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The Cultured Lymphocyte in Clinical and Experimental Medicine*

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Our understanding of the nature of the lymphocyte and its role in health and disease has undergone dramatic changes in the last decade. Known for almost one hundred years, peripheral lymphocytes had traditionally been considered short-lived with limited biologic activity and of little significance to the economy of the host. Recent studies, however, have clearly demonstrated that the circulating lymphocyte pool is composed of a spectrum of cells of varying origins, lifespans, fine structural features and capacities to mediate immunologic responses (1). A fortuitous observation by *Nowell* in 1960 (2) was a major impetus for this continuing series of investigations in lymphocyte biology. He noted that phytohemagglutinin (PHA), a crude extract of the common red kidney bean, had the remarkable ability to cause normal small lymphocytes from peripheral blood to undergo a series of morphologic changes to "blast like" cells in tissue culture. A variety of immunologic and non-immunologic stimuli were subsequently shown to initiate similar morphologic alterations in small lymphocytes associated with new RNA, protein, and DNA synthesis, followed by mitosis and cell division (3). This process, termed lymphocyte transformation, confirmed that the lymphocyte is a resting cell capable of further differentiation and proliferation and provided an exceedingly ver-

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satile and accessible *in vitro* model for the orderly analysis of human immunologic reaction, genetic variation, and cellular differentiation. The profound impact of this discovery on medical genetics, immunology, cell biology and biochemistry is immeasurable, recognized by clinicians and researchers alike. Work with lymphocytes in short term culture and in the more recently developed established suspension cultures has produced a wealth of information to be assimilated and put to use. The versatility of these systems for the detection, definition, and solution of problems in clinical and experimental medicine has been gratifying and the limits of their potential usefulness are yet to be defined. Our Progress in Lymphology will highlight some of the advances made possible by the study of cultured lymphocytes and will consider new directions for future research and clinical application.

Short-Term Culture of Peripheral Lymphocytes

The earliest and most widely useful by-product of the *in vitro* study of lymphocytes has been the expansion of the field of cytogenetics and the understanding of the role of chromosomal abnormality in the etiology of human disease. Techniques for chromosomal analysis available before 1960 required fetal tissues, bone marrow cells or the culture of skin biopsy specimens. These were arduous methods with very limited application for widespread use. The ability to produce large numbers of normal mitotic cells from a drop of blood with phytohemagglutinin (PHA) permitted the wholesale investigation of the gross genetic basis of disease. Hundreds of chromosomal disorders have been defined by this simple technique which provides the foundation for modern cytogenetics and genetic counseling. Study of the PHA stimulated lymphocyte has led to an understanding of the possible mechanisms of chromosomal abnormality (4, 5) and of the potential teratogenic effects of a variety of common drugs (6) and viruses (7). Most of the genetically determined complement of lysosomal enzymes produced by the host may be found in the peripheral lymphocyte, which has become useful in the detection and diagnosis of patients with inborn errors of metabolism as well as heterozygous carriers of these disorders (8). PHA stimulation of lymphocytes from heterozygous individuals can unmask the carrier state as has been described for Pompe's disease (9). Even in diseases where the carrier state can be detected in cultured fibroblasts, the culture of lymphocytes appears to be preferable in terms of time, ease, and expense of materials and handling. These considerations have made the short-term culture of lymphocytes the most practical and useful genetic screening procedure.

The *in vitro* culture of peripheral small lymphocytes has become a model system for the controlled investigation of derepression, differentiation, and mitosis as a general biologic phenomenon. Despite our lack of understanding of the actual biochemical mechanisms whereby PHA attaches to the cell membrane and induces transformation and mitosis, study of the stimulated lymphocyte has greatly expanded our knowledge of the biochemical events of the cell cycle associated with synthesis, growth and mitosis in normal cells and cells from individuals with a variety of diseases (10). Early in the stimulation of lymphocyte growth a number of biochemical phenomena have been described which seem to be related to altered gene activity (11). After an initial interaction of PHA with lymphocyte membrane (12) the following changes have been found: altera-

tions of membrane phospholipid metabolism (13); acetylation of histones (14); increases in tRNA (15) and ribosomal RNA (rRNA) synthesis (16) including decreased 18S rRNA wastage (17); increases in nuclear template activity for RNA transcription and increases in protein synthesis (18); increase in lysosomal acid hydrolases and shifts of these enzymes to the cytoplasm (19); induction of uridine kinase (20) and DNA polymerase (21); alterations of glucose metabolism (22); increases in LDH (23), G6-PD, acid phosphatase and α-glucosidase activities (24); and increases in DNA synthesis as well as induction of tRNA methylases later in transformation but before mitosis (25, 26).

Although these events associated with increased template activity of nuclei will occur in the absence of protein synthesis, these biochemical changes in stimulated cells usually lead to the synthesis of new protein, lysosomal enzymes, immunoglobulins and other products associated with host immune defense. Many of the newly synthesized products which arise as a consequence of transformation are secreted into the supernatant and are readily accessible for study and characterization (27). Short term lymphocyte culture supernatants show precipitation bands in the γ1A and γ2-globulin regions on immunoelectrophoresis and contain all classes of immunoglobulins (28). Measurement of the production of immunoglobulins has been useful clinically to characterize immunologic disorders such as congenital and acquired agammaglobulinemias (29, 30). Stimulated lymphocytes from patients with macroglobulinemia are said to secrete increased amounts of macroglobulins into the culture medium (31), while lymphocytes from patients with myeloma can be induced to synthesize the specific paraprotein (28). Production of "long acting thyroid stimulator" (LATS), a 7S γ-globulin in patients with Grave's disease was first described in PHA-stimulated lymphocyte cultures from such patients (32).

Although delayed-type hypersensitivity, cell-mediated immunity, had been known since the time of Jenner and the object of considerable study for the past 70 years, little headway was possible within the limits dictated by *in vivo* skin testing. The similarity of transformed lymphocytes in short-term culture to the large pyroninophilic cells of the paracortical regions of lymph nodes draining areas of antigenic challenge in animals sensitized for delayed-type hypersensitivity (DTH) responses quickly captured the interest of immunologists and heralded the beginning of the modern era in cellular immunity. Indeed, the past decade has witnessed a revolution in our understanding of the immunologic capacities of the lymphocyte arising in great measure from the extensive investigations provided by the *in vitro* lymphocyte transformation model. It is quite clear that the PHA responsiveness of lymphocytes in short term culture generally reflects the integrity and competence of the cellular immune system of the host (33). PHA testing has become a standard clinical procedure in the evaluation of patients with suspected immuno-deficiency, anergic states, and malignancy and has found wide use in the monitoring of induced immunosuppression. Antigenic stimulation of lymphocytes *in vitro* has been shown to correlate closely with *in vivo* skin test reactivity (33) and has been used in the evaluation of hypersensitive states, allergies, collagen-vascular disorders, autoimmune phenomena, and cancer. Whether the cause or a result of the disease process, altered cell-mediated immune responses as detected by lymphocyte transformation have been shown in such diverse maladies as drug sensitivities (34), chronic liver disease (35), multiple sclerosis (36), acute rheumatic fever (37), and infantile eczema (38). In addition, the demonstration that allogeneic lymphocytes

induce blastogenic responses in resting cells of normal individuals (39) has provided the most sensitive tool for the evaluation of HL-A histocompatibility differences and the likelihood of homograft retention by recipients of organ transplants.

Perhaps the most exiting developments in the investigation of DTH resulting from the *in vitro* study of lymphocytes has been the detection of potent extracellular factors with capacities to recruit non-sensitized cells and to cause cell damage in ways that rather closely reflect the various responses associated with DTH *in vivo* (40). Activation of small lymphocytes by antigens as well as phytomitogens is attended by the release of a macrophage migration inhibitory factor (MIF) (41), a cytotoxicity factor (42), blastogenic factor (43), chemotactic factors (44) transfer factor (45), and interferon (46). It is postulated that these are the soluble mediators which orchestrate *in vivo* the sequence of events resulting in induration and augmented response to antigenic differences on a cellular level. Selected deficiencies for these factors have already been noted in clinical disease (47). The further documentation of their *in vivo* reality should provide the understanding for effective immunotherapy of a wide variety of disorders.

Lymphoid Cells in Continuous Culture

A logical progression of interest in the investigation of cultured lymphoid cells has led to the development of permanently established human lymphoid cell lines from patients with varied benign (48) and malignant (49, 50) lymphoproliferative disorders and from normal individuals (51, 52). Unlike conventional lines, these cells grow in suspension culture as pleomorphic forms with a fundamental lymphoid character, resembling the immature "blast-like" cells seen following the *in vitro* stimulation of peripheral small lymphocytes by phytomitogens and antigens. The enormous proliferative potential of these established systems was recognized early. They double their numbers every 24–48 hours, reaching a stable population of 1–2 $\times$ 10^6 cells/ml of culture fluid and many have been maintained in continuous culture for five years or more without signs of senescence or termination. Innumerable human lymphoid cell lines have been derived from peripheral blood, lymph node tissue, thoracic duct fluid, and spleen and several lines from subhuman primates are now available (53). Details of these lines were quickly disseminated with the discovery of a herpes-like virus (*Epstein-Barr,* EBV) in a portion of the cultured cells, amid speculation that a human oncogenic virus had been uncovered. Intensive study in many laboratories has found them to be remarkably versatile and is contributing much to our rapidly expanding concepts of the multiple functions of lymphoid cells in the immune response. While controversy still clouds the significance of the EBV and it is unknown what relationship exists for cells in culture and a particular disease state in the donor, it is clear that the study of human lymphoid cells in long-term culture will have a lasting imprint on the biology of the 1970's. They have every possibility as recent advertisements proclaim of becoming the "new E. coli" of the geneticist and immunologist (54).

Human lymphoid cells in long-term culture demonstrate capacities for synthesis and secretion of a wide variety of products of immunologic significance (55). Synthesis of intact immunoglobulins of all major heavy chain classes and light chain types has been amply demonstrated (56, 57, 58, 59). Although antibody specificity has never been confirmed for these proteins, sequential study of several cell lines indicates qualitative

differences in biosynthetic profiles with time (59), suggesting that lymphoid cell lines are capable of undergoing selection and differentiation *in vitro*. Myeloma paraproteins continue to be produced in large quantities by lymphoid cell lines derived from patients with myeloma (60). Synthesis of immunoglobulins in synchronized cultures involves more than 90% or more of the cell population, implying that all cells retain the potential for this activity (61). Cloning studies (62) and immunofluorescent analysis demonstrate that many cells can produce at least two heavy chain classes of immunoglobulin, but only one light chain type. Of considerable interest is the finding that lymphoid cell lines from agammaglobulinemic patients have the capacity to synthesize all classes of immunoglobulins *in vitro* (63). Perhaps *in vivo* inhibitors prevent the expression of intact constituitive lymphoid functions in these patients.

Current interest has focused on the relationship of cultured human lymphoid cells to the lymphocytes which mediate *in vivo* delayed-type hypersensitivity. This immunity is the cornerstone of our defenses against intracellular parasitism by viruses, fungi, and protozoa and has the primary function of maintaining surveillance for somatic cell changes, ridding the body of foreign tissue and neoplasms. The similarity of lymphoid cells in long term suspension culture to phytomitogen and antigen stimulated peripheral small lymphocytes and the large pyroninophilic lymphoid cells in regional lymph nodes during acquisition *in vivo* of DTH suggested that these lines would be useful in the detection, identification, and purification of the putative soluble mediators of DTH. Progress in these areas had been limited by the inability to demonstrate these factors in *in vivo* sites and the small quantities of materials extractable from lymphocytes in short term culture. Multiple studies have shown, however, that lymphoid cells in long-term culture synthesize and secrete into the medium large quantities of migration inhibitory factor (MIF) (64, 65), lymphotoxin (LT) (64), blastogenic factor (66), chemotactic factor (67), and interferon (68). The remarkable proliferative and synthetic potential of these cell systems has already facilitated the partial isolation and characterization of some of these biologically active immunologic factors (65). Whether they are multiple and distinct or one or two molecular species with multiple biologic effects should be quickly resolved. Purified materials will allow the production of reagents so that the *in vivo* reality of these mediators can at last be documented and followed. Of interest, it has been found that lymphoid cells from lines producing MIF will migrate in ways closely mimicking guinea pig peritoneal macrophages (65, 69). These lymphoid cells respond to migration inhibitory substances produced by lymphocytes stimulated in short-term culture phytomitogens and antigens as well as to supernatant culture fluids from syngeneic and allogeneic lymphoid cell lines. The migration of these lymphoid cells appears to be more sensitive than guinea pig macrophages to human migration inhibitory products, suggesting that there is species specificity for the extracellular mediators of DTH.

Additional and unexpected lymphoid functions with possible consequences for host defense mechanisms have been uncovered by analysis of lymphoid cells in long-term suspension culture. Synthesis of components of complement, particularly B_{1A}/B_{1C}-globulin (C'3) (70) has been detected as well as active phagocytosis of particulate matter (71). These properties are traditionally viewed as macrophage functions. Not only are the cultured lymphoid cells highly mobile and responsive to macrophage migration inhibi-

tory factor, but studies have also detected macrophage related IgG and complement receptor sites on their cell surfaces (72). While the precursor of these cells is essentially unknown, it appears that it may be similar to the uncommitted bone marrow stem cell with potential for differentiation in multiple directions. The possibility that lymphoid cells can ingest and perhaps process antigens with the synthesis and release of specific and non-specific immune factors provides the host with a simple mobile system for localized restriction and destruction of aberrant tissues, infectious agents, and foreign material.

Lymphoid cells in long-term culture bear a close resemblance to the fresh autochthonous cells from which they were derived. Multiple studies have documented the general stability of the diploid chromosomal pattern of these cells. Unusual morphologic features observed in the donor are retained by cells in long-term culture as demonstrated by the presence of giant lysosomes in lines derived from patients with Chediak-Higashi syndrome (73). Investigations have shown the similarity of donor cells and cells in culture for the synthesis of enzymes with genetic polymorphisms (74). More than 20 such systems have been studied to date (75). Specific enzyme deficiencies of the donor appear in the cultured cells as found for patients with Lesch-Nyhan syndrome (76) and ganglioside storage disease (77). Techniques have now been developed to establish long-term lymphoid cell lines from small amounts of blood from most individuals (78), which will make available massive quantities of material for the study of specific enzyme defects in patients with inborn errors of metabolism and in asymptomatic carriers of these disorders. New enzyme deficiencies will ultimately be defined by the analysis of these cell systems and lead to better understanding of inheritable diseases and to more effective genetic counseling.

Of considerable interest has been the demonstration in multiple laboratories that there is virtual identity of the HL-A surface antigens of cultured lymphoid cells and fresh autochthonous donor cells (79). New antigenic determinants have been found on the culture cells as well (79, 80). The concentration of HL-A antigens appears to be much greater on the cultured cells and soluble fractions are found in large quantities in the supernatant culture fluid (81). These cell lines should provide exquisitely sensitive target cells for tissue typing prior to homotransplantation as well as the ideal tool for the isolation of highly purified soluble HL-A antigens for characterization and eventual immunologic preparation of homograft recipients by recipient specific anti-lymphocyte antibody and/or the induction of immune tolerance.

The interest generated by the findings of *Epstein, Achong* and *Barr* (82) in 1964 of a heretofore unknown virus (EBV) within the lymphoid cells of a line derived from a biopsy specimen from a young African with Burkitt's lymphoma has sustained a major research effort to uncover at long last a human oncogenic virus responsible for malignant lymphoproliferative disorder. While the etiologic significance of the EBV remains unclear, without question these investigations have contributed to our understanding of host-viral interactions and expanded our clinical knowledge of a variety of lymphoproliferative diseases including infectious mononucleosis (83), lymphoma (84), nasopharyngeal carcinoma (85) and sarcoidosis (86). It seems likely that the presence of this viral information is required to establish the lymphoid cell in long term culture (87) and

perhaps is responsible for the *in vivo* lymphoproliferation in infectious mononucleosis. Even if etiologically unrelated, determination of the actual mechanism of EBV induced benign lymphoproliferation and its reversibility is certain to have far-reaching consequences for our understanding and therapy of malignant disease. Early findings with sarcoidosis and leprosy (88) suggest that lymphocyte-mediated immunologic competence may be a decisive factor in the outcome of this infectious process.

Areas for Future Research

While cultured lymphoid cells have already demonstrated remarkable versatility in the investigation of immunologic and genetic problems, many questions about their growth and function remain unanswered and some potential areas of usefulness are virtually unexplored. Little is known of the actual process which brings about the self-limited activation of resting lymphocytes in response to mitogens and antigens or the unrestricted proliferation of lymphoid cells in long-term culture. The nature of surface receptor sites is unknown as well as the trigger mechanisms activated by binding and the cellular controls governing growth, synthesis, and mitosis. Herein lie the basic issues to be resolved in the ultimate understanding and control of the immune response. Resolution of these areas would define the differences between the reversible activation of lymphocytes during immunologic reactions and the malignant proliferation of leukemia and lymphoma. It seems quite likely that much of the surface phenomena and cellular responses involved in these processes is similar or identical. Cultured lymphoid cells offer the best tool for repeated investigations of these relationships.

The usefulness of cultured lymphocytes to clarify the basic mechanisms of immune reaction will be limited unless we develop antigen-sensitive lymphoid cell lines. Present information suggests that there is no immunologic specificity for the large quantities of immunologic materials synthesized by these established cell cultures. Although we have no evidence that this stage of differentiation cannot be reached by human lymphoid cells in long-term culture, no antibody function has been described for the intact immunoglobulins produced. Available studies, however, suggest that these cultured cells may have stem cell potential with T and B cell function. Attempts to induce establishment of specific lymphoid cell lines by antigen stimulation or to render established lines antigen-sensitive with transfer factor have been unrewarding, perhaps for technical reasons. The need to continue such efforts is obvious. Antigen-specific lymphoid cell lines would clearly permit the definition of specific surface receptors, antigen binding, and the interim cellular events which culminate in specific immunologic reactions. They could conceivably provide bulk *in vitro* human antibody factories for therapeutic intervention in infectious and malignant disorders of man.

Perhaps like the "old E. coli", cultured human lymphoid cells will lend themselves to genetic and molecular rearrangements. Classic transformations with DNA, RNA, reverse transcriptases and transduction by the EBV have yet to be tried, but are all within the realm of possibility. The potential uses of well-characterized, specifically modified human lymphoid cell lines for enzymatic or immunologic reconstitution of the original donor and/or HL-A matched recipients stagger the imagination. While many

problems must be resolved, including the acceptance of grafted cells by the recipient and their potential for malignancy, the magnitude of these problems could be quickly defined with the sub-human primate systems currently available.

These emerging scientific opportunities and challenges are well within our abilities to master. They cover a remarkable portion of the significant problems in current immunology, genetics, cell biology and medicine. Without question, culture of human lymphoid cells will continue to play a major role in the medicine of the future.

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EDITORIAL

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The Place of Lymphadenectomy in Cancer Surgery

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History of Lymphadenectomy

The principle of removing regional lymph nodes along with the primary cancer was established in England, in 1867, by a surgeon named *Moore*, who was the first to perform a radical mastectomy. For the next century surgeons devoted their energies to devising more and more radical operations designed to remove more and more of the nodes that drained the tumors. Few voices were raised in opposition to this policy, because it seemed too be logical. Yet in 1898 *Rudolph Matas*, in a discussion of one of Halsted's papers, said that his "3 years cures" had been 41% with the old "incomplete" operation and only 38.3% with the new Halsted type of radical mastectomy (1).