype switching may be observed during clonal proliferation of B cells in response to certain antigens or mitogens such as LPS. Constant heavy chain (CH) region isotype switching may also occur during the pre-B to B cell transition. At this stage of development the switching process is most probably independent of antigen and T cells. The function of these cells, switched at early stages, is unknown. Isotype switching may be either sequential, from IgM to other immunoglobulin isotypes in order of the CH genes on the chromosome, or direct, from IgM to any of the

other isotypes encoded by downstream CH genes. Evidence suggests that the most commonly used pathways are direct isotype switches from IgM (9, 11-13).

A number of reports have recently been published describing monoclonal antibodies to cell surface antigens present on B lymphocytes (14-18). Although these show promise in delineating different subpopulations of B cells based on surface antigens, a functional correlation analogous to that in the T lymphocyte system has yet to be shown. However, evidence does exist which suggests that there are at least two subsets of human B cells which differ with regard to their relative susceptibility to proliferative signals delivered by B cell growth factor (BCGF) (19). B cell heterogeneity is also evident from studies of their physical properties (e.g. size, tissue distribution and charge) and from their functional characteristics (reactivity to different mitogens, antigens, genetic requirements for activation by T helper cells, and susceptibility to tolerance induction). It is still not entirely clear whether this heterogeneity reflects changes associated with the clonal expansion of a single line of B cells or whether it arises as a result of the generation of distinct sublines of B cells (20-22). Support for the concept of distinct subpopulations of B cells has emerged from the study of mice with the immune deficiency determined by the X chromosomal gene Xid. Xid mice appear to lack a subpopulation of B cells that express the cell surface determinants Lyb3, Lyb5 and Lyb7, which are important in the response to type 2 antigens (e.g. soluble polysaccharides) (23).

In contrast to B cells, T cells mature in the microenvironment of the thymus. Although it is not known with certainty how T cells mature, various theories have been proposed. One of these suggests that bone marrow stem

cells migrate to the thymic cortex during ontogeny and as these cells move to the medulla they undergo a series of differentiation stages induced by thymic stromal cells. Monoclonal antibodies define a number of T cell surface antigens which are thought to correlate with thymic differentiation and the acquisition of T cell functions. Approximately 10% of the total human thymocyte pool display markers of early cortical thymocytes: T11, T10 and T9. Late cortical thymocytes (80%) maintain T11 and T10, lose T9 but gain T8, T6, T4 and T1. At the cortical stage, thymocytes do not demonstrate peripheral T cell functions. As thymocytes migrate from the cortex to the medulla, they differentiate into two distinct lineages; one displaying T4, the other T8. T11, T10 and T3 are retained in both lineages. After further medullary differentiation in which T10 is lost, cells begin to acquire functions of mature T cells. Once exported to the periphery, T cells are functionally mature and can be distinguished on the basis of T4 and T8 expression. T8+ cells primarily mediate T cell cytotoxicity. T4+ cells act as effector cells for delayed hypersensitivity, provide "help" for B cell and cytotoxicity functions and are involved in the induction of suppression. The function of most of these T cell surface molecules has not been well defined. However, T4 and T8 appear to be involved respectively in the recognition of MHC class II and class I antigens. T3 is closely associated with the T cell receptor. T9 is identical with the receptor for transferrin. T11 (sheep red blood cell receptor) may be an activation structure through which the effects of phytohemagglutinin (PHA) are mediated (9, 24-29). Postulated cellular interactions involved in T cell activation and proliferation are illustrated in Figure 2.

Recently, this classical concept of thymic T cell differentiation has been challenged and two possible independent pathways of T cell maturation

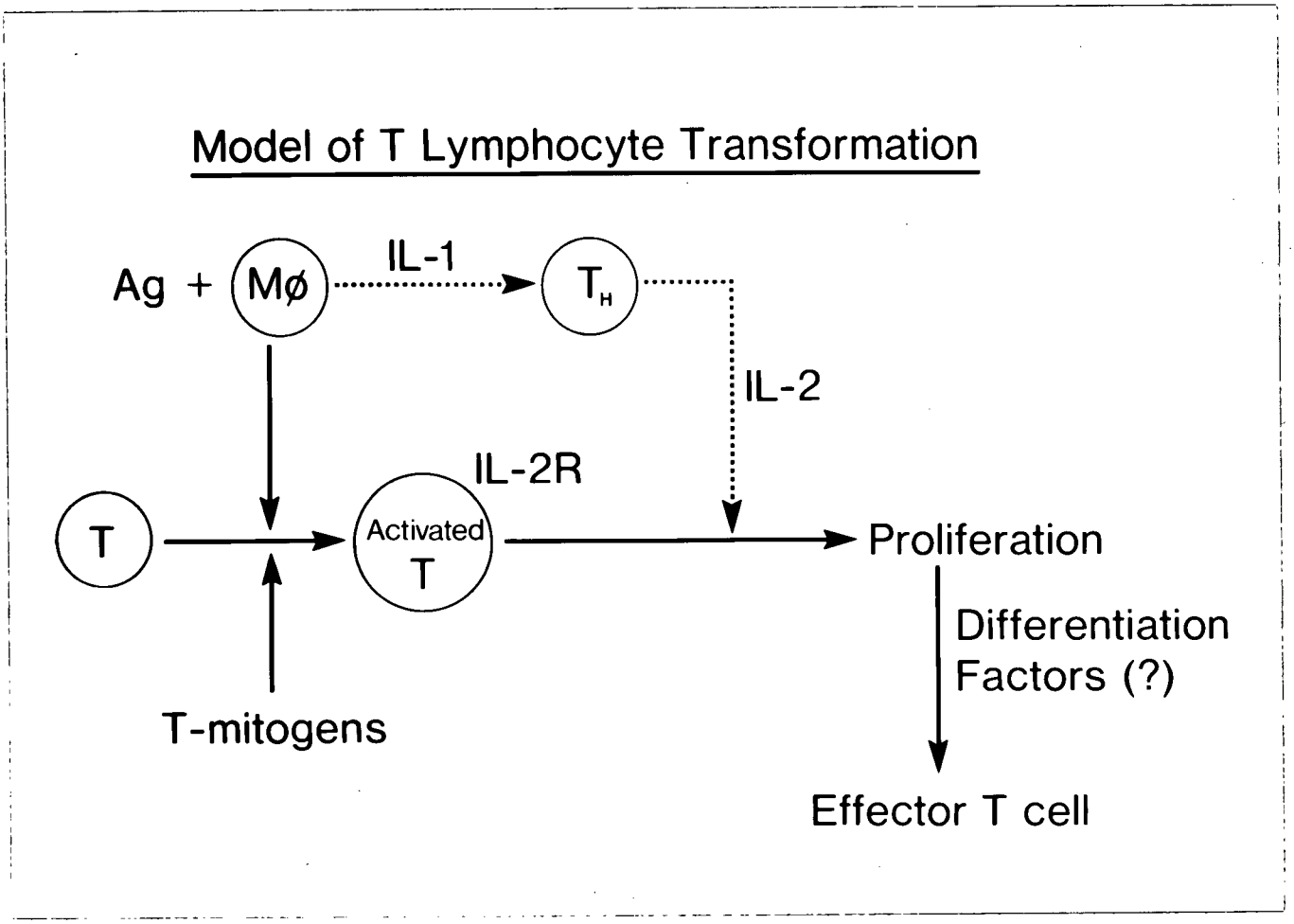

FIGURE 2 Model of T lymphocyte transformation. Resting T cells must first be activated before they are able to respond to interleukin-2 (IL-2).

have been proposed. In the first of these, superficial thymic cortical lymphoblasts divide and differentiate to give rise to small deep cortical thymic lymphocytes, medullary lymphocytes and cells leaving the thymus. The second hypothesis suggests that the medulla contains an independent self-renewing cell population that contains the precursors of the peripheral T-cell pool (30,31). These issues remain to be resolved.

(D) Lymphocyte Cell Surface Receptors and Gene Rearrangement

The B lymphocyte surface receptor involved in antigen recognition is immunoglobulin (Ig). The T cell receptor for antigen has been characterized as a class of cell surface heterodimers, termed Ti, that are membrane associated with the T3 glycoprotein complex. Ti molecules are present on all mature T cells and confer exquisite specificity to these cells in terms of their ability to recognize antigen (32, 33). Genes coding for both B cell receptors (Ig) and T cell receptors (Ti) have been cloned (34-36) and found to exhibit considerable homology (37).

During the process of B and T cell differentiation Ig and Ti genes are rearranged. Rearrangement precedes the cell surface expression of the respective receptors (34-39). Using appropriate DNA probes, a distinction can be readily made between monoclonal and polyclonal populations of B and T cells based on the pattern of Ig or Ti gene rearrangement. This technology has recently been applied to determine the cell of origin of lymphoid malignancies of uncertain histogenesis, such as acute lymphoblastic leukemia and hairy cell leukemia. Moreover, immunoglobulin and T cell receptor gene rearrangement can be used as a diagnostic criterion for malignancies of B and T cell type that lack characteristic cell surface markers (40-45).

The earliest stage of B cell development involves the commitment of pluripotent hematopoietic stem cells to the B cell pathway, rearrangement of

immunoglobulin (Ig) genes, and expression of cell-surface Ig which serves as the antigen receptor. Cells must migrate to appropriate peripheral lymphoid tissues where they can be activated by antigen in concert with secondary factors. Finally, selected and activated B cells must expand in number and be induced to secrete immunoglobulin (46).

Along the differentiation pathway from pluripotent hematopoietic stem cell (PHSC) to functionally mature B cell, a series of changes occur which may be conveniently delineated based upon detectable cellular events. The PHSC remains a hypothetical cell whose characteristics have yet to be determined. Upon commitment to the B lineage, the committed lymphoid stem cell undergoes a process of immunoglobulin gene rearrangement, detectable by in vitro molecular hybridization. Studies comparing Ig gene arrangement in B cells with nonlymphoid embryonic tissue have provided insight into the mechanism by which immunoglobulin diversity is generated. These studies have shown that light chain genes are organized in a discontinuous system of germ line variable (VL) regions, joining (JL) segments and constant (CL) regions. Heavy chain genes are organized similarly (VH, JH, CH) but appear to have an additional diversity (DH) segment located between their VH and JH regions. At some point early in B cell differentiation a cell rearranges its genome to form the sequences that encode the heavy (VH/DH/JH) and light (VL/JL) chain variable regions. Further differentiation, transcription and RNA splicing results in the linking of variable sequences with heavy (CH) or light (CL) chain constant region sequences. It appears that immunoglobulin heavy chain variable region gene formation precedes that of light chain, and kappa light chain gene formation precedes that of lambda (47, 48).

Cells at an early stage of B cell commitment lack detectable immunoglobulin. First to appear is cytoplasmic IgM which defines the pre-B

cell. Subsequently, IgM appears on the cell surface (immature B cell) often with IgD (mature B cell). These stages in normal B lymphocyte differentiation have their counterpart in leukemic B cells. Acute lymphoblastic leukemia (ALL) may be subdivided into prognostically significant subtypes based on the expression of cell surface markers and the presence of Ig gene rearrangement. These include the common ALL antigen (CALLA) positive subtype (Ig genes rearranged, lack of detectable Ig), pre-B ALL (cytoplasmic IgM positive) and B ALL (surface Ig positive) subtypes (49).

2) MECHANISMS OF B CELL ACTIVATION

(A) Cell Surface Interactions

Human B cells, like those of the mouse, can be activated by a number of triggering signals acting at the cell surface. Under appropriate influences, B cells once activated, proliferate and differentiate to immunoglobulin secreting plasma cells. The prevailing view of how this process occurs may be summarized as follows (19, 50-73, Figure 3):

Step 1. Helper T cells interact with antigen presented by accessory cells in the context of MHC class II determinants. These T cells are then activated to produce diffusible molecules (lymphokines) that can influence the growth and differentiation of various hematopoietic cell lineages. During the activation process both T cells and accessory cells produce lymphokines to which B lymphocytes respond. T cells produce B cell growth factor (BCGF), interleukin 2 (IL-2), B cell maturation factors, interferons and various erythropoietic and myelopoietic growth factors. Accessory cells produce interleukin 1 (IL-1) and other less well characterized factors. Lymphokines act on essentially all lymphocytes, of appropriate lineage and differentiation state, without regard to antigen specificity (29, 50, 53, 54, 56-59, 69).

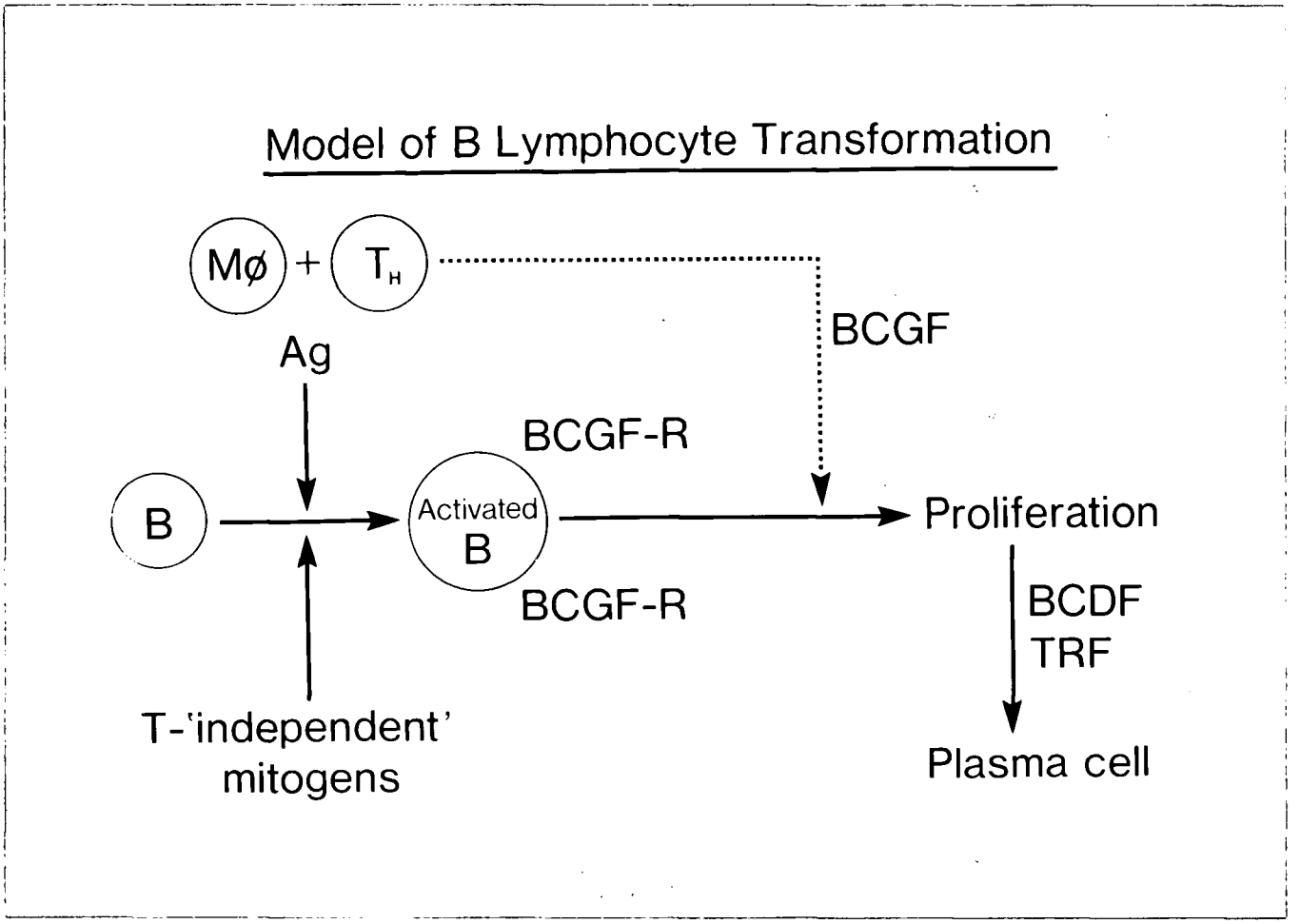

FIGURE 3 Simplified schematic diagram of B lymphocyte transformation. Figure shown illustrates the classical concepts of how normal B cells transform into B immunoblasts in response to antigen or mitogens. According to this model B cells must first be activated by antigen or mitogen before being capable of responding to B cell growth factor (BCGF). Recently, this concept has been questioned. Newer evidence suggests that BCGF (B cell stimulatory factor-1, BSF-1) may induce resting B cells to become more responsive to stimuli such as anti-immunoglobulin. See text for details.

Step 2. The second step in B cell activation involves the transition of the B cell from a resting G0 state to an activated G1 state. B cells may be activated from G0 to G1 by a number of mechanisms including:

(1) activation by specific antigen, (2) activation by polyclonal activators (e.g. lipopolysaccharide, anti-μ), (3) selective activation by antigen-specific, Ia restricted helper T cells, (4) activation by alloreactive T cells, (5) activation by T-independent antigens (e.g. bacterial polysaccharides and Ficoll) (19, 50, 52, 55, 58, 60, 61, 63, 66-68, 70). B cells may also be stimulated by anti-idiotype antibodies (72).

B cells require T cell help to produce specific antibody. Classically, this has been thought to occur through a mechanism by which antigen-specific helper T cells interact with antigen-specific B cells via an antigen bridge. By this concept B cells bind to one determinant on an antigen molecule (the hapten), while the T cells recognize simultaneously another determinant (the carrier). T helper cells may also bind specifically to antigen presenting cells (APC) which have picked up and processed the appropriate antigen (Figure 3). This interaction, like the interaction of T-helper cells with specific B cells is restricted by products encoded by the major histocompatibility complex (MHC class II products). Recent work, however, suggests this model may be oversimplified. It appears that antigen must first be internalized and processed by specific B cells before it is presented to T cells in an MHC-restricted manner, analogous to presentation by conventional APC (e.g. macrophages) (29, 58, 59, 61).

Step 3. This step involves the control of subsequent B cell proliferation and maturation events. Various T cell and accessory cell derived soluble factors act on responsive B cells to induce their proliferation and differentiation into clones of immunoglobulin secreting plasma cells (50, 62, 64, 65, 70 and also see below).

Binding of specific antigen to immunoglobulin receptors on the surface of B cells initiates the activation process leading to proliferation and immunoglobulin production. Anti-immunoglobulin antibodies (especially anti-μ) have been employed as polyclonal B cell activators on the assumption that they mimic the action of multivalent antigens on B cells, by cross-linking cell surface immunoglobulin (74). Lipopolysaccharide (LPS) likewise induces B cell activation by cross-linking surface receptors. However, LPS receptors and surface IgM appear to be distinct molecules, not physically linked to each other in the plane of the cell membrane (69).

Anti-μ antibody binds to and cross links surface membrane IgM on B cells resulting in cellular activation. Depending on the concentration of anti-μ used, two B cell responses may occur (75). At low concentrations of anti-μ, B cells are induced to enlarge and increase RNA synthesis but do not progress into S phase without the addition of T cell derived stimulatory factors. At high concentrations, anti-μ is thought to initiate a direct proliferative effect on B cells independent of T cells, monocytes or their products (13, 63, 75-78). Accessory cells appear able to play a synergistic role in in vitro stimulation of B cells (79-83) but the mechanism(s) involved are not clear.

The biochemical events which occur at the membrane and within the cell after cross-linking of B cell surface immunoglobulin, also remain to be fully elucidated. However, recent evidence suggests that $F(ab')_2$ fragments of antibodies specific for IgM are able to induce changes in B cell physiology that are indicative of cell activation. These include membrane depolarization, followed by increased I-A expression, G0 to G1 transition and thymidine uptake (84). Furthermore, evidence has been provided which supports the hypothesis that cross-linking of B cell surface immunoglobulin

leads to subsequent activation through a series of events including phosphatidylinositol hydrolysis, followed by the generation of diacylglycerol and protein kinase C activation (5, 6). These findings, suggesting that protein kinase C is involved in the regulation of normal B cell activation and proliferation, are intriguing since a number of oncogene products are protein kinases (85). It is tempting to speculate that lymphomatous transformation of B cells may be somehow linked to abnormal regulatory control of these enzymes.

(B) Growth Factors

Growth factors (interleukins, lymphokines), as applied to the lymphoid system, are genetically unrestricted peptides that nonspecifically modulate immunologic and inflammatory responses by regulating the growth and differentiation of a wide variety of cell types (86). These factors are produced primarily by lymphocytes and monocytes. Those factors most relevant to B cell growth and differentiation include interleukin 1 (IL-1), interleukin 2 (IL-2, T-cell growth factor), B cell stimulatory factor (BSF, B-cell growth factor) and a number of less well characterized B cell differentiation factors.

Interleukin 1

The principal sources of IL-1 production are blood monocytes, phagocytic cells that line the liver and spleen, and other tissue macrophages (87). IL-1 has diverse effects on multiple organ systems including the activation of T and B lymphocytes and neutrophils, the induction of the acute phase response and fever. The production of IL-1 may be initiated by microorganisms or their products, antigen-antibody complexes, toxins, injury and various inflammatory processes. IL-1 enhances the production of lymphokines by T lymphocytes. Its effects on B lymphocytes are less clear. However, it

appears that IL-1 can directly augment B lymphoproliferative and antibody-producing responses as well as indirectly augmenting these responses via effects on T cells (86-88).

Interleukin 2

Interleukin 2 (formerly T cell growth factor) is produced by mature helper T lymphocytes appropriately stimulated by antigens or mitogens. IL-2 has been purified and its gene cloned. The molecular weight of IL-2 is approximately 15,000 as judged by SDS-polyacrylamide gel electrophoresis. The major direct function of this T-cell derived factor is to stimulate the proliferation of activated T cells. Using IL-2 it has been possible to continuously propagate normal and neoplastic T cells *in vitro* (86, 89, 90). In order to respond to IL-2, T cells must be induced to express receptors specific for this product. This occurs through an initial signal supplied by a lectin, or antigen in conjunction with accessory cells (90-92). A monoclonal antibody (anti-Tac) has been raised to the IL-2 receptor (93) and the gene coding for this molecule has been cloned and sequenced (94, 95). It appears that the IL-2 receptor may coincidentally be the receptor for human T-cell leukemia/lymphoma virus (HTLV-1) (96).

Recently, activated normal B cells as well as some B cell lines have been reported to express receptors for IL-2 (97). B cells have significantly fewer sites and lower affinity receptors compared to mitogen stimulated normal T cell blasts (98). However, IL-2 can promote the growth and differentiation of normal B cells *in vitro* (99-100). A physiologic role for IL-2 in B cell responses has not yet been demonstrated *in vivo*.

Interleukin 3

Interleukin 3 (burst promoting activity, BPA), a factor whose predominant effects are on the differentiation of cells of myeloid and

erythroid lineages, has been suggested to have a role in the regulation of lymphocyte differentiation and growth (101). However, the significance and relative importance of these observations is as yet unclear. Murine IL-3 has recently been purified to homogeneity and its gene cloned also (102).

B Cell Stimulatory Factors

B cell growth and stimulatory factors have not been as well characterized as the other interleukins. Recently, a committee met and revised the nomenclature related to B cell factors (103). This group proposed that factors that had been well characterized functionally and chemically be given the designation B cell stimulatory factor (BSF) followed by a consecutive number (i.e. 1, 2...n). Unless the factor had been purified to homogeneity or its structure determined from gene cloning and sequencing a "p" for provisional would precede this number. It was decided that the factor previously referred to as B cell growth factor (BCGF) had been sufficiently well characterized to warrant the designation BSF-p1. This factor (human) has a molecular weight of approximately 12,000 by SDS-PAGE. When its purification is complete or its gene cloned, it would be given the designation BSF-1 (103).

BCGF or BSF-p1 has been defined as a T cell derived lymphokine that acts as a co-stimulator of polyclonal B-cell growth in B cells cultured with anti-immunoglobulin (e.g. anti-μ) (104). B cell stimulatory factors have been derived from mitogen stimulated normal T cells, T hybridomas, T leukemia cell lines and HTLV-1 transformed T cell lines and are distinct from IL-2 (104-110). Most early studies supported the concept that anti-μ induces cell enlargement, transition from G0 to G1 of the cell cycle, and expression of receptors for BSF-p1. BSF-p1 then induces entry of the cells into S phase (50, 52, 62-64, 74-78).

This concept has recently been challenged. Evidence has been provided which suggests that BSF-p1 may be a differentiation or activation factor instead of, or in addition to, a growth factor. These studies have shown that BSF-p1 acts upon resting B cells in the absence of anti-immunoglobulin (Ig) antibodies, to prepare these cells to respond to anti-Ig. Anti-Ig, possibly in conjunction with BSF-p1 then induces entry of the cells into S phase (111-114). The differences between earlier and more recent studies apparently relate to methodological considerations. These include culture conditions, cell washing, and timing of the addition of the various factors and antisera to purified populations of B cells. The complete significance of these findings remains to be elucidated. They may, however, account for the inability to clone and reproducibly propagate, normal factor dependent B cells in long term culture, as have been T cells (115-118).

Recently, a monoclonal antibody to BSF-p1 has been prepared. This antibody has allowed characterization of this B-cell factor and clearly shown that it is distinct from IL-1, IL-2 and IL-3. It was recommended that the 'p' for provisional be removed and this lymphokine be henceforth referred to as BSF-1 (119).

B Cell Differentiation Factors (BCDF)

B cell differentiation factors act on post-activated B cells to induce differentiation to immunoglobulin secreting cells. These factors have received a variety of names and may be a family of molecules rather than a single lymphokine. These include: B cell differentiation factor (BCDF), T cell replacing factor (TRF), B maturation factor (BMF) and B cell-derived enhancing factor (BEF) (106, 120-131). These factors, in general, are not well characterized and appear to be heterogeneous. They may be capable of inducing varied responses depending on the B cell subpopulation affected (108).

Autostimulatory B Cell Factors

The concept of autocrine secretion of growth factors in the control of cell proliferation and differentiation has recently gained popularity. B cell growth factors and differentiation factors have been reported to be products of Epstein Barr virus (EBV) transformed and neoplastic B cells (122, 132-136). It has been hypothesized that autocrine growth factors may be important in neoplastic transformation and autogenous growth of a variety of cell types (137, 138). Whether normal adult or embryonic B cells are capable of producing their own autostimulatory factors is not known. A recent report has shown that mycoplasmal contamination of cell lines may also result in a "lymphokine-like" soluble product that induces proliferation and maturation of B cells (139). Therefore, these and other reports claiming to show growth factor activity from cell line supernatants, should be viewed with some skepticism until confirmed free of mycoplasma.

3) CELL SURFACE ANTIGENS ON NORMAL AND NEOPLASTIC HUMAN B-LYMPHOCYTES

(A) Definition Using Monoclonal Antibodies

Monoclonal antibodies have shown considerable application to the study of hematologic diseases. In conjunction with flow cytometry they have provided useful clinical information with regard to: 1. Diagnosis of immunodeficiency disorders. 2. Subtyping of acute lymphoblastic leukemia (ALL). 3. Distinguishing between ALL and acute myelogenous leukemia (AML) and between lymphoid and myeloid blast crises of chronic myelogenous leukemia (CML). 4. Immunologic phenotyping of T and B chronic lymphocytic leukemia and differentiating these entities from reactive lymphocytoses.
5. Immunologic phenotyping of the non-Hodgkin's lymphomas and the separation of reactive from malignant, tissue based lymphoproliferative diseases.

6. Monitoring treatment and detecting residual disease post-treatment for leukemias and lymphomas (140-146). In addition, monoclonal antibodies, in conjunction with immunotoxins or complement, have shown promise in the treatment of hematologic neoplasia using both in vivo and ex vivo treatment protocols (147-149).

Despite major improvements in the objectivity of classifying hematologic neoplasia with monoclonal antibodies, this approach has contributed relatively little to our understanding of the biology of these diseases (150, 151). This is especially true of the B lymphoid system. Recently, a number of monoclonal antibodies have been raised toward B lymphoma cells. These have been evaluated as diagnostic reagents, for their ability to subclassify the lymphomas and to determine if they are able to improve our understanding of this heterogeneous group of diseases. Most of these monoclonal antibodies fall into one of the following categories: B cell restricted (react only with cells of B lymphocyte lineage), B cell associated (react with B cells but also cells of other lineages), blast associated (define antigens present on normal B-blasts but absent from resting B cells), and antibodies whose principal reactivity is with Burkitt's lymphoma cells or EBV transformed cell lines (14-18, 152-170) (see Table I).

The function of the antigens defined by these antibodies is largely unknown with few exceptions. These include: monoclonal antibody B2 which defines the membrane receptor for the complement fragment C3d (CR2) (171, 172); B1 which binds to a 35 Kd B cell activation antigen (173); and AB1 which may react with a receptor for BCGF (161).

TABLE I

Monoclonal Antibodies Defining B Cell and
B Cell Related Cell Surface Determinants

Antibody	Specificity	Reference
AA4.1, GF1.2	B cell subset	McKearn et al, 1984 (14)
LN-1, LN-2	B cell associated	Epstein et al, 1984 (15)
B1, B2, B4, PC-1	B cell restricted	Anderson et al, 1984 (16)
Ia, CALLA, T1, T10	B cell associated	Anderson et al, 1984 (152)
PCA-1, PCA-2		
OKB1	B cell subset	Mittler et al, 1983 (17)
OKB2	B cell associated	Knowles et al, 1984 (18)
OKB4, OKB7	B cell restricted	Nadler et al, 1981 (157)
CB2	SIg positive B cells	Jephthah et al, 1984 (153)
FMC7	B cell subset	Zola et al, 1984 (154) Brooks et al, 1981 (155)
HK-9, HK-19, HK-20	Mature B cells (DR related)	Shipp et al, 1983 (156)
B220	B cells, some bone marrow cells	Sarmiento et al, 1982 (170)
AB1	Activated B cells (BSF receptor)	Jung and Fu, 1984 (161)

TABLE I (continued)

Monoclonal Antibodies Defining B Cell and
B Cell Related Cell Surface Determinants

Antibody	Specificity	Reference
Tac	Activated B cells (IL-2 receptor)	Tsudo et al, 1982 (158)
33.1	Activated B cells	Marti et al, 1982 (159)
BB-1 LB-1	B cell blasts B and T cell blasts	Yokochi et al, 1982 (160)
4F2	Activated lymphocytes, monocytes, embryonic fibroblasts	Kehrl et al, 1984 (163) Sutherland et al, 1981 (164)
5E9	Dividing cells (transferrin receptor)	
B532	Activated B cells	Frisman et al, 1983 (162)
GB 1,2,3,5,6 8,10,11,13,14	Some B cell lymphomas variable reactivity with B cells	Funderud et al, 1983 (165)
38.13	Burkitt lymphoma cells, some B lymphoma/leukemia cells some EBV transformed cell lines	Klein et al, 1983 (166) Lipinski et al, 1982 (167)
41H.16	Normal and EBV transformed B cells, B-CLL	Zipf et al, 1983 (168)
AB89	B cell lymphomas (10%)	Nadler et al, 1980 (169)

(B) The Human Major Histocompatibility Complex (HLA System)

Nomenclature and Genetic Organization

Understanding the human major histocompatibility complex (MHC) began in the 1950's when it was observed that sera from multiply transfused patients and multiparous women contained antibodies reactive with leukocytes of non-identical donors. Since that time our appreciation of the complexity of the HLA system has increased enormously. The human HLA complex is located on the short arm of chromosome 6. Genes located in the MHC region encode at least three families of molecules. Class III molecules are elements of the complement system and will not be further discussed. Class I molecules (HLA-A, B and C antigens) and class II molecules (HLA-D or D-related antigens) comprise what is commonly referred to as the HLA system (174-177).

Class I antigens are located on all nucleated cells and are composed of a 44 Kd transmembrane glycoprotein noncovalently associated with the 12 Kd protein β_2-microglobulin (encoded on human chromosome 15). Class I antigens are principally detected in vitro by complement mediated antibody dependent cytotoxicity (174-177). Using serologic techniques HLA class I antigens have been shown to be extremely polymorphic with at least 20 distinct alleles at the A locus, over 40 distinct alleles at the B locus and approximately 8 alleles at the C locus (177). They are the principal antigens recognized by the host during graft rejection. The physiological role of class I antigens appears to involve the restriction of recognition of cell surface antigens (such as viral antigens on infected cells) by cytotoxic T lymphocytes; i.e. T cells which are exposed to virus infected cells must see those cells in the context of both the virus and identical class I antigens in order to generate cytotoxicity. Evidence has suggested the cell surface antigen defined by monoclonal antibodies OKT8/Leu-2 may be involved in the recognition of class I molecules (26, 178, 179).

The HLA class II antigen system appears to be even more complex. Class II antigens are heterodimeric transmembrane glycoproteins composed of a heavy (α) chain of 33-35 Kd and a light (β) chain of 27-29 Kd. Class II antigens are found principally on B lymphocytes, activated T lymphocytes and antigen presenting cells (macrophages, monocytes, dendritic cells, etc). They are also present on some myeloid cells and their precursors. Low levels of class II antigens have been reported on renal tubule cells and on endothelial cells. Class II antigens have also been reported on tumor cells including melanomas and gliomas (174, 180, 181). Factors present in mitogen activated T cell supernatant as well as pure γ-interferon are capable of inducing class II antigen expression on class II negative macrophages, endothelial cells and melanoma cells (174, 182, 183).

Biochemical and molecular genetic analyses of the genes and gene products associated with the HLA-D region indicate the existence of at least three groups of products (180). The nomenclature for these products was recently revised (177). The designations now include HLA-DR, HLA-DQ (formerly DC, DS, MB) and HLA-DP (formerly SB). The genes coding for many of the class II products have been cloned and amino acid sequences of their protein products determined. A combination of serology, biochemistry and molecular biology indicates there are multiple class II genes coding for at least seven light (β) chains and at least six heavy (α) chains within the D region (184). Recent evidence supports the existence of at least one alpha and two or three beta chain genes within HLA-DR, two alpha and two beta chain genes within DQ, two alpha and two beta chain genes within DP, as well as an additional alpha chain which has previously been termed DZ-alpha (180, 185-188). It appears that the same heavy chain may associate with light chains from more than one locus and that determinants may be shared between

different chains (180). The extensive polymorphism of class II antigens detected by serological, functional and structural studies appears to be mainly restricted to the β subunits (187).

Due to random matings, the frequency of association of one HLA allele with another located at a different locus should simply be the product of the frequencies of each allele in the population. However, certain combinations of alleles are found with a frequency much greater than expected. This phenomenon is termed "linkage disequilibrium". Several hypotheses have been proposed to explain the occurrence of linkage disequilibrium, although none of these is entirely satisfactory. These include: (1) a selective advantage of a given haplotype, (2) migration and admixture of different populations, (3) inbreeding, and (4) random drift (174).

Part of the difficulty in sorting out the individual products of the HLA-D region has been due to the occurrence of DR and DQ antigens in close linkage disequilibrium (i.e. an unexpected association of linked genes in a population). DP does not appear to be in linkage disequilibrium with DQ or DR (180). By biochemical criteria, human DR antigens appear homologous to murine I-E antigens; DQ antigens appear homologous to I-A. DP antigens appear intermediate in homology, although a murine counterpart for DP has not yet been identified (180, 181).

The HLA-D region was originally defined by cellular typing using the mixed lymphocyte response (MLR). The major determinants inducing the MLR probably reside on HLA-DR molecules. However, class II antigens other than HLA-DR may also induce an MLR. Therefore, what has previously been termed HLA-D may actually be the sum total of responses to several different class II antigens and not, by itself, a distinct entity (174).

DP (SB) antigens were originally defined by the secondary MLR (primed lymphocyte typing, PLT). Now, at least some DP antigens may be detected by monoclonal antibodies (184, 189).

The functions of class II antigens remain to be fully elucidated. However, they appear to be key elements in the control of immune responses and determine several immunologic phenomena including: control of the level of the immune response, delayed type hypersensitivity, susceptibility to certain diseases and primary (MLR) and secondary (PLT) allogeneic T cell proliferation (174, 180, 181, 190). HLA class II antigens appear particularly important in the recognition of antigens by regulatory T lymphocytes. In order to respond, T helper cells must recognize antigens in the context of appropriate class II molecules (i.e. they show MHC class II restriction) (191). This ability to recognize class II determinants may be related to the T lymphocyte molecule defined by OKT4/Leu-3 monoclonal antibodies (192).

Monoclonal Antibodies Directed Against HLA Determinants

A number of monoclonal antibodies directed against HLA class I and class II antigens have been described. Most of these have been directed against monomorphic determinants. Some detect polymorphic class I and class II determinants (193-203). Most early reports of monoclonal antibodies directed against class II antigens reported them as "anti-DR". With the increased appreciation of the complexity of the MHC it is preferable to refer to these as anti-class II monoclonal antibodies until it is known whether these react with DP, DQ or DR antigens. This can be determined by sequential immunoprecipitation, cross-blocking studies and by testing these antibodies against appropriate panels of DR homozygous cell lines or cell lines transfected with DP, DQ or DR genes (189, 198).

Three anti-HLA-DQ monoclonal antibodies have been described and characterized in detail (198). These are Genox 3.53, BT3/4 and anti-Leu-10. These antibodies were shown to react with a different population of molecules (DQ) than did the HLA-DR specific monoclonal L243. When tested against DR homozygous cell lines Genox 3.53 reactivity correlated with DR1,2,6; BT3/4 reactivity correlated with DR1,2,4,6,8 and anti-Leu-10 reactivity correlated with DR1,2,4,5,6,8 and 9. These antibodies most likely define different polymorphisms of DQ molecules (197, 204).

Monoclonal antibodies against HLA class II antigens have been very useful in delineating expression of these antigens on various cell subpopulations, as well as in increasing our understanding of the structure and function of these molecules. Different roles for DP, DQ and DR molecules in the immune response have not been definitely established (205-214). However, these differences may be important since studies of DQ antigen expression on monocytes and macrophages have revealed functional differences between DQ^+ and DQ^- subpopulations. DQ positive monocyte subpopulations are involved in antigen presentation and stimulation in the autologous mixed lymphocyte reaction, while DQ negative subpopulations are not (196, 215, 216). In addition, a unique pattern of expression of class II antigens on myeloid progenitor cells has been reported. These cells appear to be DR positive but DQ negative (217-220). The physiological significance of this latter observation is not yet clear.

In summary, the MHC codes for two classes of cell surface molecules which are involved in a variety of crucial immunologic responses. The inherent complexity of this system is just now beginning to be appreciated. Recent advances have allowed the determination of the genetic and molecular structure of these antigens and their genes. Monoclonal antibodies have been

very useful in delineating subpopulations of HLA class I and class II molecules and correlating these with various immune functions.

(C) Lymphocyte Function Associated Antigen (LFA) Family of Molecules

Lymphocyte function-associated antigen 1 (LFA-1), Mac-1 and p150,95 cell surface molecules constitute a novel family of structurally and functionally related glycoproteins. Each molecule contains a common beta chain (mw = 95 Kd) noncovalently associated with an alpha chain. The alpha subunits have molecular weights of 177 Kd (LFA-1), 165 Kd (Mac-1) and 150 Kd (p150,95), different isoelectric points, and are immunologically non-cross-reactive. The α and β subunits appear to be synthesized intracellularly as distinct α^1 and β^1 precursors. These then associate into $\alpha^1\beta^1$ complexes, are processed, and transported to the cell surface in the mature $\alpha\beta$ form. The α subunits have been suggested to bear determinants that govern the specificity of cell interactions, while the identical beta subunits may mediate a common function such as signal transduction (221-223).

LFA-1 is expressed on lymphocytes, monocytes, large granular lymphocytes, weakly on granulocytes and on approximately 35% of bone marrow cells. Mac-1 (Mo-1, OKM-1) is found on monocytes, granulocytes and large granular lymphocytes. P150,95 is expressed on monocytes, lymphocytes and strongly on granulocytes. Human LFA-1, like mouse LFA-1 is present on both B and T lymphocytes, although quantitatively greater amounts are found on T cells. Also, there is a 3 to 4-fold difference in the quantitative expression of LFA-1 on lymphocytes. Whether there are functional implications to these differences is not known (224-226).

The Mac-1 molecule (also identified by OKM-1 and Mo-1 monoclonal antibodies) appears to be identical to the complement receptor type 3 (CR3).

The identities of LFA-1 and p150,95 have not been established. However, the cellular distribution of these molecules suggest they may be important in a wide variety of T cell, B cell, granulocyte and monocyte functions (221).

Monoclonal antibodies directed against determinants on the LFA-1 molecule inhibit a number of in vitro immune functions. These include: cytotoxic T lymphocyte (CTL) killing, natural killer (NK) cell activity, T cell proliferative responses to antigen, mitogens and allogeneic cells, and antibody dependent cellular cytotoxicity (ADCC). The role of LFA-1 in B cell function has not been well characterized. However, monoclonal anti-LFA-1 antibodies have been shown to inhibit T cell dependent plaque forming cell responses, but not T cell independent responses. Also, in the mouse these antibodies did not inhibit lipopolysaccharide (LPS) induced B cell proliferation (227-236).

Most detailed studies on the role of LFA-1 in immune function have centered on the relationship of this molecule to CTL mediated killing. Anti-LFA-1 monoclonal antibodies appear to block CTL mediated killing by inhibiting adhesion between the CTL and the target cell. It has been hypothesized that LFA-1 may participate in the Mg^{+2}-dependent adhesion step of CTL mediated killing and that the LFA-1 cell surface structure is involved in strengthening effector-target cell adhesion. Quantitative differences in the ability of different anti-LFA-1 monoclonal antibodies to block cytolysis indicate distinct functional and antigenic epitopes exist on the LFA-1 molecule. With respect to CTL function anti-LFA-1 blocks killing by binding to effector cells rather than target cells (227-236).

Recently, the structure of the α subunit of LFA-1 has been partially determined by N-terminal amino acid sequencing. Sequence homology shows that the α subunits of all members of the family are related and suggests their

evolution occurred by gene duplication. A further unexpected homology was found between LFA-1 and leukocyte (α) interferon. The significance of this homology is not known (237).

Clinical Implications

Recently a hereditary disorder in which patients manifest multiple recurrent bacterial infections, progressive periodontitis and impaired wound healing has been described. These patients have an inherited deficiency of the Mac-1, LFA-1, p150,95 glycoprotein family on their cell surfaces. Patients have severe impairment of adherence and adhesion dependent cell functions. Immune abnormalities described include: defective antibody dependent cellular cytotoxicity (ADCC), natural killer (NK) cell function, phagocytosis, neutrophil migration and mitogen stimulation (238-244). In some studies the defect in phagocyte function has been more profound than lymphocyte function (238). However, increasing evidence supports the clinical pathologic importance of lymphoid cell function in this disorder (221).

Monoclonal antibodies directed against this family of molecules have been able to reproduce in vitro many of these defects when co-cultured with normal cells (238-242). Family studies suggest this disorder is inherited as an autosomal recessive. Biosynthetic experiments have shown the presence of normal amounts of α^1 (LFA-1) intracellular precursor. This in conjunction with the absence of all three members of the family, each with a different α chain but a common β chain suggests the primary deficiency is of the β subunit (221).

The importance of this family of molecules is further emphasized by the recent observation that granulocytes may markedly increase their expression of Mo-1 after appropriate stimulation. During degranulation Mo-1α , located

in specific neutrophilic granules, is translocated to the plasma membrane. This results in a 5-10 fold increase in surface expression of this glycoprotein. Clinically, patients may show a 5 fold increase in Mo-1 expression within minutes after beginning renal dialysis. This enhanced expression of Mo-1 may provide a mechanism for initiating leukocyte aggregation and sequestration and explain the neutropenia of dialysis (243, 244).

4) NEOPLASMS OF THE IMMUNE SYSTEM: THE NON-HODGKIN'S LYMPHOMAS

Classification

The concept of a pluripotential progenitor cell (the reticulum cell) was invoked in the earlier part of this century to attempt to conceptually explain morphologically and clinically diverse types of lymphoid malignancies (245, 246). The distinction between the various types of lymphomas was based primarily on cell size. The terms "reticulum cell sarcoma", "lymphosarcoma" and "giant follicular lymphoma" were in popular usage. Malignancies of small lymphocytes were termed lymphosarcoma; those composed of larger cells were designated as reticulum cell sarcoma (247). There was considerable difficulty correlating the various subtypes of lymphoma with patient survival. In addition, these subcategories each included biologically unrelated disease entities.

In 1956, Rappaport proposed a classification of the non-Hodgkin's lymphomas which was prognostically relevant and made pathologic subtyping relatively easy (248). The reasoning behind this revision of the older classification was that the "reticulum cell" could not be identified as a precise entity and that previous classifications failed to provide sufficient prognostically and therapeutically useful information.

Rappaport's classification was based on morphology. The degree of presumed differentiation and the similarity of the malignant cells of the various lymphomas to what was thought to be their normal cellular counter-

parts were the criteria by which these malignancies were subtyped. Individual cells of large cell lymphomas were thought to resemble histiocytes; "histiocytic lymphoma" replaced "reticulum cell sarcoma" as a diagnostic category. Lymphomas composed of small normal appearing lymphocytes were termed "well-differentiated lymphoma" replacing the older term "lymphosarcoma". Lymphomas were classified as either well differentiated or poorly differentiated depending on whether the cell size and nuclear configuration more or less resembled that of normal lymphocytes. Further categories were created to include lymphomas that appeared to be composed of more than one cell type (mixed lymphocytic-histiocytic lymphoma) and those that appeared especially primitive or "undifferentiated". Rappaport also demonstrated that a nodular (follicular) pattern of growth within a given subgroup of lymphoma was a prognostically favorable feature (248).

Subsequent clinicopathologic studies demonstrated that the histopathologic classification proposed by Rappaport was relevant prognostically and useful in the clinical management of patients with non-Hodgkin's lymphomas (249-251). Generally, lymphomas composed of larger, more "poorly differentiated" cells carried the worst prognosis. Well-differentiated lymphocytic lymphoma carried the best prognosis, histiocytic or undifferentiated lymphomas the poorest, poorly differentiated and mixed lymphomas were intermediate in their clinical outcome.

Advances in immunology have markedly changed older concepts of the malignant lymphomas. These neoplasms are now known to involve T and B lymphocytes (252-254). The Rappaport classification was proposed before T and B cells were defined as distinct functional subpopulations and the phenomenon of lymphocyte transformation recognized. These older classification schemes became obsolete in light of the modern appreciation of the functional complexity of the immune system.

Lymphocytes may be functionally divided into cells of T and B lineage. These exist as small cells with dense nuclear chromatin, round nuclei and barely discernable nucleoli until stimulated to transform by antigen or mitogen. Transformed cells develop characteristics of "blasts" (large size, fine nuclear chromatin, prominent nucleoli). Individually, transformed lymphocytes appear similar or identical by light and electron microscopy to cells of various non-Hodgkin's lymphomas. Lymphoid neoplasms are characterized by the clonal proliferation of cells. In contrast, reactive cell populations tend to be morphologically and functionally heterogeneous (252-254).

In the past decade, major advances have taken place in the understanding of the biology of normal lymphocytes. These changes have taken place pari passu with advances in immunologic techniques that have permitted the dissection of the immune system into its functional components. Newer classifications of the lymphomas have evolved in parallel with greater appreciation of the functional complexities of normal lymphocytes (252-261). These proposals remain hypotheses based on data indicating: (1) that morphologically homogeneous populations of lymphoid cells are functionally heterogeneous (B cells, T cells and their subsets) and, (2) that lymphocytes during the course of their immunoregulatory and effector cell functions may undergo a variety of morphologic changes reflecting their state of activation and differentiation (262-267). Recent reports suggest that immunologic studies may assist in recognizing clinically relevant subgroups of non-Hodgkin's lymphomas (268-274).

The revolution in the terminology of lymphomas began with the work of Lukes and Collins in the United States and Lennert in West Germany in the early 1970s (252-254, 259). Since that time many others (Dorfman, Rappaport etc) have contributed significantly toward increasing the understanding of the biology of the lymphomas. However, in North America the classification

of Lukes and Collins has gained the most widespread popularity. They proposed a new concept that related the malignant lymphomas to the T and B lymphocytic systems and alterations in lymphocyte transformation. The in vivo counterpart of B lymphocyte transformation in vitro was hypothesized to occur in the follicular centers of lymph nodes - from which is derived the term follicular center cell lymphoma. T-cell transformation occurs outside of the follicles (Figure 4). According to this new reasoning, cell size and nuclear configuration are not necessarily related to the degree of differentiation of lymphoma cells, but instead reflect the point along the lymphoid transformation continuum that malignant change occurs. A "block" or "switch-on" of cells at discrete stages of normal B lymphocyte differentiation may, by this concept, result in the morphologic expression of malignancy. Thus, histiocytic lymphomas are not composed of histiocytes but rather are the neoplastic counterpart of the large transformed lymphocyte. Immunologic marker studies and other techniques have supported these observations (254, 264, 266).

The Lukes' classification attempts to synthesize morphology and function. Using immunologic markers, lymphomas are separated into those of T cell, B cell and true histiocytic (macrophage) types. A small proportion are undefined or unclassifiable by currently available techniques. The T and B cell lymphomas are further subdivided based upon their morphologic appearance and relationship to lymphocyte transformation (252-254, 259, 265). A comparison of the major classifications is found in Table II. Recently, a prognostically relevant synthesis of the leading classifications (based on morphology alone), has been proposed. This was termed the "international working formulation" and was not intended to be a classification scheme, but rather a vehicle by which different classifications may be compared (256, 274).

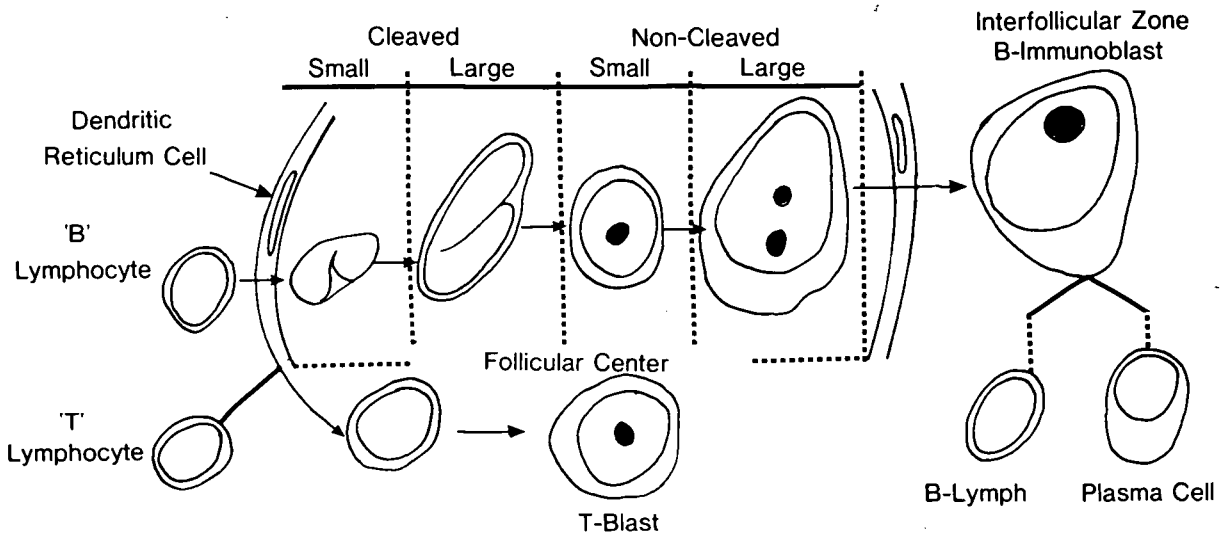

FIGURE 4 Follicular center cell concept of lymphocyte transformation. According to this hypothesis, normal B cells pass through a series of morphologic stages within the follicular centers of lymph nodes. B cell lymphomas may be classified according to which subtype of cell predominates. The predominant cell type, within a given lymphoma, may correspond to one of the stages in normal B cell transformation illustrated (252-254).

TABLE II

A Comparison of the Proposed "Working Formulation" with Classifications for Non-Hodgkin's Lymphomas (274)

WORKING FORMULATION	RAPPAPORT	LUKES-COLLINS
Low Grade		
A. Small lymphocytic	A. Well differentiated lymphocytic	**? U Cell (Undefined)**
		B Cell
B. Follicular, small cleaved	Poorly differentiated lymphocytic	A. Small Lymphocytic
	B. nodular	A. Plasmacytoid Lymphocytic
C. Follicular, mixed small cleaved and large cell	E. diffuse	Follicular Center Cell Types (follicular or diffuse)
Intermediate Grade	Mixed histiocytic-lymphocytic	
D. Follicular, large cell	C. nodular	(B or E) Small cleaved
	F. diffuse	(D or G) Large cleaved
E. Diffuse, small cleaved		(D or G) Large noncleaved
	Histiocytic	(J) Small noncleaved
F. Diffuse, mixed, small and large cell	D. nodular	H. Immunoblastic Sarcoma
	G., H. diffuse	**T Cell**
G. Diffuse, large cell	I. Lymphoblastic	
High Grade	Undifferentiated	A. Small lymphocytic
H. Large cell, immunoblastic	J. Burkitt's	I. Convoluted lymphocytic
	J. pleomorphic	? Cerebriform (cutaneous)
I. Lymphoblastic (convoluted or nonconvoluted)		F.G. Lymphoepithelioid cell
		H. Immunoblastic sarcoma
J. Small noncleaved cell		**Histiocytic**
Others		
Hairy Cell, Cutaneous T-Cell, etc		

Etiology

Very little is known about the etiology of the non-Hodgkin's lymphomas (275). Epstein-Barr virus (EBV) has been shown to be present within the tumor cells of patients with the endemic form of Burkitt's lymphoma; a causal relationship is suspected but not proven (276). Also, the majority of Burkitt's lymphomas are associated with a characteristic chromosomal abnormality t(8;14) in which the myc oncogene, located on chromosome 8, is translocated to the region of the immunoglobulin heavy chain gene on chromosome 14. Variants occur in which myc is translocated to the region of the kappa t(2;8) or lambda t(8;22) light chain genes (277-283). These three different chromosomal rearrangements result in a deregulation of c-myc so that it is expressed at high levels, while the normal c-myc oncogene on the uninvolved chromosome 8 is transcriptionally silent (284-286). The existence of enhancer elements has been postulated, within the three immunoglobulin loci genes. These enhancers are thought capable of activating transcription of the translocated c-myc which then may result in neoplastic transformation (284). Other oncogenes (e.g. ras) and less well characterized transforming sequences may also play a role in certain lymphoid malignancies (287, 288).

A number of characteristic chromosomal abnormalities have been described in B cell lymphomas and leukemias. Some of these show a tendency to associate with specific histologic subtypes (289-292). However, these associations are not invariant. Recently, recombinant DNA probes were utilized to detect DNA rearrangements in cases of follicular (B cell) lymphoma. These probes detected a gene (bcl-2 gene) which seems to be interrupted in most cases of follicular lymphomas carrying the t(14;18)

chromosomal translocation. It was speculated that the bcl-2 gene may have a role in the pathogenesis of this subtype of lymphoma (293). Whether there are analogous chromosomal defects in other lymphomas remains to be determined.

The concept of autocrine secretion of growth factors as a major contributor to the evolution of neoplastic cell populations was first postulated by Todaro (294). More recently, B cell growth factors and differentiation factors have been reported to be products of neoplastic and EBV transformed cell lines (122, 132-136). Whether autocrine secretion of growth factors is important in the pathogenesis of B cell malignancy is not yet known.

The putative causative agent of some human T cell leukemias and lymphomas is the human T lymphocytic virus (HTLV-1). Infection with this retrovirus does not invariably result in malignancy. The mechanism by which HTLV-1 might induce neoplasia is not known (295-297).

In summary, the non-Hodgkin's lymphomas are a pathologically, clinically and immunologically diverse group of diseases. There is very little known about the mechanisms involved in the neoplastic transformation process. The growth requirements of the non-Hodgkin's lymphomas are also unknown. Whether lymphoma cells secrete autostimulatory growth factors or are factor independent remains to be determined. Since many cellular, growth factor and viral interactions occur at the cell surface, identification and characterization of molecules characteristic of or unique to lymphoma cells is of obvious biologic interest.

5) THESIS OBJECTIVES

The cell surface is involved in many events which are crucial to the function of normal B lymphocytes. These include: cell activation and proliferation, cell-cell interactions, growth factor-receptor binding and the regulation of metabolic processes. A role for cell surface molecules in the neoplastic transformation of B lymphocytes has been postulated but not documented. Monoclonal antibodies are powerful tools for delineating cell surface determinants due to their exquisite specificity.

The purpose of this research is to:

1. Characterize cell surface antigens on normal, activated and neoplastic lymphocytes utilizing monoclonal antibodies as probes.

2. Determine the functional importance of these molecules in B lymphocyte activation.

3. Raise monoclonal antibodies specific for neoplastic or blast associated lymphocyte antigens, with the object of defining those events underlying neoplastic transformation.

4. Ultimately utilize these monoclonal antibodies in the classification and therapy of the non-Hodgkin's lymphomas.

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C H A P T E R II

MATERIALS AND METHODS

"Beware that you do not lose the
substance by grasping at the shadow."

Aesop, 550 B.C.

1) CELLS

Light density mononuclear cells were obtained from peripheral blood from normal volunteers after separation on 1.077 gm/cm^2 Ficoll-Hypaque (LSM, Litton Bionetics, Bethesda, MD). Cells were obtained from tissues (lymph nodes, spleens etc) by gently teasing these apart, using forceps, into RPMI 1640 + 10% FCS under aseptic conditions. The larger fragments were then gently pressed through a fine wire mesh to further release cells and remove larger pieces of tissue. Bone marrow specimens were obtained in Heparin and the mononuclear fraction separated as above on Ficoll-Hypaque. Tissues and bone marrows were obtained from patients as part of their routine diagnostic evaluation. Cells were then washed in RPMI 1640 with 10% FCS and prepared for cell culture or FACS analysis (see below).

B-blasts were prepared by culturing splenic lymphocytes from a kidney donor in Falcon flasks at 10^6 cells/ml in RPMI 10% FCS with 100 µg/ml of lipopolysaccharide (LPS, E. coli, Sigma, St. Louis, MO) for four days. T cells were depleted by E-rosette sedimentation using aminoisothiouronium bromide (AET) treated sheep red blood cells (SRBC) (1). The remaining cells were 90% surface immunoglobulin positive B cells and were large in size as

judged by forward scatter on the FACS. Morphologic examination of stained cytospins showed that these cells were predominantly large lymphoblasts. Cells prepared in this way were < 5% T cells and monocytes as shown by staining with Leu-5 and Leu-M3 (Becton Dickinson, Mountain View, CA).

9 EBV cell lines were the generous gifts of Drs. John Hansen and Paolo Antonelli (Genetic Systems, Seattle, WA) and the 8 lymphoma lines the kind gifts of Drs. Alan Epstein (Univ. S. California, Los Angeles, CA), Jun Minowada (VA Medical Center, Hines, IL) and the late Dr. Henry Kaplan (Stanford Univ., Stanford, CA) (2-5). All cell lines were maintained in RPMI 1640 medium plus 10-15% FCS and subcultured twice weekly or as required.

2) MONOCLONAL ANTIBODIES

(C3HXBALB/c) F1 (bred in our colony) or BALB/c (Charles River Laboratories) mice were immunized with DHL-10 cells (DH-84, DH-224), DHL-10 membranes (LM-26, LM-155) or LPS stimulated spleen cells from a patient with B cell lymphoma and splenic involvement (NB-29, NB-65). Cells or membranes (10^7 cells or equivalent membranes) were homogenized in Freund's complete adjuvant (Difco Labs, Detroit, MI) and injected intraperitoneally (IP) into mice. Two IP injections were given at least 3-4 weeks apart. Cells (2 x 10^7) or equivalent membranes in saline were administered intravenously (IV) 3-4 weeks after the last IP injection. Cell fusion took place the fourth day after IV boost according to established procedures (6).

Cell membranes were prepared by washing 2 x 10^9 DHL-10 cells with phosphate buffered saline (PBS) then resuspending these cells in 10 mM Tris HCL pH 8.0 plus PMSF (phenylmethylsulfonyl fluoride, Sigma, St. Louis, MO) (7). Cells were homogenized with a syringe and 21 gauge needle, spun at 2,000 RPM for 15 minutes to remove nuclei and intact cells. The supernatant

was overlayed on 40% sucrose in dH_2O and spun at 25,000 RPM for 60 minutes. Membranes were removed from the interface and washed with Tris HCL. The protein content of the pellet was measured at 280 nm, and the crude membrane material aliquoted and frozen at -70°C until used.

Prior to fusion, NS-1 cells were grown in Dulbecco's minimal essential medium (DMM) plus 5% fetal calf serum (FCS) in spinner flasks. Cells were washed 3 times in DMM immediately prior to fusion. The spleen was removed aseptically from an immune mouse. Cells were gently teased into DMM, filtered to remove large clumps and washed. Spleen cells and NS-1 cells were mixed at a ratio of 10:1, diluted to 50 ml in DMM and spun. After removing the supernate, 1 ml of 50% polyethylene glycol 1500 (PEG)/DMM was added slowly over one minute with constant agitation. Nine ml of DMM was then added slowly over the next five minutes. Fused cells were spun at 1,200 RPM for 5 minutes and resuspended slowly in 5 ml DMM + 15% FCS. Cells were then resuspended in 100 ml DMM + 15% FCS and 1 ml aliquoted into each well of plastic multiwell (24) plates (Flow Labs, McLean, VA). One ml of normal syngeneic spleen cells used as feeders were dispensed into each well at a concentration of 2×10^5 cells/ml. Cells were cultured in a 37°C incubator at 5% CO_2 and 100% humidity. On day one post fusion approximately 1 ml of supernate was removed from each well and replaced with hypoxanthine-aminopterin-thymidine (HAT) media. This procedure was repeated on days 3 and 6. On day 8 or 9 post fusion cells were switched by a similar procedure to hypoxanthine-thymidine (H-T) media. Reagents for HAT and H-T media were obtained from Sigma, St. Louis, MO. Stock HAT was made by dissolving 0.65 g hypoxanthine, 0.0095 g aminopterin and 0.195 g thymidine in 500 ml of 0.01 N NaOH dH_2O. Stock H-T was prepared by dissolving 0.135 g hypoxanthine and 0.039 g thymidine in 100 ml of NaOH. Stock solutions were diluted 1:100 with

media prior to use. Final concentrations in culture of reagents were hypoxanthine (13 μg/ml), aminopterin (0.19 μg/ml) and thymidine (3.9 μg/ml). Wells were observed daily for hybridoma growth. When hybrids appeared well established, supernatants were screened for antibody activity using the binding assay described below. Antibody positive cups were transferred to DMM 15% FCS in individual flasks and cups and rescreened prior to cloning.

Hybridomas were plated in methylcellulose media (40 ml 2% methylcellulose in alpha media + 1 ml glutamine + 1 ml 2 mercaptoethanol + 10 ml FCS + 30 ml DMM). Visible colonies were detected in approximately one week, plucked and transferred to individual multiwell plates. When grown, cultures were rescreened for antibody activity and the cloning process repeated. Hybrids were grown in DMM gradually reducing the concentration of FCS to 5%. Cells were frozen at appropriate stages of the cloning process and after double cloning, thawed and viability checked. Antibody subclasses were determined by Ouchterlony immunodiffusion in 1.2% agar using goat anti-mouse IgG subclass specific antisera (Tago Inc., Burlingame, CA). Antibodies which preferentially bound to large B-lymphoma cells (DHL-10) compared to small B-lymphocytes (CLL cells) by binding assay or FACS analysis (see below) were selected for further characterization.

3) PREPARATION OF ASCITES

Hybrid clones were thawed and grown in DMM + 5% FCS. Cells were washed four times with normal saline, resuspended in saline and then injected IP into pristane (2, 6, 10, 14-Tetramethylpentadecane, Aldrich Chem. Co., Milwaukee, WI) primed mice (2×10^7 cells per mouse). Mice were primed with 0.5 cc of pristane IP 1 week prior to being injected with hybridomas. After 1 to 2 weeks ascites were collected, spun at 2,000 RPM for 10 minutes and stored at $-20^{o}C$.

4) PURIFICATION OF ANTIBODY

Equal volumes of immune ascites and saturated ammonium sulfate were mixed and stirred for one hour at room temperature. The mixture was spun at 10,000 RPM for 15 minutes and the supernate removed. The precipitate was then dissolved in 20 mM phosphate buffer pH 8.0 and dialyzed overnight against the same buffer. A DEAE Affi-Gel Blue column (Bio-Rad, Richmond, CA) was prepared and washed sequentially with 0.5 M phosphate buffer pH 8.0 and 20 mM phosphate buffer pH 8.0. Dialyzed antibody was loaded onto the column followed by 20 mM phosphate buffer. Samples were collected in a fraction collector and each fraction individually analyzed for protein concentration at OD 280 nm, binding activity to appropriate cell lines, and SDS-PAGE gel electrophoresis. Aliquots containing antibody were pooled and frozen at -20°C. Preparation of rabbit anti-mouse immunoglobulin $F(ab')_2$ (RaMIg) was by affinity purification using mouse immunoglobulin conjugated to sepharose beads (8).

5) BINDING ASSAYS

Target cells (e.g. DHL-10, CLL) were washed in Earl's balanced salt solution (EBSS) + 0.5% BSA + 10 mM HEPES + 0.1% azide (binding assay media, BAM). Cells were resuspended at 10^7 per ml in the above media. 10^6 cells per well were aliquoted into microtiter wells, spun, and the supernate removed. 50 µl of hybridoma supernate was added per well in duplicate. After a 1 hour incubation at 4°C, cells were washed twice in BAM. 10^5 cpm of ^{125}I labeled $F(ab')_2$ rabbit anti-mouse immunoglobulin in 50 µl of BAM was added to each well. After a one hour incubation at 4°C, cells were washed three times and resuspended in 100 µl of BAM. They were then transferred to tubes and counted on a Beckman model 5500 gamma counter. Supernates showing

> 1,000 cpm were considered positive relative to negative control values of < 300 cpm.

6) ANTIBODY COUPLING PROCEDURE

One ml of Affi-Gel-10 beads (Bio-Rad, Richmond, CA) were packed into a column per 5 mg of antibody available for conjugation. Beads were washed with 3 bed volumes of isopropyl alcohol, followed by 3 bed volumes of cold deionized water and 0.1 M $NaHCO_3$ pH 8.0. Purified antibody and beads were mixed and incubated overnight at 4^oC with continuous shaking. Beads were then washed with at least 10 volumes of coupling buffer, resuspended in binding assay media and stored at 4^oC.

7) ANTIBODY LABELING

^{125}I labeling was performed according to established procedures (9). A p30 column was prepared by placing a small piece of glass wool in a pasteur pipette which was then filled with a p30 sizing gel (Bio-Rad, Richmond, VA). The gel was washed with BAM to saturate protein binding sites, followed by phosphate buffered saline (PBS). 25-50 µg of antibody in 25-50 µl was added to 10 µl of chloramine T solution (0.5 mg/ml in dH_2O, Sigma) in a small microfuge tube. To this was added 1 mC: ^{125}I (as NaI, Amersham Int. Ltd., Amersham, U.K.) followed by a 15 minute incubation at room temperature. 50 µl of sodium bisulphite (20 µg/ml in PBS, Sigma) was added to stop the reaction. The mixture was added to the column of p30 followed by PBS. Fractions were collected in tubes and a gross estimate of the radioactivity made using a Geiger counter. Fractions containing labeled antibody were pooled diluted with BAM and stored at 4^oC.

^{3}H-lysine labeling: 5 x 10^{6} hybridoma cells were washed in lysine free DMEM media containing glutamine + 10% dialyzed FCS. Cells were resuspended in 5 ml of the same media containing 1 mCi ^{3}H-lysine (New England Nuclear, Boston, MA) and incubated 6-8 hours at 37°C. An additional 5 x 10^{6} cells were then added and the mixture incubated overnight at 37°C. Cells were removed by centrifugation. To the supernatant an equal volume of saturated ammonium sulfate was added, the mixture microfuged and the precipitate dissolved in saline. This procedure was repeated 3 times. Finally, the precipitate was redissolved in BAM and dialyzed to remove unbound ^{3}H-lysine.

8) STIMULATION ASSAYS

Mitogen stimulation assays were performed by modifying existing procedures (10, 11). Cells were separated as above and resuspended in RPMI 1640 + 2 mM glutamine + Na pyruvate (110 mg/liter) + 10 mM HEPES buffer + 5% FCS at a concentration of 2 x 10^{6} cells/ml. 0.1 ml (2 x 10^{5} cells) were placed in wells of flat bottom microtiter plates (Linbro, Flow Labs, McLean, VA). LPS (150 μg/ml) (E. coli, Sigma, St. Louis, MO) PHA (2%) (Gibco, Chagrin Falls, OH) or anti-μ (150 μg/ml) (Cappel Labs, Cochranville, PA) were diluted in the above media. 0.1 ml of mitogen was added to each of triplicate wells to give a final volume of 0.2 ml. Control wells contained cells without mitogen in the same volume. Cultures were incubated for 3 days (PHA) or 4 days (LPS, anti-μ) at 37°C, 5% CO_2 and 100% humidity. One μCi of ^{3}H-thymidine (Amersham Int. Ltd., Amersham, UK, specific activity 2.0 Ci/mM) was added to each well for 4 hours. Cells were harvested onto glass filter disks using a multiple automated sample harvester, dried, dissolved in scintillation fluid and counted on a Beckman liquid scintillation counter.

Stimulator cells for one-way mixed lymphocyte cultures (MLC) were prepared by suspending PBMC at a concentration of 10^7 cells/ml in the above media and adding 0.1 ml stock mitomycin C (Sigma, St. Louis, MO) per ml of cell suspension. Cells were incubated 1 hour at 37°C and washed 3 times with media. 1.5×10^5 responder cells (untreated, washed PBMC) and 1.5×10^5 mitomycin treated stimulator cells were added to each well of a microtiter plate in triplicate followed by 50 µl of antibody or media as appropriate. Cells were cultured 4 days, pulsed with ^{3}H-thymidine, harvested and counted, as above.

9) INHIBITION ASSAYS

Anti-µ, LPS, PHA or MLR cultures were set up as described under stimulation assays. Each well of the microtiter plate contained cells in media or cells in media plus mitogen. To each of these was added 50 µl of hybridoma supernate or purified antibody. Purified antibodies were titrated for their ability to inhibit stimulation over a concentration ranging from 0.3 µg/ml to 50 µg/ml. All tests were set up in triplicate and included negative controls (cells + media, cells + test monoclonal antibody), positive controls (cells + mitogen) and test samples (cells + mitogen) and test samples (cells + mitogen + antibody). Results are expressed as mean counts per minute (cpm) with standard error of the mean (SEM) of triplicate wells. Percent inhibition was calculated by dividing mean test cpm by positive control cpm after subtracting background counts according to the following equation: $\left(1 - \frac{\text{(Test) - (antibody control)}}{\text{(Positive control) - (Negative control)}}\right) \times 100$. Those instances in which the numerator of this equation exceeds the denominator are reported as 0 percent inhibition.

Cell lines used for inhibition assays were cultured at an initial density of 2 x 10^4 cells per ml in RPMI 1640 + 10% FCS either with or without 50 µl of antibody supernatant or 50 µl of purified antibody at various concentrations (1 to 20 µg/ml final concentration). Cells were pulsed with tritiated thymidine, harvested on day three and counted as above.

10) COLONY ASSAYS

Erythropoietic (CFU-E and BFU-E), granulopoietic (CFU-GM), and pluripotent (CFU-G/E) progenitors were assayed in 0.8% methylcellulose in Iscove's medium, supplemented with 30% FCS, 1% deionized BSA, 10^{-4} M 2-mercaptoethanol, 200 mM L-glutamine, 3 units/ml of human urinary erythropoietin (purified to a specific activity of > 100 u/mg) (12) and agar-stimulated human leucocyte conditioned medium (13) with or without the addition of purified NB-107 at a final concentration of 5 µg/ml. Histologically normal fresh marrow cells from two different donors were assayed by plating 2 x 10^5 washed buffy coat cells per 1.1 ml of culture and mature erythroid, granulocyte-macrophage, and mixed colonies identified and scored according to standard criteria (13).

11) PURIFICATION OF B CELLS

PBMC from one unit (500 ml) of freshly drawn blood were separated over Ficoll-Hypaque. T cell depletion was then accomplished by incubation of these cells with aminoethylisothiouronium bromide (AET) treated sheep red blood cells (S-RBC) followed by Ficoll-Hypaque separation (1). Monocytes were depleted by adherence to glass petri dishes overnight at 37°C. Using these two procedures B cells were enriched to 60-70% as determined by positivity for surface immunoglobulin. Partially purified B cells were then

stained using OKT11-FITC (Ortho Diagnostics, Raritan, NJ) which detects the sheep-RBC receptor on T cells. Sort gates on a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Sunnyvale, CA) were set to exclude T cells (fluorescein positive) and monocytes (on the basis of light scatter). The final B cell enrichment resulted in 85-90% B cells, ~5% monocytes and < 1% T cells as determined by FACS analysis and staining for surface immunoglobulin, Leu-M3 and OKT11 respectively. Purified B cells were then set up in culture as described above.

12) FACS ANALYSIS

Cells (1×10^6) were washed with RPMI 1640 containing 0.5% bovine serum albumin, 0.1% NaN_3 and 10 mM HEPES buffer and were resuspended in 50 µl of undiluted culture supernatant or purified antibody. After a 1 hour incubation at 4^oC, the cells were washed twice and resuspended in 50 µl of pretitrated FITC conjugated goat anti-mouse IgG $F(ab')_2$ (Tago, Burlingame, CA). After an additional incubation at 4^oC for 1 hour, cells were washed three times with PBS containing azide, fixed in formalin and analyzed on a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Sunnyvale, CA). Appropriate positive and negative antibody controls were performed with each experimental run. These included antibodies of irrelevant specificity (e.g. anti-Thy 1.2) as negative controls, and antibodies known to react with hematopoietic cell populations of interest (e.g. anti-leukocyte, Becton Dickinson, Mountain View, CA) as positive controls.

13) IMMUNOPRECIPITATIONS

At least 2×10^7 viable DHL-4, DHL-10 or WALK cells were washed 3 times with PBS. The cells were counted, resuspended in 0.5 ml PBS and transferred

to an iodogen (Pierce, Rockford, IL) vial. 10 μCi of ^{125}I were added and the mixture incubated on a rocker at room temperature for 1 hour with constant shaking. Cells were then washed 3 times with PBS, once with BAM and resuspended in 1.5 ml of 50 mM Tris-saline containing 0.5% BSA. 0.5 ml of 2% NP40 in 50 mM Tris-saline was added and the cells observed for lysis. Lysed cells were microfuged for 10 minutes at 4°C, the supernate removed and 10 μl counted in a gamma counter. A minimum of 2×10^6 counts per tube of labeled cell lysate was utilized. To the lysate was added 30 μl of antibody supernatant followed by a one hour incubation at 4°C. Rabbit anti-mouse IgG (RaMIg) conjugated to beads was washed 3 times with Tris-saline buffer containing 0.5% BSA and 0.5% NP40. 40 μl of 50% suspension of washed beads was transferred to each antibody-lysate containing tube. This mixture was rocked at 4°C 4 hours to overnight. Beads were then washed 4 times with 0.5% NP40 Tris-saline and 150 μl of non-reducing sample buffer added to each tube. Samples were boiled 5 minutes and the supernate removed. Half of each supernate was transferred to a microfuge tube and 5 μl of mercaptoethanol added and the sample boiled again for 5 minutes. Reducing and non-reducing samples were stored at 4°C until ready for gel electrophoresis.

Sequential immunoprecipitations were performed with cells labeled as above. Cell lysate was divided into an appropriate number of tubes. 50 μl of negative control (Thy 1.2, NHL 30.5) (14), positive control (NB65, anti-transferrin receptor) (15), or test antibodies were added to each tube followed by a 1 hour incubation at 4°C. 100 μl of 50% RaMIg beads were added to each tube which were shaken for 1 hour, spun and the supernatant removed. This process was repeated three times with the last bead incubation lasting overnight. Precleared supernates were then processed as above for ordinary immunoprecipitations. In some instances (the anti-class II antibodies)

complete removal of immunoreactive material could not be accomplished using this indirect technique. Therefore, these antibodies were purified, conjugated to beads and preclearing performed using conjugated beads. Under these circumstances 100 µl of 50% suspension of beads was added to labeled cell lysate followed by a 1 hour incubation and removal of supernate after centrifugation. This process was repeated up to 5 times, with the last incubation lasting overnight. Following this direct preclearing, immunoprecipitation was performed in the usual way as described above.

Reduced and nonreduced samples were run in a 10% polyacrylamide gel using a slab gel electrophoresis apparatus (Bio-Rad, Richmond, CA).

14) ANTIBODY BLOCKING STUDIES

Antibodies were labeled as above with ^{125}I or ^{3}H-lysine. Labeled antibodies were titrated and saturating levels utilized for blocking experiments according to modifications of previously described methods (16). Unlabeled ascites of the test antibodies were titrated and each shown to have significant reactivity with DHL-4, DHL-10 or WALK cells at titers in excess of 1:30,000. 10^4 cells per well (e.g. DHL-4) were placed into round bottom microtiter wells. Labeled antibody was mixed with an equal volume of serially diluted ascites. 50 µl of labeled-unlabeled antibody mixture was added to each well and incubated 1 hour at $4^{o}C$. Cells were washed 3 times with binding assay media, then counted in a Beckman liquid scintillation counter. Controls for non-specific inhibition of binding included ascites fluid containing antibody against unrelated determinants present on test cells. The percentage inhibition was calculated as a ratio of counts per well, containing cold antibody, to control wells without cold antibody.

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C H A P T E R III

LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN (LFA-1) IS INVOLVED IN B CELL ACTIVATION

"Whatever you do will be insignificant, but
it is very important that you do it."

Gandhi

1) INTRODUCTION

An interrelated family of three different cell surface molecules, expressed on hematopoietic cells of diverse types, has recently been identified. These molecules, termed LFA-1, Mac-1 (OKM1, Mo-1), and p150,95, are defined by different monoclonal antibodies (1-3). Each possess a common, apparently identical, β subunit of Mr 95,000 and variable α subunits of approximately Mr 177,000, 165,000 and 150,000 respectively. The antigen defined by OKM1 appears to be the complement receptor type three (CR3) (1). The specific functions of LFA-1 and p150,95 are unknown. LFA-1 is a widely expressed human leukocyte antigen present on lymphocytes, monocytes, thymocytes, granulocytes and some bone marrow cells (2). LFA-1 has been shown to play an important role in a variety of T cell interactions. Monoclonal antibodies to LFA-1 have been shown to inhibit various T cell functions including: antigen-specific cytotoxic T lymphocyte (CTL) mediated killing, natural killer (NK) cytolysis, T cell proliferative responses to antigen, mitogen and allogeneic cells, and T cell dependent plaque-forming cell responses (1-6). The mechanism of action of monoclonal anti-LFA-1 in

inhibiting the diverse cellular immune processes is largely unexplored, although evidence that anti-LFA-1 monoclonal antibodies block CTL mediated killing by inhibiting adhesion between the CTL and the target cell has been reported. According to these studies LFA-1 may participate in the Mg^{+2}-dependent adhesion step of CTL mediated killing (2). Despite the demonstrated widespread importance of LFA-1 in T cell responses a role for LFA-1 in B cell function has not been documented.

B cells may be induced to proliferate in response to a variety of stimuli including specific antigens as well as polyclonal mitogens such as lipopolysaccharide (LPS) and anti-IgM (μ) antibodies. The molecular interactions underlying B cell activation are largely unknown. At high concentrations, anti-μ is thought to initiate a direct proliferative effect on B cells independent of T cells, monocytes or their products (7,8). In this chapter the inhibition of normal B cell activation by a monoclonal antibody to LFA-1 is reported, and evidence provided that this effect is mediated via action of the antibody on accessory cells or T lymphocytes. These findings document a previously unrecognized role for LFA-1 in the regulation of B cell proliferation and suggest a more generalized role for LFA-1 in the regulation of immune function.

2) RESULTS

(A) Monoclonal Antibody NB-107 Defines a Distinct Epitope on the LFA-1 Molecule

Monoclonal antibody NB-107 is an IgG_1 monoclonal antibody which immunoprecipitates a noncovalently linked heterodimer (170 and 95 Kd) from the cell surface of ^{125}I labeled DHL-4 B-lymphoma cells (Fig. 5). The molecules immunoprecipitated by NB-107 appear identical to the two subunits

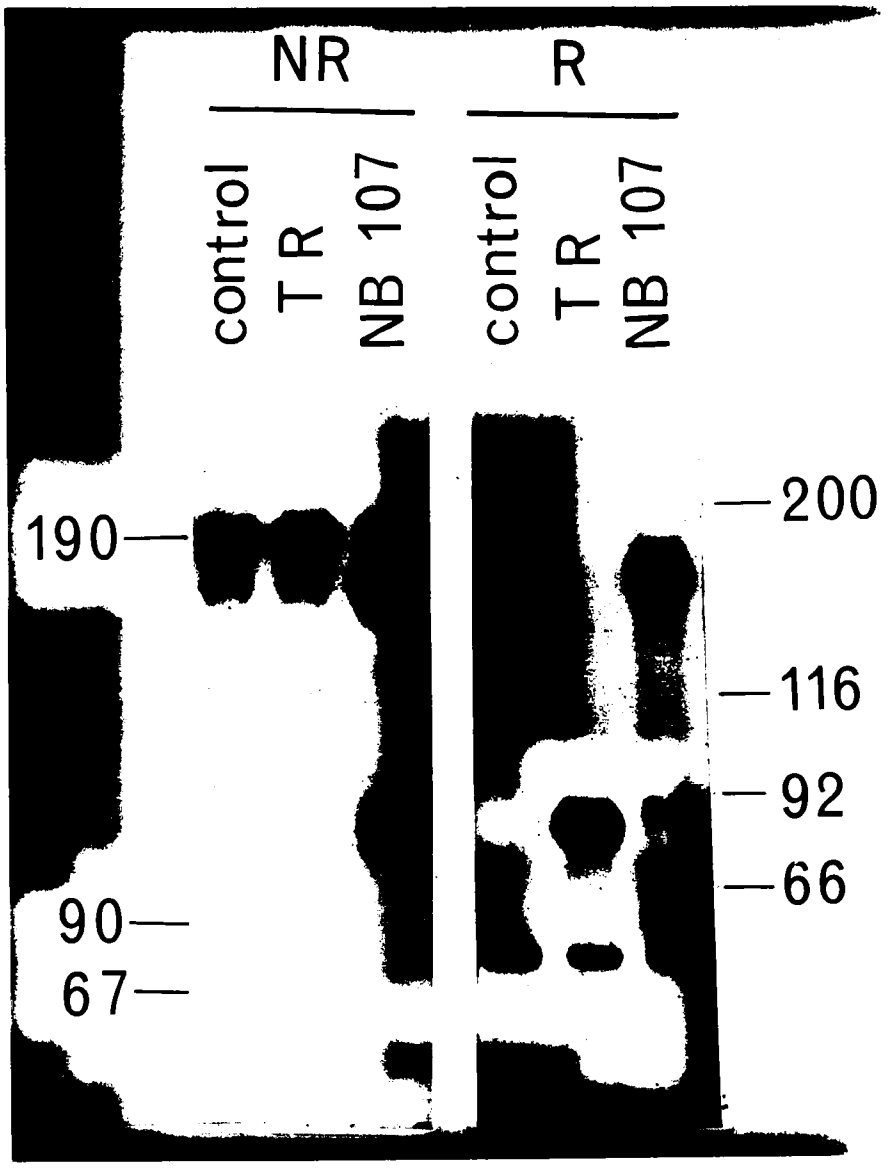

FIGURE 5 The molecular weight of the antigen precipitated from DHL-4 cells by NB-107 is approximately 170 and 95 Kd under reducing conditions (R) and 170 and 115 Kd under non-reducing conditions (NR). Negative control (antibody to Thy 1.2) and positive control (antibody to Transferrin receptor, TR) are included for comparison.

immunoprecipitated by monoclonal antibodies TS1/18 and TS1/22, which react preferentially with the β and α chains respectively of the LFA-1 molecule (1). Removal of material in DHL-4 cell lysates reactive with NB-107 by preclearing with this antibody, completely eliminated lysate reactivity with TS1/18 and TS1/22 indicating these antibodies react with identical molecules. In contrast, binding of antibody NB-65 to the transferrin receptor was not significantly decreased by preclearing with NB-107. Preclearing with the same antibody to the transferrin receptor, however, did remove all detectable transferrin-receptor and did not affect immunoprecipitation using the anti-LFA-1 monoclonal antibodies (Fig. 6).

That NB-107 reacts with a distinct epitope on the LFA-1 molecule was demonstrated by the antibody inhibition studies shown in Table III. The binding of radioactively labeled NB-107 to B lymphoma cells was nearly completely inhibited by cold purified NB-107 even at very high dilutions of cold antibody. In contrast, no significant inhibition of labeled NB-107 binding was observed by TS1/18 or TS1/22 even at high concentrations of these antibodies. These findings demonstrate that NB-107 reacts with an epitope on the LFA-1 molecule that is distinct from those defined by TS1/18 and TS1/22.

(B) Expression of NB-107 on Peripheral Blood Mononuclear Cells, Neoplastic and Non-Neoplastic Cell Lines

FACS analysis of NB-107 binding to peripheral blood mononuclear cells from 27 normal individuals revealed a mean ± SEM reactivity of 83 ± 13 percent. Mean reactivity with bone marrow cells from 7 patients (3 CML, 1 AML, 1 myelodysplasia, 1 monocytosis, 1 hairy cell leukemia) was 45 percent (range 21-72 percent). Figure 7 shows a FACS analysis of NB-107 tested against normal PBMC. Cells with the greatest amount of light scatter (larger cells, predominantly monocytes) displayed the most intense staining by

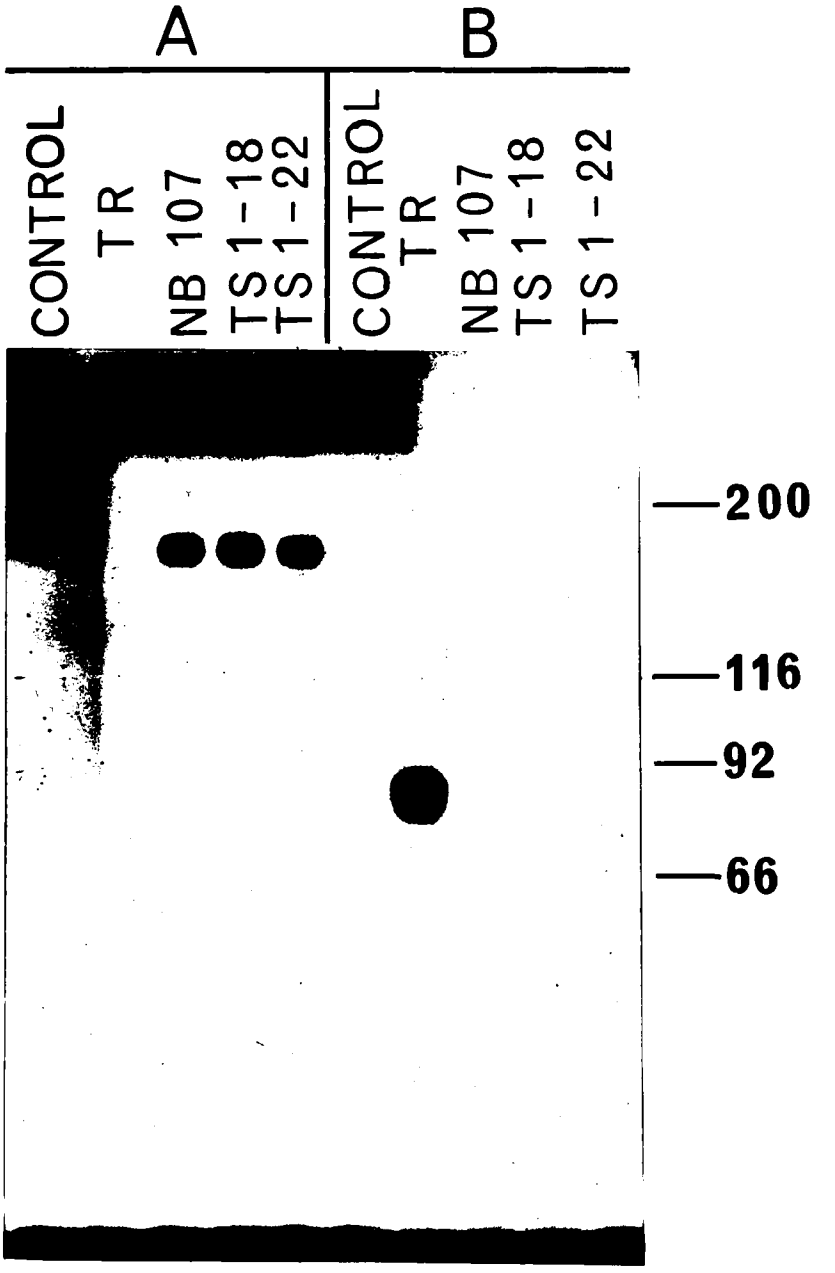

FIGURE 6 Sequential immunoprecipitation ("preclearing"): Antibody to transferrin receptor (NB-65), completely removes transferrin receptor from DHL-4 lysate. LFA-1 detected by TS1/18, TS1/22 and NB-107 remains (A). Preclearing with NB-107 (B) removes material reactive with TS1/18, TS1/22 and NB-107, while leaving transferrin receptor unaffected. Note that molecules immunoprecipitated by NB-107, TS1/18 and TS1/22 have an identical appearance and mobility.

TABLE III

Competitive Inhibition of ^{3}H-lysine Labeled NB-107 Binding to DHL-4 Cells

Antibody	Ascites Dilution	NB-107 Binding (CPM)	% Inhibition
NB-107	1:200	201	94
	1:400	240	93
	1:800	372	90
	1:1600	621	87
	1:3200	1003	72
TS1-18	1:200	3208	9
	1:400	3583	0
	1:800	3692	0
	1:1600	3549	0
	1:3200	3500	1
TS1-22	1:200	3529	0
	1:400	3616	0
	1:800	3681	0
	1:1600	3706	0
	1:3200	3171	10
Control	none	3542	

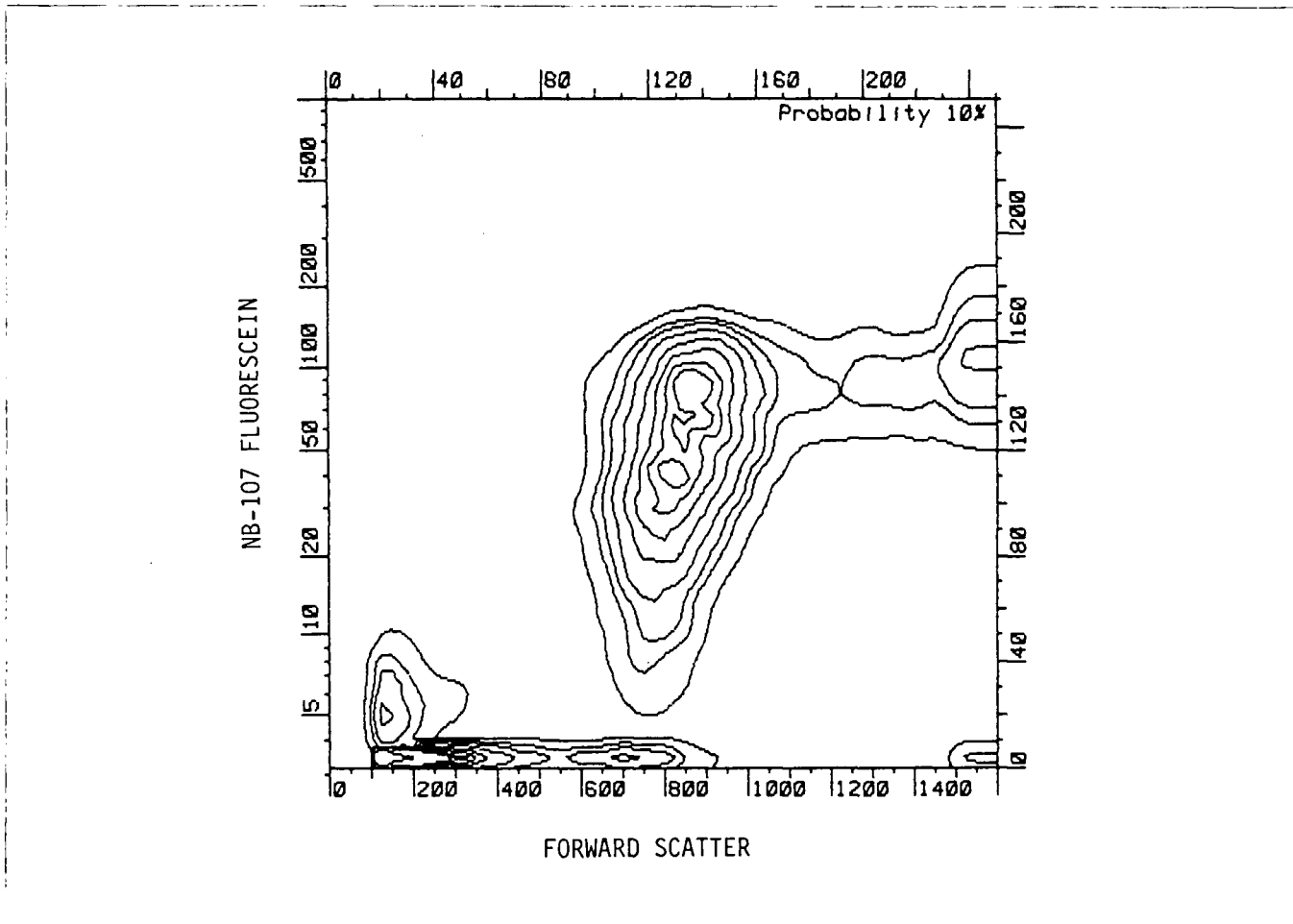

FIGURE 7 FACS analysis of NB-107 tested against normal peripheral blood mononuclear cells. Cells with the greatest amount of light scatter (larger cells, predominantly monocytes) display the most intense staining by NB-107. Cells of intermediate size (lymphocytes) show a spectrum of reactivity from strong to weak.

NB-107. Cells of intermediate size (lymphocytes) showed a spectrum of reactivity from strong to weak. Dual labeling of PBMC with phycoerythrin anti-DR and fluoresceinated NB-107 revealed that a major fraction of both DR positive cells (B cells and monocytes) and DR negative cells (principally T cells) were positive for NB-107 (Fig. 8). Whether there is any functional significance to the quantitative heterogeneity of LFA-1 expression on lymphoid cells is not known.

We also measured the expression of this antigen on neoplastic and EBV transformed B cell lines. Table IV shows that LFA-1 defined by NB-107 was present on all 9 EBV transformed DR-homozygous cell lines examined and 3 of 7 B lymphoma cell lines. Noteworthy was its absence on the other 4 B lymphoma cell lines tested.

(C) NB-107 (Anti-LFA-1) Inhibits B Cell Activation

NB-107 profoundly inhibited both LPS and anti-μ induced proliferative responses of PBMC (Table V). This was true for both hybridoma supernate and purified antibody. Significant inhibition was observed over a wide range of antibody concentrations (Table VI). In all experiments isotype identical negative controls were performed and showed no significant inhibition. Antibody to transferrin receptor, a known inhibitor of cell proliferation served as a positive control (10). NB-107 inhibited the MLR, as previously reported for anti-LFA-1 monoclonal antibodies (11). In contrast to other studies, we did not observe inhibition of PHA stimulation by anti-LFA-1 monoclonal antibody (Table VII) (6).

In order to determine whether anti-LFA-1 has a direct effect on B cells, we attempted to inhibit the growth of EBV transformed B cell lines by addition of purified NB-107 to cultures of these cells. Two different EBV cell lines were tested and in neither case was evidence of significant

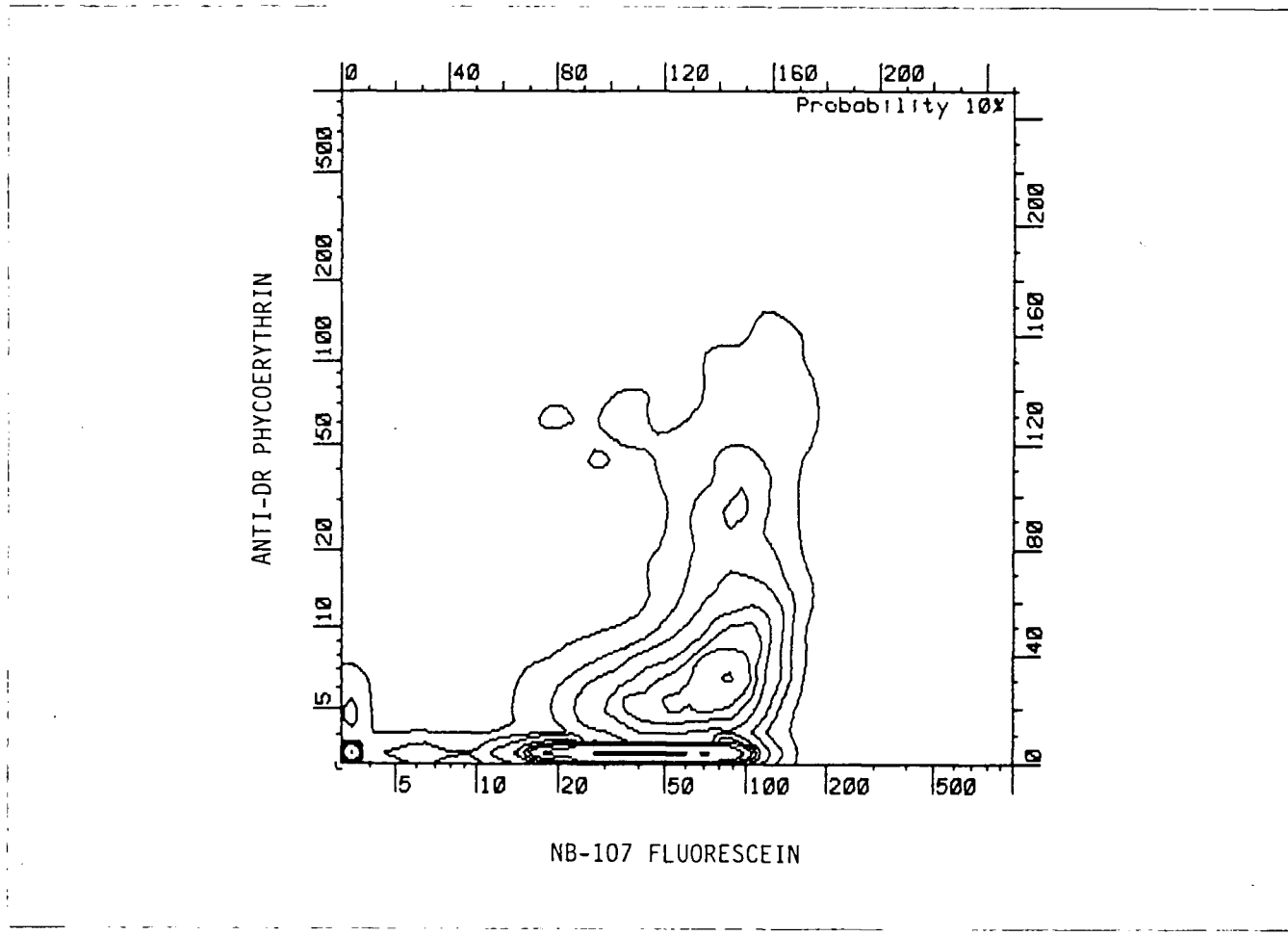

FIGURE 8 Dual fluorescence of normal peripheral blood mononuclear cells using phycoerythrin labeled anti-DR and fluorescein labeled NB-107. Cells with the greatest intensity of DR staining (principally monocytes) also express the highest amounts of LFA-1 defined by NB-107.

TABLE IV

Cell Line Reactivity of NB-107 (FACS Analysis)

Cell Line	Type	Reactivity
JREE	EBV (DR1)	+
CMG	EBV (DR2)	+
RMG	EBV (DR3)	+
WAIK	EBV (DR4)	+
SWEI	EBV (DR5)	+
ELD	EBV (DR6)	+
BN	EBV (DR7)	+
MAD	EBV (DRW8)	+
KOZ	EBV (DRW9)	+
SU-DHL-1	B lymphoma	-
SU-DHL-4	B lymphoma	+
SU-DHL-6	B lymphoma	-
SU-DHL-8	B lymphoma	-
SU-DHL-10	B lymphoma	+
BALM-5	B lymphoma	+
U-698-M	B lymphoma	-
Jurkat	T leukemia	-
HL-60	Myeloid leukemia	+
K562	Erythroleukemia	-

TABLE V

Inhibition of B Cell Activation by Anti-LFA-1[a]

Mitogen	Antibody	^{3}H-Thymidine Incorporation (Mean CPM ± SEM)		% Inhibition
		Control[b]	Test[c]	
Anti-μ[d]	None	1167 ± 192	3248 ± 402	
	NB-65[e]	586 ± 92	1735 ± 129	69 ± 21
	NB-107	566 ± 173	1456 ± 119	71 ± 21
LPS	None	1946 ± 752	4028 ± 426	
	NHL 30.5[f]	1338 ± 190	3150 ± 475	12 ± 7
	NB-65	491 ± 21	901 ± 125	68 ± 16
	NB-107	725 ± 320	1349 ± 83	80 ± 14

[a]Results from a representative experiment are expressed as mean counts per minute (CPM) ± standard error of the mean (SEM) of triplicate wells containing 2 x 10^5 peripheral blood mononuclear cells (PBMC). Per cent inhibition was calculated as described in materials and methods and is the mean ± SEM of six individual experiments.

[b]Cultures without mitogens.

[c]Cultures with mitogens.

[d]Affinity purified polyclonal goat anti-human IgM.

[e]Antibody to transferrin receptor used as a positive control.

[f]NHL 30.5 is a subclass identical monoclonal antibody directed against a myeloid differentiation antigen (9); one of several monoclonals used as negative controls.

TABLE VI

Inhibition of LPS Stimulation: Titration Using Purified NB-107

Antibody[a] (μg/ml)	^{3}H-Thymidine Incorporation (Mean CPM ± SEM)		% Inhibition
	Control[b]	Test[c]	
None	310 ± 27	4469 ± 839	
NHL 30.5 (10)	387 ± 85	4390 ± 1528	4
NB-107 (20)	137 ± 25	1109 ± 375	77
NB-107 (10)	157 ± 31	1159 ± 111	76
NB-107 (5)	228 ± 66	1959 ± 307	68
NB-107 (2.5)	180 ± 31	2071 ± 227	55
NB-107 (supernate)	212 ± 40	1407 ± 374	71
NB-65 (supernate)	246 ± 25	997 ± 97	82

[a]See Table V for description of antibodies.

[b]Without mitogen.

[c]With mitogen.

TABLE VII

Inhibition of T Cell Proliferation

Antibody[a]	Mean ± SEM Control[b]	Mean ± SEM Test[c]	% Inhibition[d]
		MLR	
None	459 ± 204	17,998 ± 797	
NHL 30.5	679 ± 141	19,810 ± 1941	0
NB-65	343 ± 93	4,028 ± 502	83 ± 6
NB-107	219 ± 104	3,811 ± 826	85 ± 7
		PHA	
None	519 ± 62	85,767 ± 1,665	
NHL 30.5	272 ± 48	87,022 ± 3,951	0
NB-65	305 ± 82	48,373 ± 3,083	43 ± 1
NB-107	164 ± 60	98,442 ± 12,123	3 ± 3

[a]See Table V for description of antibodies.

[b]Culture without stimulator cells or mitogen.

[c]Cultures with stimulator cells or mitogen.

[d]Mean ± SEM of two individual experiments.

inhibition obtained (Table VIII). This was in contrast to the marked inhibition achieved by the addition of anti-transferrin receptor antibody. The proliferative response of highly purified normal B cells to anti-μ was, likewise, not inhibited by NB-107 (Table IX) nor did the presence of NB-107 have any inhibitory effect on colony formation by bone marrow CFU-E, BFU-E, CFU-GM or CFU-G/E assayed in standard methyl cellulose cultures (Table X).

3) DISCUSSION

We have demonstrated that LFA-1 may be important in the regulation of B cell proliferation. Monoclonal antibody to LFA-1 (NB-107) profoundly inhibited stimulation of human peripheral blood mononuclear cells (PBMC) by LPS and anti-μ. In order to further investigate whether NB-107 inhibits B lymphocyte mitogen induced activation via a direct action on B cells or by inhibiting an essential accessory cell function or T-B cell interaction, the effect of NB-107 on activation of highly purified B cells was tested. NB-107 antibody did not inhibit the anti-μ stimulation of the B enriched populations, which contained less than 1% T cells ($OKT4^+$), approximately 5% monocytes ($OKM1^+$) and 85-90% surface immunoglobulin positive B cells. Furthermore, NB-107 also did not inhibit ^3H-thymidine uptake of several EBV transformed B cell lines. Therefore, anti-LFA-1 does not seem to inhibit B cells directly.

These findings raise the question of how the B cell response to anti-μ involves other cells (such as T cells or monocytes) and how anti-LFA-1 inhibits the B cell response. Because anti-μ stimulated highly enriched populations of B cells, either this stimulation does not require T cells and/or monocytes, or those cells still contaminating the B enriched population may be sufficient and also necessary for the stimulation of B

TABLE VIII

Lack of Inhibition of EBV Cell Line Growth

Antibody[a]	(μg/ml)	WALK (DR4) Cells Mean CPM ± SEM	% Inhibition
None		1339 ± 289	
NB-65		494 ± 19	69
NHL-30.5	(20)	1563 ± 881	0
NHL-30.5	(5)	1663 ± 152	0
NHL-30.5	(1.25)	1602 ± 152	0
NHL-30.5	(0.3)	1581 ± 256	0
NB-107	(20)	1226 ± 48	9
NB-107	(5)	1067 ± 947	22
NB-107	(1.25)	1071 ± 149	22
NB-107	(0.3)	1293 ± 359	4
		ELD (DR6) Cells	
None		7492 ± 270	
NB-65		671 ± 62	90
NHL-30.5	(20)	5644 ± 279	25
NHL-30.5	(5)	8036 ± 823	0
NHL-30.5	(1.25)	7244 ± 2282	3
NHL-30.5	(0.3)	9075 ± 1826	0
NB-107	(20)	5829 ± 384	22
NB-107	(5)	6341 ± 1520	15
NB-107	(1.25)	5858 ± 1734	22
NB-107	(0.3)	6702 ± 109	11

[a]See Table V for description of antibodies.

TABLE IX

Inhibition of Anti-μ Stimulation: Purified B Cells

Antibody (10 μg/ml)	Mean CPM ± SEM	% Inhibition
None[a]	969 ± 85	
None[b]	13,225 ± 156	
NHL 30.5	11,379 ± 724	15
NB-107	12,661 ± 550	5

[a]Cells without anti-μ

[b]Cells with anti-μ

TABLE X

Effect of Anti-LFA-1 on Bone Marrow Progenitor Cells

Progenitor	NB-107	Colonies[a] Exp 1	Exp 2
CFU-E	-	409	107
(late erythroid)	+	399	110
BFU-E	-	204	169
(primitive erythroid)	+	149	156
CFU-GM	-	286	128
(granulopoietic)	+	254	109
CFU-G/E	-	1.5	1
(pluripotent)	+	2	0

[a]Number of colonies per 2 x 10^5 normal human marrow buffy coat cells. Values are means of counts from 2 replicate 1.1 ml methylcellulose cultures each initially containing 2 x 10^5 cells.

cells. If anti-LFA-1 inhibits B cell activation by affecting T cells and/or monocytes, and if the contaminating T cells and monocytes are involved in the stimulation of B cells, the antibody should also inhibit the stimulation of purified B cells. However, the present study clearly showed that anti-LFA-1 does not inhibit the stimulation of purified B cells by anti-μ. This apparent paradox may be explained as follows: At low cell densities (unfractionated PBMC) B cell stimulation by anti-μ requires factors produced by T cells and/or monocytes. These latter cells are inhibited by anti-LFA-1 which results in inhibition of PBMC stimulation by anti-μ. At high cell densities (purified B cells) anti-μ stimulates B cells in the absence of accessory cells and/or monocytes perhaps due to a critical cell density which is achieved or required for endogenous release of BCGF and autostimulation (see below).

The mechanisms involved in B cell activation are currently being actively researched. LPS stimulation of B cells appears to be macrophage but not T cell dependent (13). Anti-μ antibody is thought to exert its effects on B cells by two concentration dependent mechanisms. At low concentrations, anti-μ induces cell enlargement, RNA synthesis and cell surface expression of B cell growth factor receptors. Proliferation of B cells in this case then requires a second signal, mediated by BCGF secreted by activated T helper cells. At high concentrations (such as used in this study), anti-μ has been thought to initiate a direct proliferative effect on B cells independent of T cells, monocytes or their products (7,8,14). Recent studies have shown that EBV transformed B cells require either a critical cell density or supplementation with exogenous factors for proliferation. Furthermore these cells appear to secrete their own autostimulatory B cell growth factor (17,18). Whether normal stimulated B cells at high cell densities are capable of autostimulation is not yet known.

Previous studies have not shown an inhibitory effect of anti-LFA-1 monoclonal antibodies on LPS stimulation of mouse lymphocytes (12). The differences between these and our findings may be related to species differences between mouse and human or relative concentrations of B cells. Human LFA-1 expresses multiple unique antigenic epitopes that reside with varying degrees of spatial proximity to the functional region(s) of the LFA-1 molecule (19). NB-107 clearly reacts with a different epitope on the LFA-1 molecule than does TS1/18 and TS1/22 as shown by competitive inhibition studies (Table III). Monoclonal antibodies to different LFA-1 epitopes appear to vary in their ability to inhibit T lymphocyte function (20). The region defined by NB-107 may be uniquely involved in the cooperative interaction of B cells with monocytes and/or T cells and thus susceptible to inhibition by NB-107 but not other anti-LFA-1 monoclonal antibodies. Inhibition of the MLR by NB-107 (Table VII) is similar to that previously described for other anti-LFA-1 monoclonal antibodies. In contrast, PHA stimulation of T lymphocytes was not inhibited by this antibody (6).

Our findings of the expression of LFA-1 (defined by NB-107) on B and T lymphocytes and most intensely on monocytes of normal peripheral blood is consistent with previous reports (Fig. 7,8) (6,21). LFA-1 was present on all EBV cell lines examined (9/9), but absent on 4 of 7 neoplastic B lymphoma lines. The latter finding implies the loss of LFA-1 may be somehow associated with the development of B cell malignancy. Loss of normal B cell growth regulatory cell surface molecules may be a step in the development of autonomous (neoplastic) cell growth. Alternatively, the surface expression of LFA-1 on B lymphoma cells may simply be lost by altered gene expression or product modification. The functional implications of the loss of LFA-1 on B cells are not known.

Although approximately half of bone marrow cells are positive for LFA-1 (2) the identity of these cells has not been established. In order to test whether LFA-1 is important in hematopoietic stem cell differentiation, purified NB-107 was added to cultures of normal human marrow buffy coat cells. No inhibitory effect of NB-107 on the growth of CFU-E, BFU-E, CFU-GM or CFU-G/E was identified suggesting that LFA-1 is not important in the formation of colonies of diverse myeloid cell lineages.

Recently, the family of glycoproteins LFA-1, OKM1, p150,95 have been found to be deficient on the cells of patients with chronic bacterial infections and multiple immune abnormalities (22-24). It has been postulated that these abnormalities result from the abnormal function of granulocytes, monocytes and/or T cells and that these defective functions may be attributable to the limited expression of LFA-1, OKM1 and p150,95 on these cells. We report the inhibition of B lymphocyte activation by a monoclonal antibody to LFA-1, and provide evidence that this is due to effects on monocytes and/or T cells. These findings suggest that this class of cell surface molecules has a more diverse functional role in the regulation of the immune response than previously realized. We are currently investigating B cell function and LFA-1 expression in patients with hereditary immune deficiency and chronic bacterial infections.

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C H A P T E R IV

MONOCLONAL ANTIBODIES TO HLA-CLASS II DETERMINANTS: FUNCTIONAL EFFECTS ON THE ACTIVATION AND PROLIFERATION OF NORMAL AND EBV TRANSFORMED B CELLS

"Probable impossibilities are to be preferred to improbable possibilities."

Aristotle 384-322 B.C.

1) INTRODUCTION

HLA class II molecules are heterodimeric transmembrane proteins consisting of a heavy (α) chain of 33-35,000 MW and a light (β) chain of 25-28,000 MW (1). Genetic and biochemical analyses of the genes and gene products encoded in the HLA-class II (D) region have revealed the existence of three groups of molecules with similar structures (1-3). DR is structurally homologous to murine I-E. DQ (DS, MB, DC) shows homology to murine I-A, and DP (SB) has been defined by primed lymphocyte typing (4). There is evidence for at least six alpha and seven beta chain genes within the D region (4). At the protein level, HLA-DR homozygous cell lines appear to express at least five class II molecules: two DR molecules, two DS molecules, and one SB molecule (5). Although it is well established that class II genes and their products are involved in a variety of immune responses, very little is known about how these molecules modulate biologic behavior (2,3).

Evidence in both mouse and man indicates that class II genes determine several immunologic phenomena. These include: control of the level of immune responses, delayed type hypersensitivity, susceptibility to certain diseases, and primary (mixed lymphocyte culture) and secondary (primed lymphocyte typing) allogeneic T cell proliferation. In addition, HLA class II molecules are involved in T cell activation, apparently by playing an associative role in the recognition of antigen (3). The relative contribution of DQ vs DR molecules to these processes is unknown. A role for HLA class II molecules in B cell activation has not been documented. However, accessory cells (which express class II antigens) and T cells are thought to have a regulatory role in the activation of B cells (6).

B cells are induced to proliferate in response to a variety of stimuli including specific antigens as well as polyclonal mitogens such as lipopolysaccharide (LPS) and anti-IgM (μ) antibodies (6,7). The molecular events underlying B cell activation by these mitogens is not understood, although both cross-link cell surface molecules (6). In order to investigate the functional role of HLA class II antigens in B cell activation we have generated a panel of anti-class II monoclonal antibodies. In this chapter are described the characteristics of three of these, that differ in their specificities for DQ and DR determinants as well as in their effects on the proliferation of normal and EBV transformed B cells.

2) RESULTS

(A) Antibody Specificity

Monoclonal antibodies to HLA class II molecules were produced using the B lymphoma cell line DHL-10 (DH-84, DH-224) or LPS stimulated splenic B lymphoma cells (NB-29) as immunogens. Monoclonal antibodies DH-84 and DH-224

reacted consistently by FACS analysis with a subpopulation of PBMC from each of 30 normal donors (14 and 11 percent of PBMC, respectively). In addition, these antibodies reacted with a panel of DR homozygous cells encompassing DR1 through DRw9 specificities (Table XI). Both immunoprecipitated heterodimers of approximately 35,000 and 28,000 m.w. (Fig.9). These values are similar to the molecular weights of the heterodimers immunoprecipitated by OKIa and BD-DR. NB-29 monoclonal antibody reacted with homozygous cell lines (HCL) DR 1,2,4,6,8,w9. This pattern of reactivity is the same as that obtained with BT 3.4, a known and well characterized anti-DQ monoclonal antibody (8). NB-29 also immunoprecipitated a cell surface heterodimer similar in molecular weight to that precipitated by BT 3.4 and Leu-10, which is slightly less for both chains than that precipitated by the known anti-DR monoclonals (Figs.9,10).

NB-29 reacted with the same class II epitope as did BT 3.4. This was demonstrated by the ability of BT 3.4 to completely inhibit the binding of ^{125}I labeled NB-29 (Table XII). NB-29 binding was also significantly inhibited by the known anti-DQ monoclonal antibody, Leu-10. Thus, on the basis of the molecular weight of its target antigen, its reactivity with DR homozygous cell lines and by cross-blocking studies, NB-29 appears to be a monoclonal antibody to a polymorphic determinant present on DQ molecules, that is similar or identical to that defined by BT 3.4.

As shown in Table XII, DH-224 binding was blocked markedly by BD-DR but only weakly by OKIa. On the other hand DH-224 did not block either DH-84 or NB-29 binding. Removal of nearly all material reactive with DH-224 by "preclearing", substantially reduced the amount of DR precipitated by BD-DR. However, the amount of Leu-10 reactive material (DQ) was not affected (Fig.11). Based upon its reactivity with normal PBMC and DR-HCL, and from

TABLE XI

Reactivity of Anti-class II Monoclonal Antibodies with Homozygous DR Cell Lines: FACS Analysis[a]

Cell Line	Specificity		Antibody					
	D/DR	HLA-ABC	NB-29	BT3.4	Leu-10	DH-84	DH-224	BD-DR
JREE	DR1, DW1	A2, BW44 (W4)	+	+	+	+	+	+
CMG	DR2, DW2	A3, B7	+	+	+	+	+	+
RMG	DR3, DW3	A1, B8	-	-	-	+	+	+
WALK	DR4, DW4	A2, BW44, CW5	+	+	+	+	+	+
SWEI	DR5, DW5	A29, B40	-	-	+	+	+	+
ELD	DR6, DW6 (DW "6.1")	A2, B40	+	+	+	+	+	+
BD	DR7, DW7	A2, B13	-	-	-	+	+	+
MAD	DR8, DW8 (LD "8.1")	A2, B40, CW3	+	+	+	+	+	+
KOZ	DRW9, LD "4x7"	AW24, 26 BW54, 40	+	+	+	+	+	+

[a]Cell lines were considered positive if greater than 10% of cells were reactive with the test antibody compared to negative control antibody of irrelevant specificity.

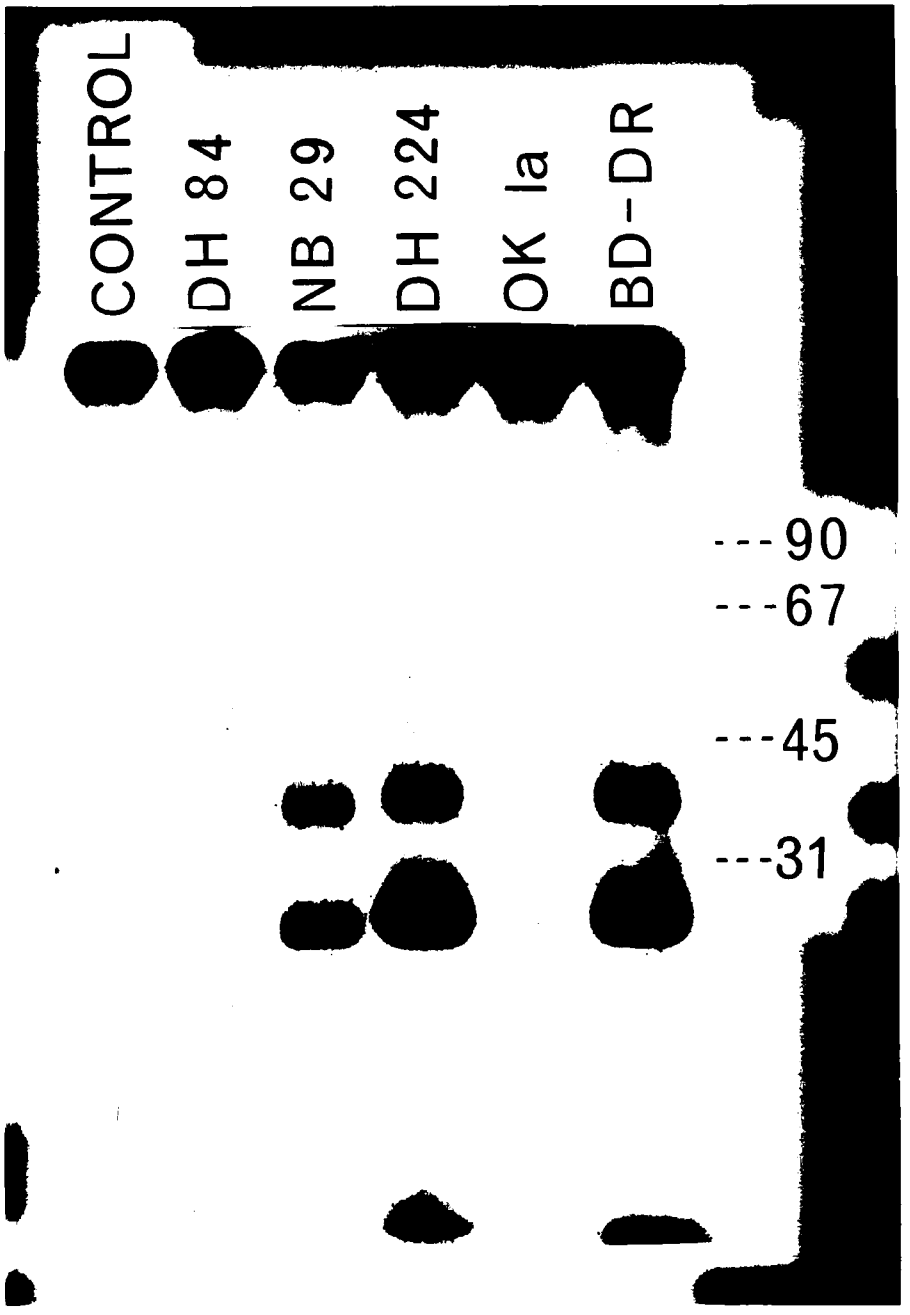

FIGURE 9 Immunoprecipitation using ^{125}I-labeled WALK (DR4) cells. NB 29 (anti-DQ), DH-224 (anti-DR) and DH-84 (anti-DQ+DR) immunoprecipitate bands of approximately 35,000 and 28,000 molecular weight. Shown for comparison are the known anti-DR monoclonals from Ortho (OKIa) and Becton-Dickinson (BD-DR). The amount of material precipitated by DH-84 and OKIa is considerably less than that of the other antibodies. This probably relates to differences in antibody affinity. The molecular weight of each chain precipitated by the anti-DQ monoclonal NB-29 is 1 to 2 kd less than that of the anti-DR monoclonals (e.g. BD-DR).

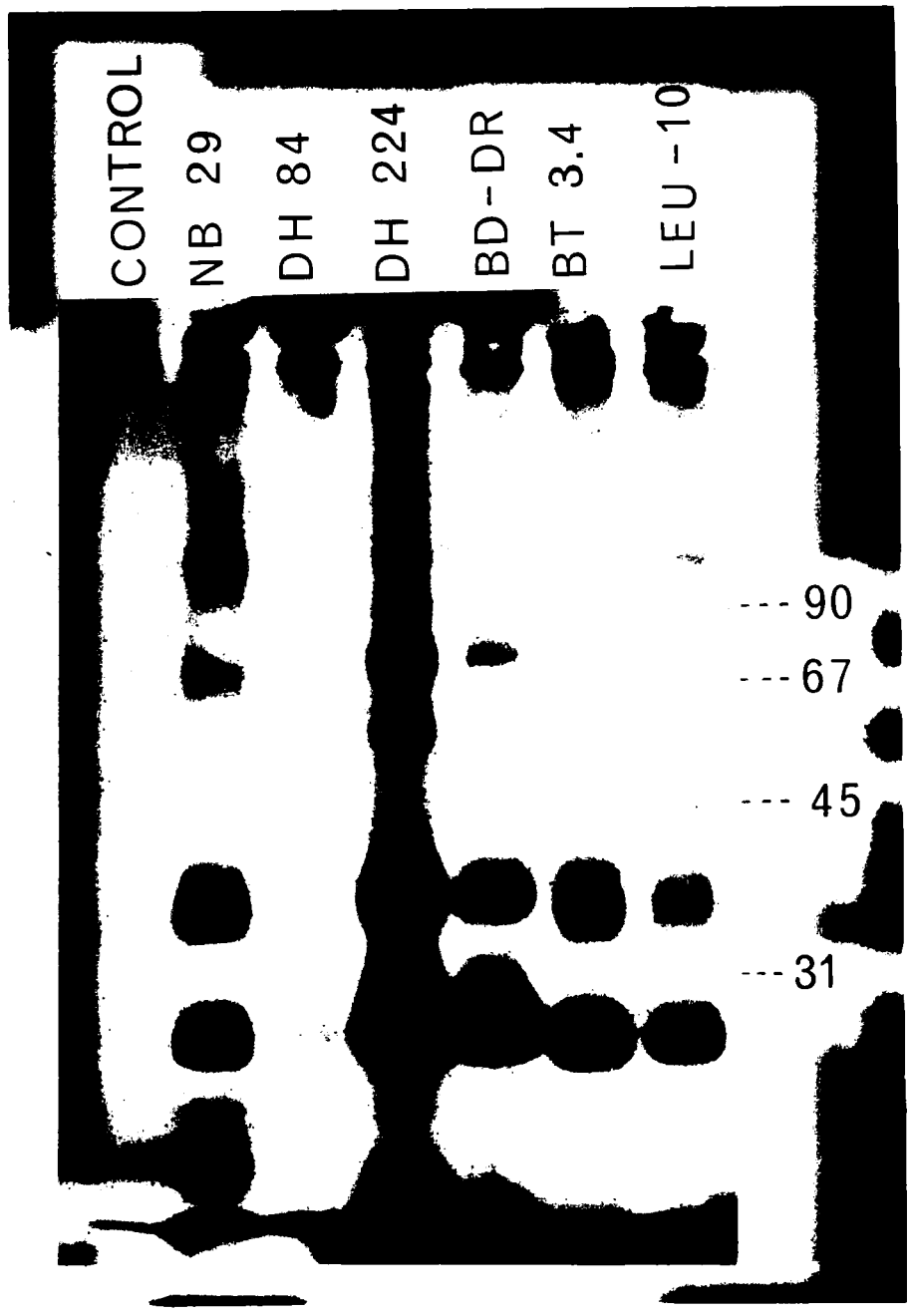

FIGURE 10 Immunoprecipitation using ^{125}I-labeled WALK (DR4) cells. Antibodies NB-29, DH-84 and DH-224 are shown for comparison with known anti-DQ monoclonal antibodies BT3.4 and Leu-10. NB-29 and BT3.4 immunoprecipitate identical bands, each of which has mobility slightly greater than those precipitated by BD-DR.

TABLE XII

Cross Blocking of ^{125}I-labeled Anti-class II Antibodies: DHL-4 Cells

	Labeled Antibody		
	NB-29	DH-84	DH-224
Blocking Antibody	% Inhibition[b]		
NHL-30.5	0	0	0
NB-65[c]	0	0	0
Thy1.2[c]	4	3	0
NB-29	99	0	0
DH-84	78	91	0
DH-224	7	0	94
BT3.4	99	0	0
Leu-10	50	0	0
BD-DR	ND[a]	ND	72
OKIa	ND	ND	18

[a]ND = not done

[b]Calculated as described in materials and methods.

[c]Hybridoma supernate

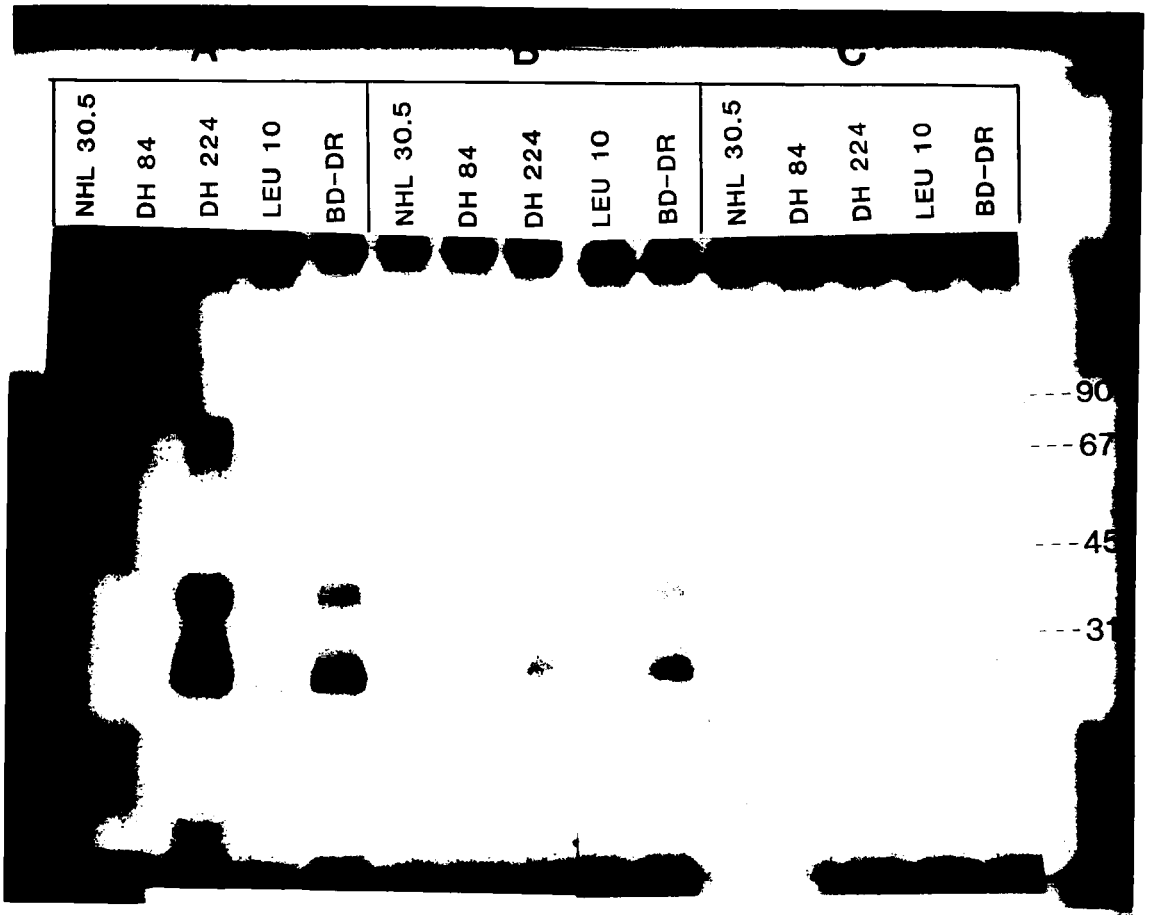

FIGURE 11 Sequential immunoprecipitation "preclearing" of ^{125}I-labeled WALK (DR4) cell lysate. "A" is precleared with antibody of unrelated specificity. DH-84, DH-224, Leu-10 and BD-DR each precipitate bands of approximately 35,000 and 28,000 m.w. "B" is precleared using DH-84 which substantially reduces the amount of BD-DR and DH-224. The marked diminution in the amount of material precipitated by the known anti-DR monoclonal antibody (BD-DR) indicates DH-84 has specificity for DR molecules. The amount of Leu-10 (anti-DQ) precipitated material is unaffected. "C" is precleared using DH-224. In addition to removing material reactive with itself, preclearing with DH-224 has markedly reduced the amount of DR precipitated by BD-DR while leaving DQ reactive material (precipitated by Leu-10) unchanged.

the cross-blocking and sequential immunoprecipitation data, DH-224 appears to react with a monomorphic DR determinant similar, but not identical in specificity, to BD-DR.

The specificity of DH-84 was more difficult to establish. DH-84 reacted with all HCL and normal control PBMC tested. Preclearing of cell lysates with DH-84 substantially diminished the amount of material reactive with BD-DR, but did not remove it completely; Leu-10 reactive material was not diminished (Fig.11). Preclearing of material reactive with NB-29 did not substantially remove DH-84 reactive molecules (Fig.12). These findings suggest that DH-84 reacts with a monomorphic DR determinant. However, DH-84 substantially and reproducibly blocked ^{125}I labeled NB-29 binding to DHL-4 cells. As described above, NB-29 is specific for a polymorphic determinant on HLA-DQ molecules. Therefore, DH-84 appears to have specificity predominantly for DR, but also reacts with a subpopulation of DQ molecules. Whether these antibodies show any cross reactivity with DP molecules has not been determined.

(B) Inhibition of PBMC Stimulation

Both DH-84 and DH-224 profoundly inhibited PBMC stimulation by antisera against IgM (mean inhibition of 70-90%), and LPS (mean inhibition of 80-90%) (Tables XIII and XIV). Titration of the inhibitory effect of DH-84 on anti-μ induced stimulation showed that purified DH-84 was effective over a wide concentration range (from 4 μg/ml to 0.16 μg/ml). Similar inhibition was not seen in cultures containing any of a number of monoclonal antibodies of unrelated specificity (e.g. NHL-30.5 Table XIV). In contrast to DH-84 and DH-224 which define monomorphic DR determinants, NB-29 (DQ polymorphic) did not show significant inhibition of LPS or anti-μ stimulation (Table XIII). Likewise DH-84 and DH-224 markedly inhibited the mixed lymphocyte response,

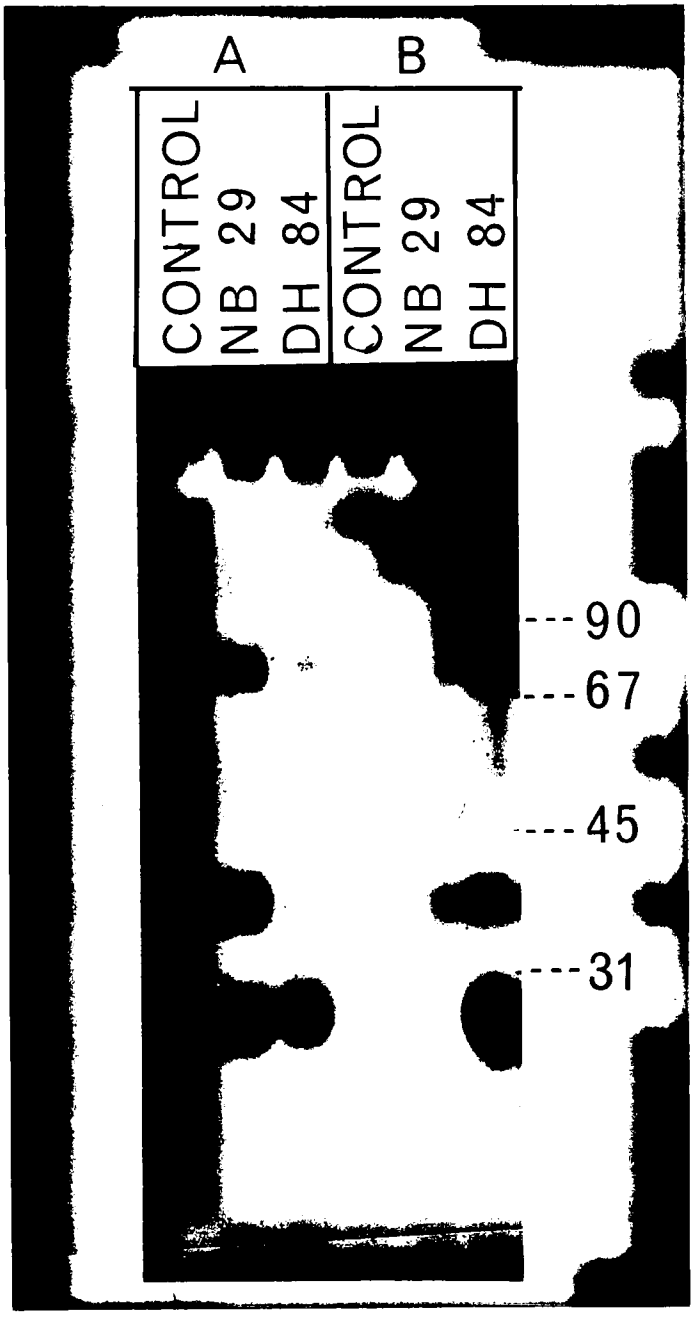

FIGURE 12 Sequential immunoprecipitation "preclearing" of ^{125}I-labeled WALK (DR4) cell lysate. "A" is precleared with control antibody of unrelated specificity. NB-29 and DH-84 each precipitate bands of approximately 35,000 and 28,000 m.w. "B" is precleared with NB-29. NB-29 preclearing removes all material reactive with itself, while not quantitatively affecting the amount of material precipitated by DH-84.

TABLE XIII

Inhibition of Stimulation of Normal PBMC[a]

Antibody (5 μg/ml)	Mean cpm ± SEM		% Inhibition
	Control[b]	Test[c]	
		Anti-μ[d]	
media	232 ± 124	3,436 ± 637	
NB-29	445 ± 159	2,421 ± 198	17 ± 10
DH-84	197 ± 46	298 ± 54	83 ± 22
DH-224	247 ± 75	939 ± 397	70 ± 29
		LPS	
media	1,520 ± 339	10,951 ± 1,323	
NB-29	1,541 ± 296	7,969 ± 237	18 ± 13
DH-84	222 ± 38	1,332 ± 256	83 ± 11
DH-224	455 ± 109	2,207 ± 249	84 ± 11
NB-65[e]	523 ± 53	2,914 ± 484	64 ± 17

[a]Results are expressed as mean counts per minute (cpm) ± standard error of the mean (SEM) of triplicate wells containing 2 x 10^5 peripheral blood mononuclear cells (PBMC). Per cent inhibition was calculated as described in Materials and Methods and is the mean ± SEM of values from four separate experiments.

[b]Cultures without mitogens.

[c]Cultures with mitogens.

[d]Affinity purified polyclonal goat anti-human IgM.

[e]Antibody to transferrin receptor (hybridoma supernate).

TABLE XIV

Inhibition of Anti-μ Stimulation of Normal PBMC: Titration Using Purified Anti-HLA Class II Antibody

Antibody (μg/ml)		Mean cpm ± SEM Control	Test	% Inhibition
media		737 ± 12	4,586 ± 252	
DH-84	(4.0)	360 ± 113	654 ± 91	92
DH-84	(0.8)	404 ± 70	892 ± 104	87
DH-84	(0.16)	348 ± 72	1,416 ± 30	71
NHL-30.5[a]	(4.0)	1,241 ± 232	4,669 ± 510	9
NHL-30.5	(0.8)	1,033 ± 156	3,984 ± 771	22
NHL-30.5	(0.16)	1,027 ± 137	4,013 ± 795	21

[a]NHL-30.5 is a monoclonal antibody directed against a myeloid differentiation antigen, one of several monoclonals used as negative controls.

while NB-29 did not, even when tested against reactive individuals (Table XV). None of these anti-class II monoclonal antibodies inhibited PHA stimulation of PBMC in culture (Table XVI).

(C) Inhibition of Purified B Cells

To test whether the inhibitory effects of these monoclonal antibodies were mediated via direct effects on B cells or through an action on accessory cells, B cells were extensively enriched by a combination of E-rosette depletion of T cells, monocyte adherence to glass and cell sorting. These extensively purified populations of B cells showed an excellent stimulatory response to anti-μ, and this was not affected by the addition of any of the monoclonal anti-class II antibodies studied here (Table XVII).

(D) Inhibition of EBV Cell Lines

To assess further whether the inhibitory effects of DH-84 and DH-224 on PBMC mitogenesis were direct or indirect, the ability of these antibodies to inhibit the growth of EBV transformed B cell lines was tested. As shown in Table XVIII, both purified antibody and hybridoma supernate substantially inhibited the proliferation of EBV cell lines. NB-29 also inhibited these lines, but the effect was less than that obtained with the monomorphic antibodies. Control antibodies, including both one that did (NB-107) and one that did not (NHL-30.5) react with the cell lines, did not inhibit their growth (Table XVIII).

3) DISCUSSION

To determine whether HLA class II molecules are involved in B lymphocyte activation and proliferation, we tested several new anti-class II monoclonal antibodies for their ability to inhibit various lymphocyte responses. Consistent inhibition of LPS and anti-μ stimulation of PBMC by two

TABLE XV

Inhibition of Mixed Lymphocyte Reaction

Antibody (5 μg/ml)	Mean cpm ± SEM		
	Control[a]	Test[b]	% Inhibition[c]
media	459 ± 204	17,998 ± 797	
NB-29	591 ± 118	16,117 ± 1,175	13 ± 3
DH-84	111 ± 20	4,748 ± 286	69 ± 8
DH-224	139 ± 69	6,654 ± 644	61 ± 3
NHL-30.5	679 ± 141	19,810 ± 1,941	0
NB-65[d]	343 ± 93	4,028 ± 502	83 ± 6

[a]Cultures without stimulator cells.

[b]Cultures with stimulator cells.

[c]Mean ± SEM of two separate experiments.

[d]Hybridoma supernate.

TABLE XVI

Inhibition of PHA Stimulation of Normal PBMC

Antibody	Mean cpm ± SEM		% Inhibition[a]
	Control	Test	
media	519 ± 62	85,767 ± 1,665	
NB-29	332 ± 101	84,530 ± 8,938	5 ± 7
DH-84	132 ± 20	89,735 ± 5,416	0.5 ± 1
DH-224	113 ± 17	83,102 ± 1,850	9 ± 9
NHL-30.5	327 ± 79	85,280 ± 6,105	0
NB-65[b]	305 ± 82	48,373 ± 3,083	43 ± 1

[a]Mean ± SEM of two individual experiments.

[b]Antibody to transferrin receptor consistently showed 40-50% inhibition of PHA stimulation.

TABLE XVII

Inhibition of Anti-μ Stimulation of Purified B Cells[a]

Antibody (10 μg/ml)	Mean cpm ± SEM	% Inhibition
Media Control	969 ± 85	
Anti-μ alone	13,225 ± 156	
NHL-30.5	11,379 ± 724	15
NB-107	12,661 ± 550	5
NB-29	17,191 ± 2,532	0
DH-84	11,558 ± 538	14
DH-224	12,764 ± 275	4

[a]Data is representative of three separate experiments all showing equivalent results.

TABLE XVIII

Inhibition of EBV Cell Line Proliferation: ELD (DR6) Cells

Antibody (5 µg/ml)	Mean cpm ± SEM	% Inhibition[a]
Media control	7,492 ± 270	
NHL-30.5	8,036 ± 823	0
NB-107	6,341 ± 1,520	15
DH-84	3,710 ± 647	51
NB-29	4,406 ± 751	41
DH-224	1,578 ± 155	79
NB-65[b]	671 ± 62	90

[a]Data is representative of four separate experiments with each of two EBV transformed cell lines (ELD and WALK).

[b]Hybridoma supernate.

anti-class II antibodies, DH-224 and DH-84, was observed. These are antibodies directed against monomorphic DR (DH-224) and DQ + DR (DH-84) determinants. In order to investigate the relative roles of DQ and DR in this phenomenon, these antibodies and an antibody to a polymorphic DQ determinant (NB-29) were evaluated further for their ability to inhibit mitogen stimulation of PBMC and purified normal B cells, and the proliferation of EBV transformed B cell lines.

Monoclonal antibodies DH-84 and DH-224 profoundly inhibited the anti-u and LPS stimulation of PBMC; NB-29 did not. This observation suggests that DR and DQ molecules differ in terms of their functional involvement in B cell activation. However, these studies could not distinguish which effects result from direct binding of the antibodies to B cells and which might result from antibody binding to accessory cells. To answer this question, B cells were extensively enriched from PBMC using a combination of E-rosette separation of T cells, glass adherence removal of monocytes, and "negative" cell sorting. The resultant highly purified population of B cells, while showing an excellent response to anti-μ, was not inhibited by any of the anti-class II monoclonal antibodies studied here. These observations suggest that the anti-μ and LPS activation of PBMC depends on a DR sensitive step in which accessory cells are involved. Since DQ molecules are expressed only on the subpopulation of monocytes capable of presenting antigen (9,10), lack of inhibition by NB-29 might be due to the inability of this monoclonal to inhibit the monocyte subpopulation which is necessary for PBMC responses to anti-μ and LPS.

Anti-HLA class II polyclonal or monoclonal antisera have been shown to inhibit a variety of cellular responses in human and mouse systems including: proliferative and plaque forming responses to Con A and pokeweed mitogen

(11-13), primary and secondary MLR (14-16), and antigen specific lymphoproliferative responses (17-19). Anti-Ia has also been shown to inhibit LPS stimulation of mouse lymphocytes (20). An inhibitory effect of anti-HLA class II monoclonal antibodies on anti-μ, LPS and PHA induced proliferation of human cells has not been previously reported. Anti-μ antibody appears to exert its effects on B cells by two concentration dependent mechanisms (6). At low concentrations anti-μ induces cell enlargement, RNA synthesis and cell surface expression of B cell growth factor (BCGF) receptors. Proliferation of B cells in this case then requires a second signal, mediated by BCGF secreted by activated helper T cells. At high concentrations (as used in this study), anti-μ is thought to initiate a direct proliferative effect on B cells independent of T cells, monocytes or their products. Surface receptors for LPS on B cells appear to be distinct from surface IgM (21). Inhibition of both LPS and anti-μ induced proliferation of human B cells suggests a fundamental role for HLA class II molecules in the regulation of B cell growth at physiological concentrations of B cells as seen in unfractionated PBMC. As a component of PBMC, B cells are present at low cell density and may be dependent upon interaction with monocytes or a monocyte derived factor in order to proliferate in response to anti-μ. At higher cell densities B cells may not require this factor or may produce sufficient endogenous factor to be independent of accessory cells, analogous to EBV transformed B cells (see below).

Inhibition of MLR by anti-HLA class II antibodies as shown in this study confirms previous reports (13). The complete lack of inhibition of PHA stimulation suggests that activation by this mitogen, which is primarily stimulatory to T cells, may not involve HLA class II antigens.

All three anti-HLA class II monoclonal antibodies exerted a direct antiproliferative effect on the growth of EBV cell lines, although inhibition was consistently greater for DH-84 and DH-224 than NB-29. Why EBV transformed cell lines should behave differently from purified normal B cells is of interest. At low cell concentrations EBV cell lines have been shown to be dependent upon exogenous growth factor, while at higher concentrations they appear to secrete a self-stimulating factor analogous to BCGF (22-25). In order to avoid overgrowth of these lines by the day three harvest, they were seeded at a concentration of 5×10^4 cells/ml or less. At this relatively low concentration, EBV transformed cells appear to be susceptible to inhibition by anti-HLA class II monoclonal antibodies. Whether this inhibitory effect can be overcome by the addition of exogenous growth factor remains to be determined. However since EBV transformed B cells are not normal, the different responses of these cell lines compared to normal purified B cells may also be due to constitutive effects resulting from EBV transformation.

In summary, these findings document a differential inhibitory effect of anti-DR and anti-DQ monoclonal antibodies on the activation of PBMC by the B cell mitogens anti-μ and LPS. Failure to inhibit the anti-μ induced stimulation of purified B cells suggests an indirect effect on accessory cells, that at higher B cell densities may be overcome. Furthermore, anti-HLA class II monoclonal antibodies (anti-DQ, anti-DR and anti-DQ + DR) were shown to exert a direct antiproliferative effect on the growth of EBV transformed cell lines.

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C H A P T E R V

TWO MONOCLONAL ANTIBODIES THAT DEFINE UNIQUE ANTIGENIC DETERMINANTS ON B-LYMPHOMA CELLS

> "The thing that hath been, it is that which shall be;
> and that which is done is that which shall be done:
> and there is no new thing under the sun."
>
> Ecclesiastes 1:9

1) INTRODUCTION

Normal B lymphocytes can be induced by specific antigens or mitogens to reenter the cell cycle, enlarge, divide and further differentiate into immunoglobulin producing plasma cells in the presence of appropriate regulatory factors (1-3). Activated B lymphocytes subsequently develop into immunoglobulin producing plasma cells. Most non-Hodgkin's lymphomas are neoplasms that arise in cells of B-lymphocyte lineage. The neoplastic cells present in a given lymphoma represent the clonal expansion of a single cell and exhibit considerable morphologic homogeneity (4,5), although the predominant cell type varies considerably from one lymphoma to another. On an individual cell basis, non-Hodgkin's lymphoma cells are indistinguishable from the various stages of activated normal B lymphocytes when examined by light and electron microscopy. This is the basis of the popular concept that most of the non-Hodgkin's lymphomas are neoplasms of B lineage cells in which the large clonal population produced consists of cells that are phenotypically "frozen" or "switched-on" at some point along the B lymphocyte transformation continuum (4-8). Nevertheless, there is little known about

the cell type initially transformed, the possibility that some lymphoma cells can be induced to differentiate further, and the role of abnormal gene expression in these malignancies.

Recently a number of monoclonal antibodies have been evaluated as diagnostic reagents for the classification of B cell lymphomas. Most of these fall into one of the following categories: B cell specific (react only with cells of B lymphocyte lineage), B cell associated (react with B cells but also cells of other lineages), blast associated (define antigens present on normal B-blasts but absent from resting B cells), and antibodies whose principal reactivity is with Burkitt's lymphoma cells or EBV transformed cell lines (9-24). To date, none of these have shown specificity for B lymphoma cells. In this chapter, two monoclonal antibodies are described which have thus far shown remarkable specificity for human B lymphoma cells. From these initial findings it appears that these antibodies may be useful in the future diagnosis, classification and treatment of the non-Hodgkin's lymphomas.

2) RESULTS

(A) Reactivity with Cell Lines

Two monoclonal antibodies, LM-26 and LM-155, reactive with the immunizing DHL-10 lymphoma cells but not with CLL cells, were initially tested for their reactivities with various cell lines by FACS analysis. LM-26 reacted with 4/7 B lymphoma cell lines; LM-155 reacted with 7/7 (Table XIX). These lines were originally derived from patients with a diagnosis of diffuse "histiocytic" lymphoma (DHL-1, DHL-4, DHL-8, DHL-10), diffuse mixed histiocytic and lymphocytic lymphoma (DHL-6), lymphosarcoma (U698-M) and an unspecified B lymphoma (BALM-5) (25-29). In contrast, 9/10 EBV transformed "normal" B cell lines were unreactive with these antibodies.

TABLE XIX

Cell Line Reactivity of Antilymphoma Antibodies: FACS Analysis[a]

Cell line	Type	LM-26	LM-155
DHL-1	B-lymphoma	-	+
DHL-4	B-lymphoma	+	+
DHL-6	B-lymphoma	+	+
DHL-8	B-lymphoma	-	+
DHL-10	B-lymphoma	+	+
U698-M	B-lymphoma	-	+
BALM-5	B-lymphoma	+	+
JREE	EBV	-	-
CMG	EBV	-	-
RMG	EBV	-	-
WALK	EBV	-	W+[b]
SWEI	EBV	-	-
ELD	EBV	-	-
BN	EBV	W+	-
MAD	EBV	-	-
KOZ	EBV	-	-
WAY	EBV	-	-
U937	Histiocytic	-	-
I937	Histiocytic	-	-
Jurkat	T leukemia	-	-
HL-60	Myeloid	-	-
K562	Erythroid	-	-

[a]Cell lines were considered positive if >10% of cells reacted with the test antibody compared to negative control antibody of irrelevant specificity.

[b]W=weak (10-15% of cells showed low intensity fluorescence).

Neoplastic cell lines of monocyte/macrophage (U937, I937), T leukemia (Jurkat), myeloid (HL-60) and erythroid (K562) origin also showed no reactivity with either antibody.

Cytogenetic analysis of the Giemsa banded metaphases from non-reactive EBV cell line WAY-1 revealed a normal 46 X,Y karyotype. DHL-10 cells, which were positive with both antibodies, had a highly abnormal karyotype: 47, XY, +7,-8, $10q^-$, $11q^+$, $14q^+$, +M.

(B) Reactivity with Fresh Tissues

Eighteen of 23 non-Hodgkin's B cell lymphomas were positive by FACS analysis for the antigen defined by LM-26 (Table XX). This included both small and large cell lymphomas, both with and without nuclear cleavages. Figures 13 and 14 show histogram and contour plot profiles respectively of cells from a small cleaved cell lymphoma. Noteworthy is the same broad pattern of staining (small, intermediate and large cells) with anti-lambda antibodies, which establishes the presence of the neoplastic clone, and LM-26. LM-155 reacted with only 5 of the 23 B cell lymphomas tested thus far. Examples of B lymphoma cells with either kappa or lambda surface immunoglobulin light chains and with differing heavy chain isotypes were found to be reactive with LM-26 and LM-155. One patient with CALLA positive ALL (a known early B cell malignancy) reacted with LM-155.

Five B cell lymphomas, three T cell lymphoma/leukemias and one lymphoma of uncertain immunologic subtype were negative with both LM-26 and LM-155 (Table XXI). Tissues containing reactive lymphoid infiltrates (lymph nodes 8, spleen 2, lung 1) also did not react with these antibodies (Table XXII). Cells in peripheral blood and bone marrow samples from normal controls, as well as patients with reactive lymphocytosis, chronic lymphocytic leukemia (CLL) and a variety of other lymphoid and nonlymphoid

TABLE XX

Analysis of Fresh Tissues: B Cell Malignancies - Positive

Patient	Age/Sex	Tissue[a]	Pathologic[b] Diagnosis	% Positive FACS						
				LM-26	LM-155	K	λ	Leu 12	Leu 5	Leu-M3
1	19F	LN	SCCL-D	79	5	79	1	78	26	2
		PB	LSCL	24	37	81	1	78	7	2
2	84F	LN	SCCL	39	14	74	3	66	32	9
3	60F	LN	SCCL-F	25	1	59	<1	60	42	2
4	40F	LN	SCCL-D	21	<1	54	6	51	22	<1
5	78F	LN	SCCL-D	13	12	58	6	66	43	22
6	72M	LN	SCCL-F	49	2	4	74	87	14	<1
7	66M	Spleen	SCCL	37	2	54	4	68	25	4
8	56M	BM	SCCL	23	<1	71	6	58	8	6
9	65M	PB	LSCL	23	1	54	6	76	7	2
10	40F	IAT	MCL-D	62	1	72	<1	72	24	2
11	18M	PB	SNC	31	<1	78	10	50	14	15
12	66M	LN	LCC-N	26	<1	2	28	36	38	<1
13	60M	LN	LCC	30	<1	50	<1	32	13	1
14	85F	LN	LCC-N	23	3	<1	34	37	41	1
15	69M	RT	HL	49	29	81	6	ND	6	ND
16	19M	BM	ALL	3	13	ND	ND	89	5	<1
17	49F	LN	NCL-D	20	1	4	55	56	21	1
18	26M	Spleen	SCCL	37	2	54	4	68	25	4
19	52F	PF	LCL/B-IBS	71	32	52[c]	68[c]	71	24	2

[a]LN, lymph node; PB, peripheral blood; IAT, intra-abdominal tumor; RT, retroperitoneal tumor; BM, bone marrow; PF, pleural fluid.

[b]SCCL, small cleaved cell lymphoma; F, follicular; D, diffuse; LSCL, lymphosarcoma cell leukemia; MCL mixed small and large cell lymphoma; SNC, small non-cleaved lymphoma; LCC, large cleaved cell lymphoma; HL, histiocytic lymphoma; ALL acute lymphoblastic leukemia; LCL, large cell lymphoma; IBS, immunoblastic sarcoma; NCL, non-cleaved cell lymphoma.

[c]Clonality not determined.

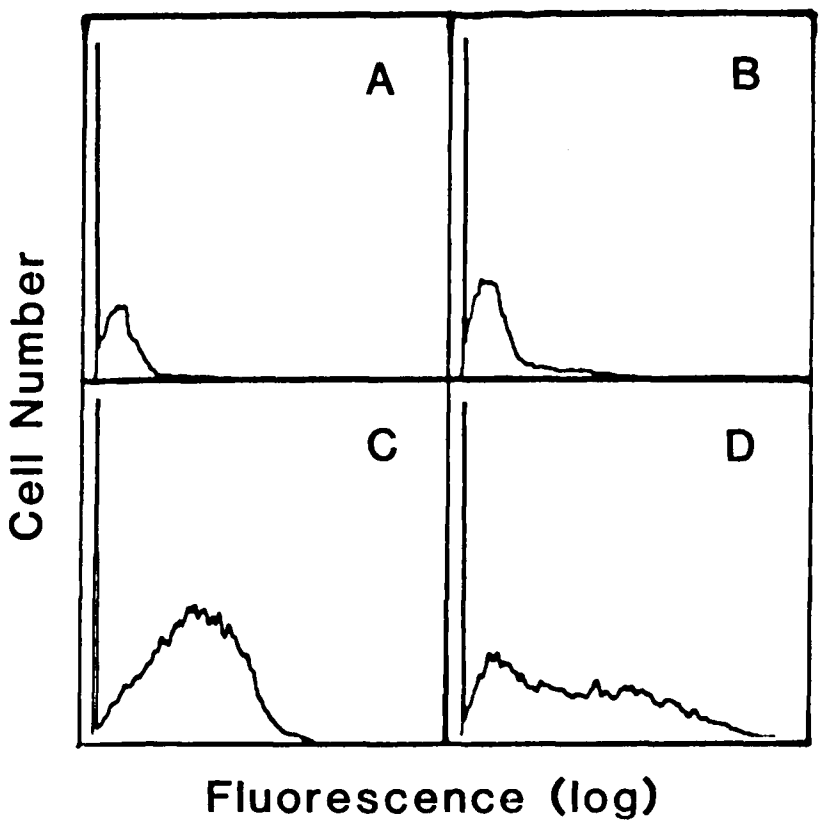

FIGURE 13 FACS histogram of small cleaved cell lymphoma stained with A) negative control antibody, B) anti-kappa, C) anti-lambda, D) LM-26. A monoclonal lambda pattern of surface immunoglobulin is identified. Staining intensity of LM-26 exceeds that of anti-lambda for some cells.

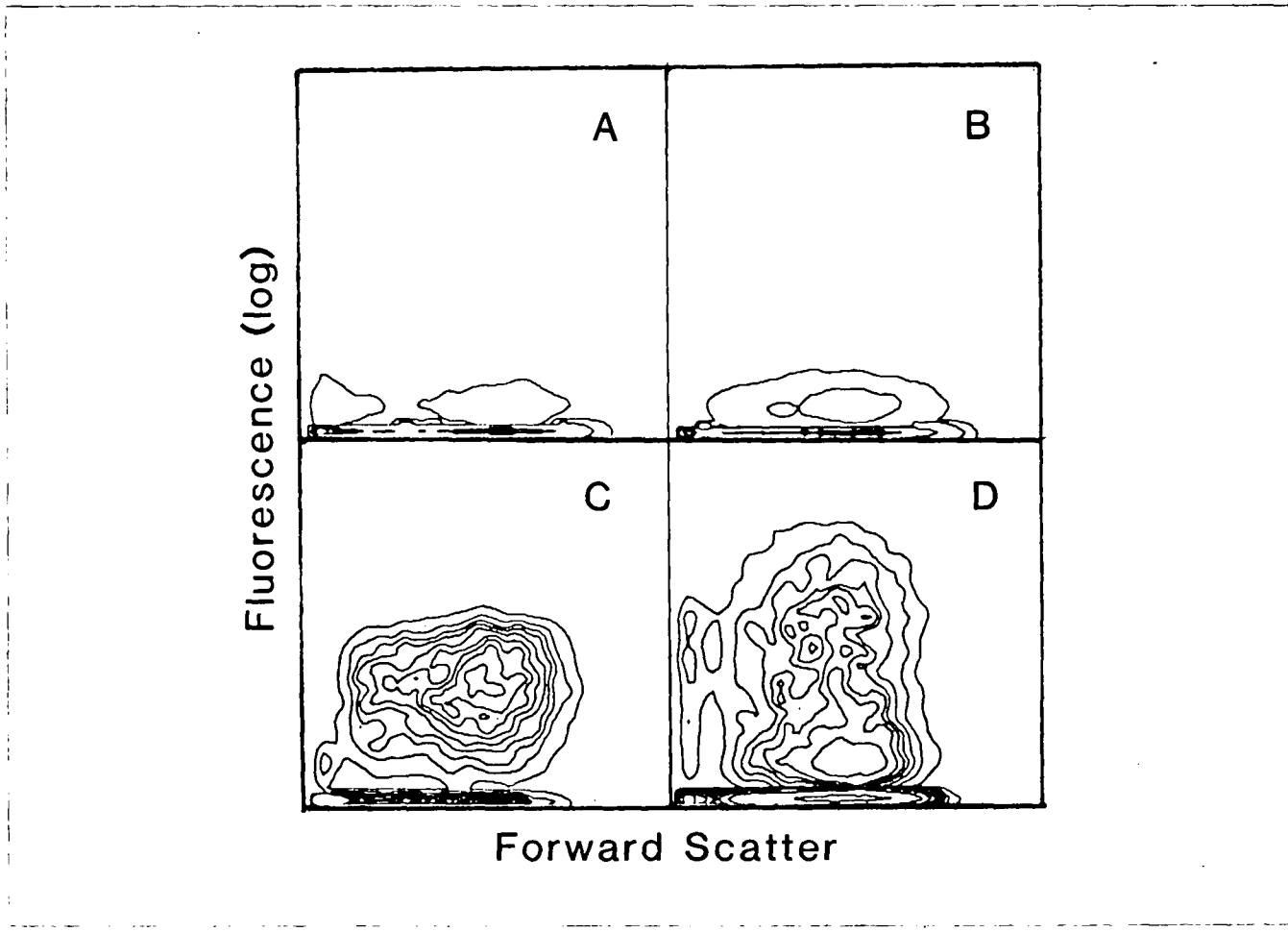

FIGURE 14 FACS contour plot of small cleaved cell lymphoma stained with A) negative control antibody, B) anti-kappa, C) anti-lambda, D) LM-26. Cell number is reflected in the 'Z' axis. Both anti-lambda and LM-26 stain lymphoma cells of all sizes. This indicates LM-26 binding is not restricted to a particular subtype of cell based on size (e.g. large transformed lymphoid cells) within a given lymphoma.

TABLE XXI

Analysis of Fresh Tissues: B and T Cell Malignancies - Negative

Patient	Age/Sex	Tissue[a]	Patnologic[b] Diagnosis	% Positive FACS						
				LM-26	LM-155	K	λ	Leu 12	Leu 5	Leu-M3
1	60M	LN	SCC-F	5	<1	41	3	45	52	4
2	44M	LN	SCC-F	<1	1	3	52	79	28	<1
3	76M	LN	SCC-D	4	4	4	2	60	15	3
4	74M	LN	SLL	3	7	61	5	72	34	6
5	63M	LN	IBS	3	<1	52	<1	49	10	3
6	53M	LN	SNC-D	<1	<1	<1	48	57	7	<1
7	84F	LN	MCL-F	<1	<1	1	1	42	44	<1
8	60M	LN	T-LL	<1	<1	2	<1	2	91	6
9	79M	LN	PTCL	2	2	19	14	28	47	4
10	48M	PB	TCL	<1	<1	18	10	1	62	17

[ab]See Table XX; T-LL, T-lymphoblastic lymphoma; PTCL, peripheral T cell lymphoma; TCL, T cell leukemia.

TABLE XXII

Analysis of Fresh Tissues: Reactive Lymphoid Proliferations - Negative

Patient	Age/Sex	Tissue[a]	Pathologic[b] Diagnosis	% Positive FACS						
				LM-26	LM-155	K	λ	Leu 12	Leu 5	Leu-M3
1	12F	LN	Lipogranulomata	2	<1	8	4	25	66	1
2	52M	LN	FH with KS	<1	<1	13	5	24	26	4
3	76M	LN	RLH	7	5	30	24	44	35	5
4	77M	LN	MCa	<1	<1	26	27	<1	<1	<1
5	10F	LN	FH with SH	4	1	14	8	24	62	<1
6	67F	LN, submax.	BLPL	7	8	50	34	52	47	6
7	29F	LN	GL	<1	<1	15	8	25	39	2
8	56F	LN	SH	4	2	14	6	25	59	2
		Spleen	CC	2	1	23	11	28	54	7
9	50M	Spleen	MCHD	<1	<1	16	12	8	16	12
10	75M	Lung	LPI	4	<1	37	28	41	48	<1

[a] See Table XX.

[b] FH, follicular hyperplasia; KS, Kaposi's sarcoma; RLH, reactive lymphoid hyperplasia; MCa, metastatic carcinoma; SH, sinus histiocytosis; BLPL, benign lymphoproliferative lesion of salivary gland; GL, granulomatous lymphadenitis; CC, chronic congestion; MCHD, mixed cellularity Hodgkin's disease; LPI, lymphoplasmacytoid infiltrate.

hematologic malignancies were all unreactive with LM-26 and LM-155 (Table XXIII).

(C) Reactivity with Normal B-blasts

In order to determine whether these antibodies reacted with lymphocyte activation-associated cell surface antigens, enriched populations of B cells were analyzed after their selective activation in vitro. For this study, normal spleen cells were stimulated in culture with lipopolysaccharide (LPS), and 4 days later the cells harvested and T cells removed by rosette sedimentation with AET treated SRBC. Remaining cells consisted of 90% surface immunoglobulin positive B cell blasts as determined by FACS analysis of fluorescence and light scatter and morphologic observation of stained cytospins. However no evidence of any reactivity of LM-26 and LM-155 with these normal B cell blasts was obtained (Figure 15).

3) DISCUSSION

Attempts to raise monoclonal antibodies to lymphoma cells have in most instances resulted in antibodies which detect normal B cell specific or B cell associated antigens (15-24). A few antibodies detecting antigens on normal B cell blasts or showing specificity for Burkitt's lymphoma cells have also been described (9,10,16,17). We have recently isolated two monoclonal antibodies, LM-26 and LM-155, that appear to show a high degree of specificity for non-Hodgkin's B lymphoma cells. To our knowledge this represents the first report of antibodies with this type of reactivity.

LM-26 reacted with some but not all B lymphoma cell lines, only weakly with one of ten EBV transformed B cell lines and was negative with all other cell lines tested. These included various cell lines with features of histiocytic, T leukemia, myeloid and erythroid origin. By FACS analysis,

TABLE XXIII

Analysis of Fresh Tissues: Miscellaneous - Negative[a]

Diagnosis[b]	Tissue[c]	FACS analysis # cases negative
Normal	PB	30
Normal	BM	3
Reactive lymphocytosis	PB	4
	BM	1
	PF	1
CLL	PB	10
T-prolymphocytic leukemia	PB	1
ALL	PB	1
	BM	1
MF/Sezary's	PB	2
CML	PB	10
	BM	6
CML-BC	PB	3
AML	BM	7
AMML	PB	1
	BM	1
HCL	PB	1
	BM	1
Aplastic anemia	PB	1
Myelodysplasia	BM	1

[a]Less than 3% of cells reacted with test antibodies (LM-26, LM-155) compared to isotype identical negative control antibody of irrelevant specificity.

[b]CLL, chronic lymphocytic leukemia; ALL, acute lymphoblastic leukemia; MF, mycosis fungoides; CML, chronic myelogenous leukemia, BC, blast crisis; AML, acute myelogenous leukemia; AMML, acute myelomonocytic leukemia; HCL, hairy cell leukemia.

[c]PB, peripheral blood; BM, bone marrow; PF, pleural fluid.

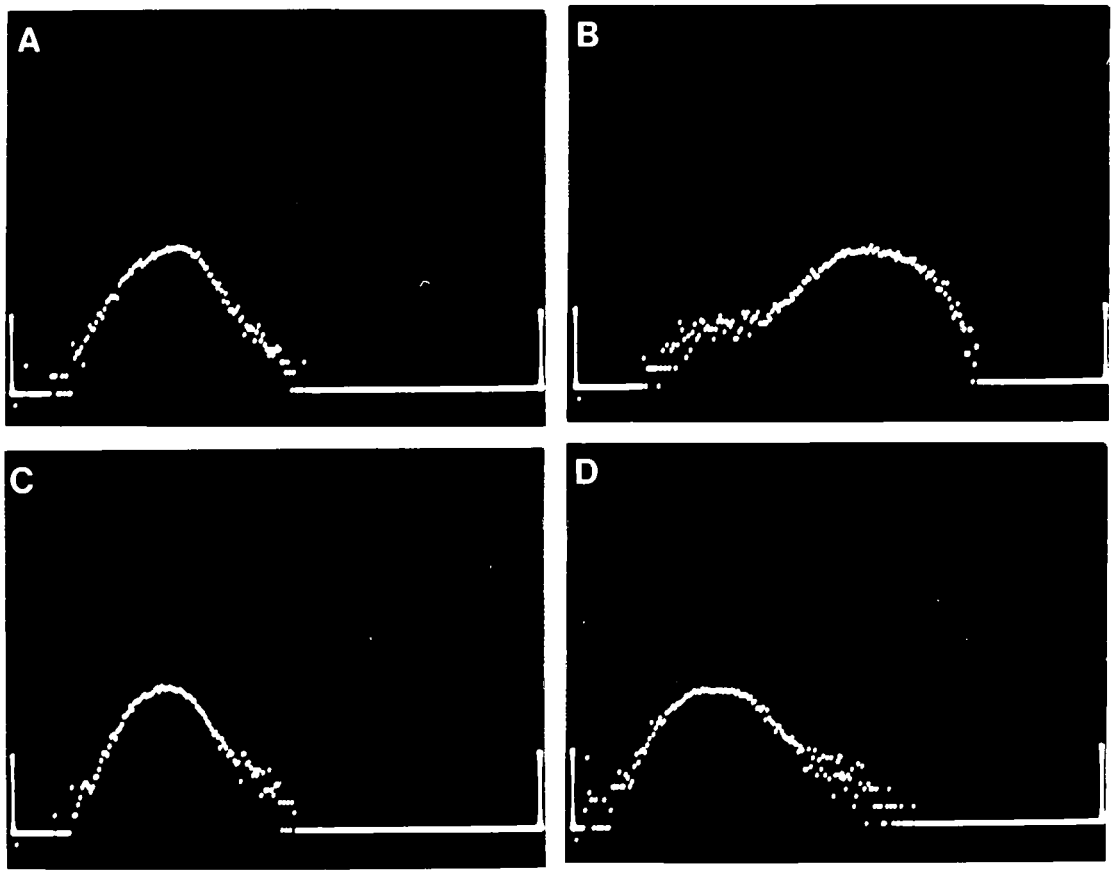

FIGURE 15 FACS histogram of purified LPS stimulated normal B cell blasts stained with A) negative control antibody, B) anti-polyvalent surface immunoglobulin, C) LM-26, D) OKT11. Ninety per cent of cells are surface immunoglobulin positive B cells (B), which by light scatter and morphologic examination of stained cytospins, are predominantly blasts. These cells do not bind LM-26 (C). There is only five per cent residual contamination with T cells (D).

LM-26 reacted with 80% of B cell lymphomas freshly explanted from patients. No reactivity was observed with T cell lymphomas or leukemias, CLL, hyperplastic lymph nodes, reactive peripheral blood lymphocytes, normal peripheral blood and marrow cells, or blood and marrow cells from a variety of non-lymphoid hematologic malignancies.

LM-155, in contrast, reacted with all B lymphoma cell lines tested, but only weakly with one EBV transformed B cell line. All other cell lines were negative with this antibody. LM-155 reacted with 20% of freshly explanted B lymphomas and one CALLA positive ALL (expressing B cell surface markers). All other normal and neoplastic tissues examined were negative for the antigenic determinant defined by this antibody.

These findings suggest that these monoclonal antibodies are detecting lymphoma specific or lymphoma associated antigens. In the past, monoclonal antibodies which were initially thought to be tumor specific have turned out after extensive examination to be normal differentiation antigens expressed at low levels or on highly restricted subpopulations of cells in normal lymphoid or myeloid tissue samples (9-24). Why large populations of malignant cells accumulate that express surface antigens found only transiently on normal cells is not well understood. Deregulation or switching-on of genes normally expressed only during embryogenesis has been documented for some tumor types (e.g. those expressing CEA or AFP). Evidence of other mechanisms, including the expression of activated, altered c-onc genes has also been reported (30). Since the non-Hodgkin's lymphomas are regarded conceptually as neoplasms of mature but activated lymphocytes, we tested the possibility that the antigens detected by LM-26 and LM-155 might also be expressed on their normal lymphoid counterparts. However, FACS analysis of highly purified populations of 4 day old LPS stimulated human

splenic B lymphocytes failed to reveal the presence of detectable numbers of LM-26 or LM-155 positive cells. Lack of expression of antigens reactive with LM-26 and LM-155 on normal cells was further supported by the negative results obtained with a variety of reactive lymphoid hyperplasias from lymph node, peripheral blood, spleen and lung. These reactive cell populations contained significant proportions of in vivo activated lymphoblasts. However, we cannot yet rule out the possibility that the antigens detected by these antibodies may still be present at low levels on minor subpopulations of normal reactive B cells. Since a small proportion (up to 7-8%) of reactive lymph node cells from some patients were positive by FACS analysis, it is conceivable that these positive cells may represent a unique B cell subpopulation expressing these antigens. Alternatively, the detection of small numbers of positive cells in reactive lymphoid proliferations may be a sign of incipient neoplasia.

Most lymphomas are clonal neoplasms and the cells present in many tumors show considerable morphologic homogeneity. However, morphologically dissimilar cell types may be admixed and are generally assumed to be part of the same neoplastic clone (4,5). It was of interest therefore to determine if LM-26 and LM-155 detected determinants expressed on a particular morphologic subpopulation of cells in a given specimen. As shown in Figure 14, cells from a small cleaved cell lymphoma that stained in a monoclonal pattern with anti-lambda light chain antisera varied in size over a considerable range including intermediate and large cells as well as small cells. Similarly LM-26 also stained cells of small, intermediate and larger size. Therefore it seems unlikely that staining patterns with this antibody are related to parameters that control cell size. Variations in antigen expression in different phases of the cell cycle is also unlikely, since in

most small cell lymphomas fewer than 10% of cells are in cycle (31). Finally it is of interest that the antigens detected by both LM-26 and LM-155 could be found on cells from B lymphomas of different morphologic subtypes, including examples composed predominantly of small or large and cleaved or noncleaved cells. At present little information about the molecular nature or function of the antigens defined by these antibodies is available. Attempts to immunoprecipitate the antigens detected by LM-26 and LM-155 using standard ^{125}I cell surface labeling techniques (32) have to date not been successful. However, it seems unlikely that LM-26 and LM-155 detect a determinant associated with immunoglobulin since both of these antibodies reacted with neoplastic cells possessing different heavy and light chain isotypes and neither reacted with normal resting or transformed immunoglobulin positive B cells.

The significance of weak reactivity of each of these antibodies with 1 of 10 EBV transformed B cell lines is uncertain. These cell lines (except for WAY-1) have been passed in culture for many years. Given the known genetic instability of EBV transformed lines and the tendency for these lines to develop karyotypic abnormalities with time (33), it would not be surprising if this reactivity proved to be associated with a genetic change that occurred after immortalization in vitro. The EBV line WAY-1 which was isolated from a patient less than six months prior to testing and shown to be karyotypically normal was negative for LM-26 and LM-155 binding. On the other hand, the B-lymphoma cell line DHL-10 which reacted with both LM-26 and LM-155 showed multiple karyotypic abnormalities as has been reported for many other B lymphoma lines (34-36). Burkitt's lymphoma cells appear to express different cell surface antigens depending on whether they possess the usual t(8;14) or variant t(8;2 or 8;22) translocations (14). Other characteristic

chromosomal abnormalities have been described in lymphoma cells freshly removed from patients (37). This raises the question as to whether there might be a particular genetic abnormality present in some lymphoma cells that confers LM-26 or LM-155 reactivity.

These findings suggest that many B cell lymphomas display on their surface common epitopes not found on the majority of resting or activated normal B cells or cells of other hemopoietic lineages. Whether these antigens are stage specific differentiation antigens, viral or oncogene products, or result from de novo alterations of surface molecules due to other processes associated with neoplastic transformation remains to be determined. Regardless of the explanation, it is noteworthy that heterogeneity of expression of such antigens in a given population of lymphoma cells was observed. It may be anticipated that antibodies such as LM-26 and LM-155 will be useful in analyzing further the biology of the non-Hodgkin's lymphomas. In addition, they are potentially valuable reagents for diagnosis, detection of residual disease, and possibly treatment in future therapeutic strategies.

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C H A P T E R VI

SUMMARY AND CONCLUSIONS

"The youth, attracted by nature and art, trusts in his vivid desire, soon to enter into the innermost sanctuary. The man realizes, after a long peregrination that he went no further than into the propylaea."

Göethe

The molecular events underlying the neoplastic transformation of normal lymphocytes are unknown. A variety of mechanisms have been postulated including: genetic mutation, chromosomal rearrangement, viral infection and inappropriate oncogene expression. The regulation of normal B-lymphocyte growth and differentiation involves the interaction of key cell surface components with extracellular molecules or surface components of other cells. Cross-linking of surface immunoglobulin or other molecules, and the interaction of receptors with growth factors provide the stimulus for B-cells to enlarge, divide and differentiate. Perturbations in normal growth regulatory controls may result in autonomous proliferation which leads to clonal selection, clonal dominance and malignancy. Clearly, through improved understanding of normal cellular regulatory processes we may be better able to appreciate and control those events that result in neoplasia.

The non-Hodgkin's lymphomas are a clinically, morphologically and immunologically heterogeneous group of diseases. Most of these are neoplasms of B-lymphocytes. Why some B cell lymphomas have a rapid clinical course while others progress only slowly is unknown, but may relate at least in part to the proliferative potential, tissue tropism and cellular morphology of the

dominant clone within a given tumor. Attempts to predict the clinical behavior of the lymphomas has largely involved subtyping based on morphologic criteria. Very little is known about how one morphologic subtype differs from another in terms of cell surface antigen expression and responsiveness to growth factors and other regulatory controls. The long-term goal of this research was to characterize the cell surface antigens of B lymphoma cells and to search for correlations between their expression and other functional differences distinguishing normal and neoplastic lymphocytes.

To achieve this goal a number of technical obstacles had to be overcome. One was obtaining sufficient numbers of lymphoma cells for immunization of mice prior to the production of monoclonal antibodies. A second was the development of a screening method for monoclonal antibodies that would allow the selection of antibodies reactive with malignant or transformed lymphocytes but not resting or normal cells. These problems were surmounted by the use of large cell lymphoma cell lines and chronic lymphocytic leukemia cells as prototypes, respectively, of transformed and resting B-lymphocytes. A third problem was how to devise methods of testing these antibodies for their ability to detect functionally relevant molecules. The approach taken was to screen monoclonal antibodies, raised against cell surface antigens, for their ability to inhibit <u>in vitro</u> mitogen stimulation of normal B and T lymphocytes. The expression of antigens detected by these antibodies on cell lines and freshly explanted neoplastic and non-neoplastic tissues was then examined using the fluorescence activated cell sorter (FACS).

Based upon these approaches, this thesis focuses on three areas of normal and neoplastic B cell function and differentiation. The first two of these describe cell surface determinants (LFA-1, HLA-class II) which may play an important regulatory function in normal B cell activation and

proliferation. The third area, and perhaps the most clinically promising, centers on the characterization of cell surface antigens which appear uniquely expressed on B lymphoma cells. This work is summarized as follows:

Lymphocyte Function Associated Antigen (LFA-1) is Involved in B Cell Activation

Human LFA-1 is a widely expressed leukocyte antigen present on cells of myeloid and lymphoid lineage. Monoclonal antibodies to LFA-1 have been shown to inhibit in vitro T cell immune functions. However, a role for LFA-1 in B cell activation has not been documented. To investigate this possibility, we examined the distribution of LFA-1 on normal, neoplastic and EBV transformed B cells as well as the ability of a monoclonal anti-LFA-1 antibody (NB-107) to inhibit B cell mitogenesis. NB-107 immunoprecipitates a noncovalently linked heterodimer of approximately 170 and 95 Kd. Sequential immunoprecipitation and cross-blocking studies showed that NB-107 identified a distinct epitope on the LFA-1 molecule. NB-107 defined LFA-1 was present on PBMC from all normal individuals (N=27) and EBV transformed cell lines (N=9), but was absent from 4 of 7 neoplastic B lymphoma lines. NB-107 was observed to profoundly inhibit the response of peripheral blood mononuclear cells (PBMC) to the B cell mitogens anti-IgM (μ) (mean 71% inhibition) and lipopolysaccharide (LPS) (mean 80% inhibition). In order to investigate the mechanism of inhibition, B cells were sequentially purified from PBMC using a combination of E-rosette depletion of T cells, monocyte removal by glass adherence and finally cell sorting. These extensively enriched populations of B cells, while still responding to anti-μ, showed no evidence of inhibition by NB-107. Growth of EBV transformed cell lines, cultured in the presence of NB-107, also were not inhibited by this antibody. When tested in assays for T cell function, NB-107 was shown to inhibit the mixed lymphocyte

response (MLR), but had no effect on PHA stimulation of PBMC, nor on the clonal growth and differentiation of granulopoietic, erythropoietic and pluripotent progenitor cells. We conclude that anti-LFA-1 monoclonal antibody inhibits B cell mitogens via indirect effects on monocytes and/or T cells, rather than by a direct antiproliferative effect on B cells.

Monoclonal Antibodies to HLA-Class II Determinants: Functional Effects on the Activation and Proliferation of EBV Transformed B Cells

Three new anti-HLA class II monoclonal antibodies were generated with differing specificities for DQ and DR determinants. Each of these antibodies (NB-29, DH-84 and DH-224) immunoprecipitates a heterodimer of approximately 35,000 and 28,000 MW from ^{125}I surface labeled B lymphoma cells as shown by SDS-PAGE. NB-29 (IgG1) detects a polymorphic DQ determinant, while DH-224 (IgG1) is reactive with monomorphic DR determinants, and DH-84 (IgG2a) has specificity for both DQ and DR. To investigate the function of HLA Class II molecules in B cell activation these were tested for their ability to inhibit various B and T lymphocyte responses. Both DH-224 and DH-84, but not NB-29, were found to inhibit significantly the stimulation of peripheral blood mononuclear cells (PBMC) by anti-μ (70-90% inhibition) and by lipopolysaccharide (80-90% inhibition), as measured by incorporation of tritiated thymidine. When added to highly purified populations of peripheral blood B cells, none of these anti-class II monoclonal antibodies inhibited anti-μ induced stimulation. This suggests that the inhibitory effect that DH-224 and DH-84 have on the stimulation of unfractionated PBMC may be due to their ability to interfere with the action of accessory cells. EBV transformed B cell lines, in contrast, showed substantial inhibition of growth when cultured in the presence of any of the three antibodies. With respect to T cells, DH-84 and DH-224 strongly inhibited the mixed lymphocyte

response (MLR); NB-29 did not. None of these antibodies inhibited stimulation of PBMC by PHA. These findings suggest that DQ and DR HLA class II molecules have differing roles in B cell activation and document a direct antiproliferative effect of anti-HLA class II monoclonal antibodies on the growth of EBV transformed cell lines.

Monoclonal Antibodies Define Unique Antigenic Determinants Expressed on B-Lymphoma Cells

The non-Hodgkin's lymphomas are a clinically, morphologically and immunologically heterogeneous group of diseases. Why lymphoma cells are unresponsive to normal regulatory growth controls and how they differ from normal lymphocytes is not well understood. In order to begin to address these questions we have developed monoclonal antibodies with specificity for neoplastic B cells. Two were found, LM-26 and LM-155, that showed a high degree of specificity for B cell lymphomas. When tested by FACS analysis, LM-26 reacted with 80 per cent (18/23) of B cell lymphomas freshly explanted from patients and LM-155 reacted with 20 per cent (5/23). The antigenic determinant detected by LM-26 was also found to be present on 4 of 7 neoplastic large cell B-lymphoma lines. LM-155 detected a determinant present on all 7 of these lines. For neither monoclonal was there any association between antibody reactivity and the morphologic subtype of lymphoma examined or the type of cell surface immunoglobulin expressed. LM-155 reacted with one case of B cell-ALL. Neither antibody reacted with normal B cell blasts, normal peripheral blood mononuclear or marrow cells, T cell leukemias or lymphomas, CLL cells, or lymphocytes from reactive lymph nodes, spleen, peripheral blood and lung. Both monoclonals were also unreactive with non-B lymphoid neoplastic cell lines, 9 of 10 EBV transformed B cell lines and cells freshly explanted from patients with malignancies of

diverse cellular origins. FACS analysis of the expression of the antigens defined by LM-26 and LM-155 on lymphoma cells and normal B cell blasts suggests that they are not normal differentiation antigens associated with lymphocyte activation or proliferation. The highly restricted expression of detectable levels of antigens reactive with monoclonal antibodies LM-26 and LM-155 on non-Hodgkin's lymphoma cells suggests a possible relation to their neoplastic properties. From a practical viewpoint these monoclonals may also prove useful in the diagnosis, classification, detection of residual disease and treatment.

General Comments

The significance of this work is two-fold. First, it demonstrates for the first time a functional role for LFA-1 and HLA-class II molecules in B cell activation and proliferation. Second, two monoclonal antibodies are described which detect unique antigenic determinants highly specific for B lymphoma cells. These findings prompt additional questions that may be answered by future research: 1) Although it was demonstrated that monoclonal antibodies to LFA-1 and HLA-DR determinants inhibit B cell activation by acting on non-B cells, what is the exact mechanism? Do these antibodies act primarily on monocytes or T cells? Why are accessory cells or T cells important in the anti-μ activation of peripheral blood mononuclear cells, but not purified B cells? What is the significance of the differential ability of HLA-DQ and DR monoclonal antibodies to inhibit B-cell activation? What is the mechanism by which anti-class II monoclonal antibodies inhibit the growth of EBV-transformed cell lines? Although technically difficult, it may be possible to determine whether LFA-1 and HLA-DR antibodies inhibit monocytes and/or T cells by performing reconstitution experiments in which antibody treated monocytes and T cells are individually "added back" to purified

populations of B cells. With the increasing availability of purified growth factors, these substances may be used in co-culture experiments of purified cell populations to determine if the mechanism of inhibition involves soluble factors. Sorting out the individual contributions of HLA-DQ and DR molecules in B cell activation and proliferation may be more difficult. However, transfection of the individual genes coding for these products may allow delineation of their separate functions. 2) Are the lymphoma antigens detected by LM-26 and LM-155 specific for malignant B cells or are they expressed on a minor subpopulation of cells at some stage of differentiation? What is the function of these antigens? Are they expressed on the putative lymphoma stem cell? Further attempts at immunoprecipitating the antigens defined by LM-26 and LM-155 will be necessary before these molecules can be characterized in detail. If LM-26 and LM-155 detect a determinant present on some normal cells, these are present either at very low antigen density or only on a small subpopulation of cells. Purification of minor subpopulations of cells using a combination of techniques (e.g. E-rosette depletion of T cells, monocyte adherence and cell sorting) may be necessary. Finally, it may be possible to clone the gene coding for the LM-26 and LM-155 defined antigens. So doing will certainly expedite evaluation of the role of these antigens in the development of B cell neoplasia.

LM-26 and LM-155 define cell surface antigens present on neoplastic B lymphoma cells which are not detectable on normal B cells or cells of other lineages, as tested by flow cytometric analysis. The question arises as to whether these antigens are expressed uniquely on neoplastic B lymphocytes, are found on a small subset of normal B cells, or are expressed during a discreet stage of B cell differentiation but are lost terminally. The absence of detectable reactivity with normal B cell blasts suggests that

these antibodies do not react with normal transformation associated or blast antigens. If LM-26 and LM-155 are uniquely expressed on malignant B cells, then this observation would appear restricted to lymphomas and unlike other human tumor systems yet studied. Such expression might be a consequence of infection by a putative virus, oncogene activation or alterations of cell surface molecules which occur in association with neoplastic transformation. In contrast, if these antibodies also detect determinants present on a minor population of normal B cells, then it would be necessary to postulate that this subpopulation is the preferential target for those events which result in the development of lymphomas. An additional possibility is that LM-26 and LM-155 detect antigens normally present on B lineage cells during embryogenesis but are later lost on normal mature B cells (oncofetal antigens). By analogy with other tumor systems studied in more detail (e.g. the acute leukemias) it is most likely that these antibodies detect antigens present at some discreet stage of B cell differentiation which are lost on normal mature resting or activated B cells, only to be reexpressed again in association with neoplastic transformation. Further work is necessary to distinguish between these possibilities.

The clinical importance of these antilymphoma monoclonal antibodies will also require additional studies. Clearly, however, the potential exists for these antibodies to be used to detect residual disease, monitor therapy, classify and treat the non-Hodgkin's lymphomas. For example, it may be possible to conjugate these antibodies to various immunotoxins such as ricin or use them in conjunction with complement in "purging" neoplastic cells from bone marrow <u>in vitro</u> to facilitate autologous marrow transplantation.

Future Prospects

The non-Hodgkin's lymphomas are clonal neoplasms of lymphoid origin. That tumors are clones has been shown conclusively by many different lines of investigation. These include: cytogenetics, glucose-6-phosphate dehydrogenase analysis, the demonstration of monoclonal surface immunoglobulin, B and T cell gene rearrangement and restriction fragment length polymorphisms (1-5). As tumors progress there tends to be the acquisition of a number of heritable characteristics such as: an increase in growth fraction, loss of differentiation, decreased antigenicity, increasing cytogenetic abnormality, the acquisition of drug resistance, elaboration of products and altered response to hormones or growth factors (2,3). How these changes are initiated and how they progress remain central unanswered questions in tumor biology.

Oncogene activation has been postulated as a possible mechanism in the genesis of many human malignancies. An initial insult or perturbation to a cell may result in the activation of a particular oncogene. This may or may not be sufficient to allow the phenotypic expression of malignancy. In fact, the concept of cooperation between two or more oncogenes has been invoked by some investigators to help explain the multistep development of human cancers (6-9). Weinberg has suggested that the reason why carcinogenesis is multistep is a requirement for activating sequentially multiple genes. There is some support for this hypothesis in animal models. For example, when either the myc or ras oncogene was introduced into normal rat embryo fibroblasts, neither caused tumorigenic transformation. When introduced together, myc and ras were able to do what neither could do alone. Cells co-transfected with these two oncogenes expanded into vigorously growing cultures and produced rapidly growing tumors in nude mice (6). Whether

oncogenes are involved in the multistage development of human lymphomas is not known. Certainly myc appears to play a role in Burkitt's lymphoma. Many Burkitt's lymphoma cells have also been reported to carry a B-lym transforming gene (7). The significance of these observations remains to be determined.

One of the more conceptually attractive theories concerning the development of neoplasia is that of clonal selection. By this concept, an initial insult to or predisposition within an apparently normal cell, confers to it an heritable growth advantage and/or increased genetic instability. This combination of an unstable genome and a relative growth advantage allows the selection of progressively more abnormal cell types. The net result is the emergence of a dominant clone of cells with features characteristic of malignancy (4).

There are clinical examples to support this concept. Philadelphia chromosome (Ph^1) positive CML tends to be a slowly progressive form of leukemia for a period of several years; until the development of blast crisis. Blast crisis is characterized by the emergence of a dominant clone with characteristics of primitive myeloid or lymphoid cells. These cells may express other cytogenic markers in addition to Ph^1. Once blast transformation develops in CML the disease is rapidly fatal.

Chronic lymphocytic leukemia (CLL) is most often a slowly progressive disease occurring in the elderly. Occasionally, this form of leukemia, in which the cells are small and relatively quiescent, may terminate in the development of large cell lymphoma/leukemia (Richter's syndrome). In one well documented case a patient with CLL during the indolent phase of her disease had a normal diploid karyotype gradually replaced by a pseudodiploid lymphocyte population. Upon the development of Richter's syndrome the

neoplastic cells became increasingly cytogenetically abnormal (hypertriploid) but retained the original pseudodiploid marker chromosomes (5). These findings demonstrated the evolution of an aggressive form of leukemia/lymphoma from one which was relatively benign.

Other examples are well documented. The non-Hodgkin's lymphomas may progress from small cell to large cell types or from a nodular to a diffuse pattern during the course of disease. The latter are associated with a more rapidly progressive clinical course. EBV infected normal B cells become immortalized in in vitro culture. Initially these cells are cytogenetically normal and polyclonal. After a period of months to years in culture these cells become monoclonal (i.e. a dominant clone emerges) and develop cytogenetic abnormalities. Rous sarcoma virus when introduced into rats produces a tumor which in its early stages is cytogenetically normal. As the tumor progresses, however, increasing karyotypic abnormalities develop (2, 4, 10).

There are a number of mechanisms possible for the development of genetic instability in tumor cells (i.e. the increased tendency to develop genetic structural abnormalities compared to normal cells). These include inherited defects (chromosomal breakage syndromes, subclinical gene defects, constitutional chromosomal abnormalities), acquired defects (gene mutations, acquired chromosomal alterations) and extracellular factors (viruses, radiation, chemical agents etc). In addition, there may be a central role for host factors in clonal evolution. These might include: immune surveillance mechanisms, nutritional status, the microenvironment, regulatory substances, exposure to infectious agents and treatment (4).

It should be emphasized that the concepts of genetic instability and clonal evolution, in the progression of most tumors, remain attractive but as

yet unproven hypotheses. However, using these concepts it may be possible to view the older notions of chemical carcinogenesis relating to initiators and promoters in a new way. An initiated cell may be one which is genetically unstable and which has a heritable growth advantage. Promotion would then be the proliferative stimulus that allows these characteristics to be expressed.

These concepts may be applied to the lymphomas. For example, Burkitt's lymphoma as discussed previously is characterized by the translocation of the myc oncogene, located on chromosome 8, to an active site of genes coding for immunoglobulin heavy (chromosome 14) or light (chromosomes 2, 22) chains. It has been postulated that this change in the regulatory environment of myc results in its abnormal expression which then leads to neoplastic transformation. There are two types of Burkitt's lymphoma; the endemic type occurring primarily in Africa and the nonendemic or sporadic type which occurs elsewhere. Both types of lymphoma are similar morphologically, both have the characteristic chromosomal translocation, but only in the endemic form is cellular infection with EB virus characteristic. It would therefore seem reasonable to postulate that there may be several "causes" of Burkitt's lymphoma. One cause may be EB virus. The others, through the interaction with constitutional or environmental factors may lead to the development of the characteristic chromosomal abnormality and the phenotypic expression of malignancy (11-14).

Much less information is presently available to suggest possible mechanisms for the development of the other subtypes of non-Hodgkin's lymphomas. In some of these, characteristic but not invariant chromosomal abnormalities may be found (15, 16). Putative oncogenes, responsible for malignant transformation in these lymphomas, remain to be identified.

It is apparent that most or all malignancies have phenotypic characteristics similar or identical to a normal cellular counterpart at some stage in differentiation. For example, $CALLA^+$ ALL appears to have as its normal cellular counterpart an early committed B lymphoid stem cell; one in which immunoglobulin gene rearrangement has occurred and which expresses B cell surface markers but lacks detectable surface or cytoplasmic immunoglobulin. Likewise, the normal cellular counterpart of pre-B ALL is the pre-B cell. This cell, in addition to expressing B cell surface antigens, contains cytoplasmic but not surface IgM. B cell ALL is probably a leukemic phase of Burkitt's lymphoma. These cells express, in addition to other markers, surface Ig. B cell type of CLL appears to have as its normal cellular counterpart the small resting (mature) B cell which expresses surface IgM with or without IgD.

The normal cellular counterparts of the non-Hodgkin's lymphomas have not been definitively established. It has been postulated that during the course of lymphocyte transformation, B cells go through a series of morphologic stages recognizable in the germinal centers of lymph nodes. These stages in normal lymphocyte development are thought to have as a neoplastic counterpart, tumors in which one particular morphologic cell type dominates (see Chapter I). One way to prove or disprove this hypothesis is by characterizing the cell surface antigens on the various subtypes of lymphomas and correlating these with subpopulations of normal cells. This is one application of monoclonal antibodies such as LM-26 and LM-155 described in this thesis.

What are the central questions relative to understanding the biology of the non-Hodgkin's lymphomas and, given existing technology, may answers be realistically expected? Clearly, it is important to understand normal B cell

function. What factors regulate the growth, transformation and differentiation of normal B cells? How do these factors interact with B cells? Via surface receptors? What is the nature of these receptors? Are they perturbed in malignant B cells? Are lymphoma cells growth factor independent? Do they rely for their growth advantage on autostimulatory factors or are they exquisitely sensitive to low levels of these factors? With respect to T cells and T cell neoplasms some of these questions are being answered. It is not unreasonable to assume that the genes coding for B cell stimulatory factors and their receptors will in the next few years be cloned and their products characterized.

Do specific subtypes of lymphomas correlate with a particular chromosomal abnormality or with the activation of a particular oncogene? For example, if Burkitt's lymphoma is caused by the activation of the myc oncogene then perhaps other morphologic subtypes of lymphomas result from the abnormal expression of different oncogenes or expression of the same oncogene at varying times in the cell cycle.

What is the nature of the replicating cell in the non-Hodgkin's lymphomas, i.e. the putative lymphoma "stem cell"? Does this cell have unique characteristics that allow it to be distinguished from associated, perhaps more differentiated, progeny? Why is this cell unresponsive to normal growth regulatory controls? Does this cell differ, depending on the morphologic subtype of lymphoma or is it the same in all B cell lymphomas, with other extrinsic factors contributing to the morphology of individual tumors? Nodular lymphomas have a less aggressive clinical course than do diffuse lymphomas. What factors contribute to the maintenance of this pattern of growth? Why is this pattern lost during the progression of many tumors?

One of the difficulties in trying to study the non-Hodgkin's lymphomas has been the inability to culture cells from these tumors in vitro over long periods. Long term culture of lymphoma cells is feasible with existing technology. To do so will require identifying those nutrient, growth factor and environmental substances which are crucial to the maintenance of cell viability and growth. This approach has already been successfully applied to the study of bone marrow myeloid and erythroid progenitors (17).

Finally, what are the causative factors which initiate malignant transformation? Viral infection? Spontaneous mutation? Hereditary or environmental factors? How can these factors be eliminated or controlled? Although much has been accomplished, much remains to be done.

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And ever, as the storey drained
     The wells of fancy dry,
And faintly strove that weary one
     To put the subject by,
"The rest next time--" "It is the next time!"

Thus grew the tale of Wonderland
     Thus slowly, one by one,
Its quaint events were hammered out--
     And now the tale is done,
And home we steer, a merry crew
     Beneath the setting sun.

Lewis Carroll
Alice's Adventures in Wonderland

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