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LYMPHOPROLIFERATIVE POTENTIAL IN INFECTIOUS DISEASES*

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THE past decade has witnessed a rapid evolution in our understanding of the nature of the lymphocyte and its role in health and disease. Whereas peripheral lymphocytes had been viewed traditionally as short-lived cells with limited biologic activity, recent studies have 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 variety of immunologic and nonimmunologic stimuli have been shown to initiate *in vitro* a series of morphologic alterations in small lymphocytes associated with new ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis, followed by mitosis and cell division.[2] This process, termed lymphocyte transformation, has confirmed the opinion that the lymphocyte is a resting cell capable of further differentiation and proliferation. Of

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TABLE I. INFECTIOUS DISEASES ASSOCIATED WITH PERIPHERAL ATYPICAL LYMPHOCYTOSIS

Infectious mononucleosis	Herpes zoster
Acute viral hepatitis	Herpes simplex
Cytomegalovirus infection	Influenza
Acute infectious lymphocytosis	Nonspecific upper respiratory illness
	Adenovirus
Primary atypical pneumonia	Rhinovirus
	Reovirus
Rubeola	Pertussis
Rubella	Brucellosis
Roseola	Tuberculosis
Mumps	Rickettsial diseases
Variola	Toxoplasmosis
Varicella	

particular interest is the striking similarity of these transformed cells to the atypical lymphocytes in the peripheral blood of patients with a wide variety of infectious, lymphoproliferative, and immunologic disorders. These observations suggest that circulating lymphoid cells in patients with atypical lymphocytosis are undergoing spontaneous differentiation and proliferation.

For at least 40 years a lymphocytosis with cells of abnormal appearance has been noted in diseases of known or presumed infectious etiology (Table I). Classified by light-microscopic features and known widely as Downey cells,[3] glandular fever cells, virocytes,[4] and atypical lymphocytes, they have been described most frequently in patients with infectious mononucleosis and acute viral hepatitis. These atypical cells are distinguished from normal lymphocytes by the quantity and tinctorial characteristics of their cytoplasm and the shape and chromatin configuration of their nuclei. They vary from cells of 10 to 15μ in diameter with scanty, deeply basophilic cytoplasm and indented nuclei with coarsely clumped nuclear chromatin to large cells 15 to 24μ in diameter with an increased cytoplasm to nuclear ratio, light blue vacuolated cytoplasm with occasional azurophilic granules, and a nucleus with finely reticulated chromatin material. Although these cells are of lymphoid character by histochemical[5] and ultrastructural analysis,[6] their function has remained obscure. The presence of atypical lymphocytes in patients with immunologic disorders such as allergic reactions[7] suggests that they play an important role in responses to immunologic challenge.

TABLE II. INFECTIOUS MONONUCLEOSIS: PERCENTAGE OF PERIPHERAL LYMPHOCYTES IN DNA AND RNA SYNTHESIS

Days postonset of illness	*8-14*	*15-21*	*22-28*	*29-35*	*36-42*	*43→*
No. of patients	11	11	7	5	5	7
Percentage of lymphocytes in RNA synthesis	3.6	3.4	2.7	1.9	1.7	0.8
Percentage of lymphocytes in DNA synthesis	3.6	3.1	2.6	1.9	1.4	1.0

Normal level of DNA and RNA synthesis <0.1%.

Spontaneous *in vivo* proliferation of atypical lymphocytes can be demonstrated in many of the viral infections, including infectious mononucleosis, acute viral hepatitis, rubeola, rubella, herpes simplex, and mumps. Dividing and binucleate forms appear in the peripheral circulation.[8] Increased cellular enzymes associated with nucleic acid synthesis have been described.[9] Autoradiographic techniques have confirmed the presence of markedly increased premitotic activity in these circulating cells with incorporation of labeled nucleoside precursors.[10] This activity reaches a peak 10- to 100-fold above normal limits in infectious mononucleosis at two to three weeks of clinical illness and returns toward normal levels by six to eight weeks (Table II). Spontaneous *in vitro* division of some of these labeled cells occurs shortly after removal from peripheral blood as well.[11]

To investigate further the proliferative potential of circulating lymphocytes from patients with a variety of virally induced lymphoproliferative states, attempts to establish long-term cultures of these cells were undertaken. Leukocyte cultures were prepared from 15 to 30 ml. of heparinized peripheral blood as previously described,[12] and 3.0×10^6 to 6.0×10^7 lymphocytes were cultured at 37° C in 2 oz. screw cap prescription bottles containing Roswell Park Memorial Institute medium 1640[13] supplemented with 20% fetal calf serum, antibiotics, and fresh L-glutamine. The cultures were replenished weekly with fresh medium. No effort was made to maintain cell concentrations and cultures were discarded when intact lymphocytes were no longer visible. Lymphocytes from most normal individuals remain intact and viable for 4 to 6 weeks of culture and then die off. About this time successful culture is manifested by a lowered *p*H of the culture me-

TABLE III. ATTEMPTS TO ESTABLISH LONG-TERM LYMPHOID SUSPENSION CULTURES FROM PERIPHERAL BLOOD LEUKOCYTES

	Number of individuals	*Number of specimens*	*Continuous suspension cultures*
No obvious clinical illness (normal)	32	39	0
Infectious mononucleosis	23	66	16
Acute viral hepatitis	7	7	3
Herpes zoster	4	4	2
Herpes simplex	2	2	1
Myxovirus parotitis (mumps)	1	1	1

dium, increase in cell numbers with clumping of cells in suspension, and the ability to pass the cell line continuously. Long-term lymphoid suspension cultures have been established with relative ease from the peripheral blood of heterophile-positive patients with infectious mononucleosis; from heterophile-negative icteric patients with typical clinical and laboratory findings of acute viral hepatitis; and from patients with herpes zoster, herpes simplex, and mumps (Table III). These established lines have been maintained for 4 to 22 months of continuous passage without loss of vitality. They grow as free floating forms and display morphologic heterogeneity with a fundamental lymphoid character on light and electron microscopic analysis.[14] With the techniques employed cultures were generally not obtained from control specimens from normal individuals.

Circulating lymphocytes with potential for long-term proliferation *in vitro* have been described in patients with a variety of lymphoproliferative disorders, particularly neoplastic diseases including lymphoblastic leukemia,[15] Hodgkin's lymphoma, lymphosarcoma,[16] and infectious mononucleosis.[12, 17, 18] If the lymphocytes from a liter of blood are cultured, the existence of peripheral cells with potential for long-term *in vitro* proliferation can be demonstrated in apparently healthy individuals as well.[13, 19] With the small blood volumes employed in the present studies, this background proliferative potential was not encountered in a control series of normal individuals. Specimens of comparably small size from patients with various acute infectious disorders nevertheless have been readily established in long-term suspension culture.

TABLE IV. ANALYSIS OF PERIPHERAL BLOOD SPECIMENS CULTURED FROM PATIENTS WITH INFECTIOUS MONONUCLEOSIS

	Unsuccessful cultures		*Established suspension cultures*	
	Number of specimens		*Number of specimens*	
Average days post-onset clinical illness specimen obtained	39	38 (Range 112-4)	16	37 (Range 112-9)
Average peripheral white blood cell count per cmm.	39	5,600 (Range 10,400-3,000)	13	7,500 (Range 10,700-4,800)
Average percent lymphocytes per 100 white cells	41	54 (Range 75-24)	13	62 (Range 76-44)
Average percent lymphocytes atypical/100 lymphocytes	35	51 (Range 79-17)	13	58 (Range 92-13)
Percent peripheral lymphocytes in DNA synthesis	27	2.2 (Range 6.8-0.1)	12	3.0 (Range 7.1-0.1)
Percent peripheral lymphocytes in RNA synthesis	27	2.2 (Range 5.7-0.1)	12	3.3 (Range 7.1-0.2)
Range GPK adsorbed serum heterophil titer	39	1:3586-Neg.	15	1:7168-Neg.

These studies demonstrate that our culture techniques are selective for proliferative potentials above the normal range and that there is an increased potential of circulating leukocytes in patients with a variety of infectious diseases for long-term proliferation *in vitro*.

Detailed study of patients with infectious mononucleosis has shown that this increased proliferative potential cannot as yet be predicted from morphologic, biosynthetic, or serologic data (Table IV). No significant differences were noted for successful and unsuccessful cultures when analyzed for percentage of atypical lymphocytosis, lymphocytes incorporating labeled RNA and DNA precursors, and guinea pig kidney absorbed heterophil titers. With the selective culture methods employed, nevertheless, we were able to monitor the increased proliferative potential in several patients during acute illness and subsequent recovery from heterophile-positive infectious mononucleosis. These studies[20] have demonstrated that the increased proliferative potential that occurs with infectious mononucleosis may last

TABLE V. ASSOCIATION OF H.L.V. WITH ACUTE VIRAL HEPATITIS

Patient	*Anti-H.L.V. antibody titer at time of sample**	*Cell line established*	*H.L.V. antigen in cell line detected by immuno-fluorescence*	*H.L.V. particles in cell line detected by electron microscopy*
1	1/160	No	.....	.....
2	1/160	Yes	Absent	Present
3	1/160	No	.....	.....
4	1/160	No	.....	.....
5	1/40	Yes	Present	Present
6	1/160	No	.....	.....
7	1/640	Yes	Present	Present

*Significant titer 1/10.

several weeks or persist for several months. It is of considerable interest that this increased potential for long-term *in vitro* proliferation appears to be a transient phenomenon, ultimately disappearing in all patients studied, with a return of clinical and laboratory parameters toward the normal state.

The stimulus for the increased proliferative potential of circulating lymphocytes in infectious diseases is presently unknown. It would be of great interest to know whether these proliferative events are caused by the etiologic agent. Replication of virus within leukocytes has been described for a number of viruses and appears to be increased in stimulated cells.[21] These cell lines derived from patients with diseases of presumed viral etiology may therefore represent the ideal tissue for isolation and propagation of viral agents which have eluded more usual methods of recovery. Recent studies have demonstrated herpeslike viral particles (HLV, Epstein-Barr virus) and antigen in cell lines derived from patients with Burkitt's lymphoma and infectious mononucleosis.[14, 18, 22] The presence of antibody uniformly in sera from these patients[23, 24] has suggested that herpeslike virus is the etiologic agent of Burkitt's lymphoma and infectious mononucleosis.[18] Antibody to HLV, however, is widely distributed among people who have no history of such disorders.[25] Further, this agent occurs in cell lines derived from a variety of unrelated lymphoproliferative disorders and from healthy "normal" individuals.[13, 19] We have recently demonstrated significant

titers of anti-HLV antibody in patients with acute viral hepatitis and the presence of HLV particles and antigen in long-term lymphoid cell lines derived from these patients (Table V).[26] Circumstantial evidence would suggest the possibility that HLV is the causative agent of acute viral hepatitis as well as Burkitt's lymphoma and infectious mononucleosis. It is equally possible, however, that the viral genome of this ubiquitous agent is present in cells of a majority of individuals in the population and that proliferative events affecting these host cells result in concomitant replication of HLV. This mechanism would explain the appearance of HLV in most long-term cell lines derived from a variety of unrelated lymphoproliferative disorders as well as from normal individuals. The presence of HLV in cell lines derived from peripheral blood, therefore, need not imply an etiologic role for this agent. Current investigation of well-defined viral diseases associated with a lymphoproliferative phase should provide additional data to evaluate the role of HLV in these systems. These studies will provide greater understanding of the underlying mechanisms of cellular proliferation in the lymphoproliferative disorders and perhaps the alterations leading to the malignant state.

It has been difficult to define the precise relation of cells in long-term culture to the lymphoproliferative states which have produced them. No long-term culture described is characteristic for a specific disease entity and in fact all cultures behave more or less alike. Cell lines derived from biopsy specimens of Burkitt's tumor synthesize immunoglobulins, yet Burkitt tumor tissue contains few cells demonstrating this activity.[27] Whereas cell lines derived from peripheral blood of patients with infectious mononucleosis synthesize intact immunoglobulin molecules, no heterophile antibody, hemagglutinin, or hemolytic activity can be demonstrated for these cell lines.[28] Cells in long-term culture derived from these disorders consistently show the presence of herpeslike virus and chromosomal abnormalities,[29] neither of which are features of freshly isolated cells. Further, the lack of appropriate animal models has seriously hampered efforts to demonstrate that cell lines from patients with Burkitt's lymphoma and infectious mononucleosis contain infectious particles capable of transmitting these disorders reproducibly. The infectivity for marmosets of the cell lines derived from patients with acute viral hepatitis is currently under study with Fritz Deinhardt. A clearer understanding

of the relation of lymphoid cell lines to the lymphoproliferative states from which they are derived may well be found in our current studies of long-term cultures induced by well-defined viral agents.

It has been suspected that circulating cells in spontaneous *in vivo* proliferation are stem cells capable of establishing the long-term suspension cultures derived from peripheral blood. This would explain the relative ease of establishing lines from small volumes of blood in patients with lymphoproliferative states, where the numbers of these cells are increased and the necessity for large volumes of blood in normal individuals, where their concentrations are low. It has been reported, however, that only a small number of DNA-synthesizing cells are capable of division *in vitro* and that cell division ceases after 18 to 24 hours in the absence of stimulation by phytohemagglutinin (PHA).[11] An imbalance between flux of cells into the S phase of mitosis and flux into the M_2 phase was subsequently demonstrated for these cells.[30] Since these studies were performed in short-term culture, the role of such cells in the establishment of long-term lymphoid suspension cultures cannot be dismissed completely. Single-cell cultures ultimately may define the cell type responsible for establishment of peripheral blood in long-term culture.

Despite a number of important missing links, the implications of the present studies are quite exciting. An increased proliferative potential has been demonstrated for circulating cells from patients with a variety of essentially benign infectious diseases associated with an atypical lymphocytosis. These findings are similar for neoplastic cells in leukemia and lymphoma, which suggests there may be common cellular potential in malignant and benign lymphoproliferative states. Long-term lymphoid cell lines offer a tool for repeated and controlled investigation of these relations and of the mechanisms leading to malignant degeneration. Further, established lymphoid cell lines derived from the peripheral blood of patients with diseases of presumed viral etiology may represent the ideal tissue for isolation and propagation of viral agents which have eluded more usual methods of recovery. They will allow us to assess the role of peripheral cells in the pathogenesis of acute viral disease and the latent viral state. The use of these systems for the production and attenuation of infectious agents remains to be explored.

In addition to these benefits from the use of long-term lymphoid

cultures, several other potentially important applications become possible. The development of techniques to establish such lines predictably from small aliquots of blood from any individual would make available massive amounts of material for the study of human genetic variations. For example, the study of specific enzyme defects in individuals affected with various inborn errors of metabolism (or carriers) would become easy in an *in vitro* system. Such studies could extend to trials of "therapy" of certain of these errors, particularly in the case of storage diseases. In the long run, with the increased use of transplantation and the efficacy of antilymphocyte sera in the suppression of homograft rejection, the availability of lymphoid cells in large quantity from potential recipients could be useful for the production of patient-specific antilymphocyte serum. Such specific antisera would be theoretically preferable to those directed against antigens not present in the donor and against other tissue components. In the long run these types of application of cell cultures may provide the greatest usefulness, although it is to be hoped that the potential benefits of this system in defining the relation between viruses, proliferation, and disease will be realized in the near future.

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