A Morphologic and Immunologic Surface Marker Study of 299 Cases of Non-Hodgkin Lymphomas and Related Leukemias

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This study relates the cytologic types of the classification of malignant lymphoma of Lukes and Collins to the results of immunologic surface marker studies as part of a systematic multiparameter study of 299 cases of non-Hodgkin lymphomas. The results support the hypothesis that malignant lymphomas are neoplasms of the immune system and involve the B- and T-cell systems and, rarely, histiocytes. The morphologic features of the cytologic types of Lukes and Collins are predictive of the subtypes of lymphoma and considerably more effective than the immunologic surface marker techniques in identifying homogeneous groups. There are considerable methodologic and interpretive problems that are evaluated in detail. The verification of the B- and T-cell subtypes of the Lukes and Collins classification indicates that the time has come to change from the terminology and classification of lymphomas of the past to a modern immunologic approach. (Am J Pathol 90:461–486, 1978)

INTRINSIC TO OUR PROPOSED FUNCTIONAL APPROACH to the diagnosis and classification of malignant lymphomas is the belief that these disorders represent neoplasms of the immune system. As such, the lymphoma cells are considered to be defective immune cells that function in, migrate to, and arise in tissues similar in varying degrees to their normal counterparts.[1,2] This approach, initially presented in 1971, is based on two related proposals: a) malignant lymphomas develop as aberrations of lymphocyte transformation and represent either a "block" or an inappropriate "switch-on" of the transformation process and b) lymphomas involve the B- and T-cell systems and can be identified as of either B- or T-lymphocytic origin by immunologic surface marker techniques. From these considerations a follicular center cell (FCC) concept was developed on the following principles: a) the reactive follicular center is a site of normal B-cell transformation; b) the FCCs are plasma cell precursors; c)

FCC lymphomas occur in both follicular (nodular) and diffuse histologic patterns; and d) FCC lymphomas are best regarded as a group of cytologic types rather than a lymphoma of follicular structures, as implied by the term "follicular lymphoma."

To test these hypotheses, a functional classification was proposed, relating the various morphologic types of lymphoma to the phases of transformation and maturation of the lymphocyte.[3,4] This classification was designed with the purpose of redefining the malignant lymphomas in modern immunologic terms according to the B- and T-lymphocyte systems. From the beginning, our approach has been to first establish a morphologic diagnosis according to the proposed classification and then to relate critically the results of surface marker methods and cytochemical techniques to the morphologic diagnosis. The techniques presently employed include detailed morphology, cytochemistry, electron microscopy, cell culture and cytogenetics, immunoperoxidase identification of cytoplasmic immunoglobulin and muramidase, and immunologic surface marker analysis.

Following morphologic reviews of several large case populations, it appears that malignant lymphomas can be related to the morphologic subtypes proposed and also to the B- and T-cell systems. For example, the majority of those lymphomas previously termed "reticulum cell sarcoma" or "malignant lymphoma histiocytic" exhibited features of transformed lymphocytes of either B- or T-cell type. That malignant lymphomas, for the most part, mark as B or T cells and only rarely as histiocytes was demonstrated in a recent report of 384 cases of non-Hodgkin lymphomas and related leukemias, presented in collaboration with Dr. Robert Collins and his associates from Vanderbilt University and our group from the Los Angeles County–University of Southern California Medical Center.[5] Similar findings, albeit in smaller groups of cases, have been reported by a number of other workers, using cytologic terms from various classifications.[6-12]

The purpose of this presentation is therefore twofold: a) to report the results of our surface marker studies of 299 cases of lymphoma and leukemia, 198 of which were previously included in the report of Lukes and Collins [5] and in which the diagnosis was initially established on a purely morphologic basis according to the Lukes–Collins classification and b) to critically evaluate the immunologic methods employed with particular reference to reported variations in technical procedure and problems of interpreting the results in relation to morphologic findings.

Table 1—Techniques Most Commonly Used For Identification of T Cells, B Cells, and Histiocytes

	T cells	B cells	Histiocytes Monocytes	Comments
Rosette methods				Cytocentrifuge preparations are of value in evaluating lymphomas.
Spontaneous sheep red cell rosette				
E	+	–	–	Most useful T-cell marker
EAC (IgM) (Compl. rec.)	(+)	+	+	Probably some T cells. Convoluted T-cell lymphomas are observed with complement receptors.
EA (IgG) (Fc receptor)	–	+	+	Limited usefulness
Surface Ig*	–	+	(+)	By immunofluorescence Monocytes may mark because of Fc receptors (Text-figure 4)
Cytoplasmic Ig	–	+	–	Immunoperoxidase more useful than immunofluorescence in lymphomas because can be used on paraffin sections.
Antiserums				
HTLA	+	–	–	Antigen source: thymus, brain; specificity is a problem.
HBLA	–	+	–	Antigen source: CLL cells, B lymphoblastoid cell lines; specificity is a problem.
Cytochemistry				
α-Naphthyl butyrase (NSE)	(+)	–	+	Focal staining reported in T cells. Specificity for T cells is not proved.
Acid phosphatase	(+)	(+)	–	Reported in convoluted T cell and T-cell ALL
Tartrate-resistant acid phosphatase	–	–	–	Hairy cell leukemia
Muramidase (lyzozyme)	–	–	+	Immunoperoxidase method on paraffin sections or imprints

* T cells have a small amount of surface Ig not detected by immunofluorescent methods.
Ig = immunoglobulin; HTLA = human T-lymphocyte antibody; HBLA = human B-lymphocyte antibody; NSE = nonspecific esterase. Parentheses indicate that the finding lacks specificity, is controversial, or is only seen with certain types of lymphoma-leukemia. Other available methods are described in the section concerning evaluation of these methods.

Materials and Methods

The application of immunologic methods to the study of lymphomas has added a new dimension to our understanding of this complex field. We began our studies with the point of view that cytomorphologic differences between lymphomas indicated their origin from T or B lymphocytes or U cells and that this would be confirmed with the appropriate immunologic surface marker and cytochemical techniques as they became available and were applied. A variety of methods are available for distinguishing T and B lymphocytes and monocytes, and a number of these have found wide application. Others have been used by only a few investigators. None of the methods provides absolute criteria for distinguishing the cell types. For this reason many laboratories, including our own, use a panel of several methods for the assessment of cell populations for the presence of T and B cells. The most commonly used techniques, including cytochemistry, are listed in Table 1. Particular emphasis will be placed on spontaneous sheep RBC rosette formation (E rosettes) for T cells and surface immunoglobulin for B cells, since these are the most widely employed and reliable procedures. The pitfalls and factors influencing these various tests have been reviewed recently by Taylor [13] and others [9,11,14] and are discussed below. (See *Evaluation and Interpretation of Surface Marker Methods.*)

Pretherapy lymphoid material for our studies has been collected from lymph node biopsies, spleens, bone marrow, and peripheral blood. It is important to emphasize that the results reported here are from studies of tissues from untreated patients, in contrast to some other published studies. In addition, cells from spinal fluid, pleural fluids, or peritoneal fluids have been studied using the same techniques.

To make reliable morphologic diagnoses, the tissues must be optimally fixed, processed, and sectioned. Two-millimeter thick slices of tissue are fixed in Zenker's or B-5 fixative.[15] Paraffin-embedded tissues are sectioned at 4 to 6 μ and stained with hematoxylin and eosin (H & E) and methyl green pyronin (MGP).[16] Identification of cytoplasmic immunoglobulins (Ig) and muramidase is accomplished by immunoperoxidase staining.[17-20] Samples of the same tissues are also fixed for transmission electron microscopy.

Specimens were obtained from the patients at the Los Angeles County–University of Southern California Medical Center, the Children's Hospital of Los Angeles, and participating hospitals of the Southern California Lymphoma Group, which includes 20 major hospitals. The results reported here were obtained over 3 years (1974 through 1976) and include 299 patients.

Cell suspensions of lymphoid tissues are prepared in our institution by gently teasing the specimen through stainless steel wire mesh in RPMI 1640. Pathologists or technologists who have been instructed in our laboratory similarly prepare suspensions in the other hospitals and a messenger delivers the cell suspension to our laboratory. Peripheral blood specimens are separated on Ficoll–Hypaque.

Spontaneous sheep erythrocyte rosette (E rosette) formation for identifying T cells as well as sheep erythrocyte rosetting techniques for detecting complement (EAC) and Fc receptors (EA) are performed on cell suspensions using the methods of Jondal et al [21] with minor modifications. Only the results of studies involving E rosettes are reported here, and the percentages recorded are for SRBC-lymphocyte suspensions incubated 18 hours at 4 C. Slide preparations of E rosettes are also prepared by cytocentrifugation (Shandon Centrifuge, Shandon Southern Instruments, Inc., Sewickley, Pa). These are stained with Wright's stain or the stains listed below. The importance of identifying the rosette-forming cells is discussed later.

Surface immunoglobulins are detected with fluorescein–isothiocyanate labeled poly-valent ($\alpha,\gamma,\mu,\kappa,\lambda$) and monospecific antiglobulin serums. When a sufficient number of cells is present, the cells are examined for heavy chains α,γ , δ, μ and, occasionally, ϵ and light chains κ and λ. We have used commercial antiserums, which have been characterized for specificity by immunoprecipitation techniques, and antiserums prepared and characterized in our own laboratory. The percentage of cells showing typical surface fluorescence is

determined by counting 100 to 200 mononuclear cells with a Zeiss photomicroscope III equipped with a mercury lamp and Ploem illuminator. Phase optics are used to discriminate between mononuclear cells and granulocytes.

Cytocentrifuge preparations are prepared from the cell suspensions for cytochemical determinations, as are touch imprints of the cut surfaces of the lymphoid tissues. The cytochemical procedures routinely applied include Wright's stain, periodic acid–Schiff (PAS),[22] methyl green pyronin (MGP),[16] granulocyte stains (peroxidase,[23] Sudan black,[24] and chloroacetate esterase [25]), acid phosphatase with and without tartrate,[26] and non-specific esterase =NSE)[23] (α-naphthyl butyrate) for histiocytes (Table 1).

All these studies are performed on every case in which there is sufficient tissue or cells. We will continue to emphasize the importance of this multiparameter approach throughout this report.

Evaluation and Interpretation of Surface Marker Method

Variations in Procedures

Lymphocyte Separation Procedures. The first variable is the mode of preparation of cells for study. Since many surface marker methods are routinely performed on separated lymphocytes, it is important to use a separation procedure that will give the least bias in terms of selecting for either T or B lymphocytes. Practical recommendations for separation and surface marker analysis are given in a paper sponsored by WHO.[27] The most widely used method, the Ficoll–Hypaque density gradient technique, allows reasonable lymphocyte separation from peripheral blood, with little T- or B-cell bias. Enrichment of peripheral blood monocytes does occur but does not represent a practical problem in our experience. Nevertheless, it is always useful to prepare smears or cytocentrifuge slides of separated cells for differential counts and specialized cytochemistry to determine accurately the cell population on which subsequent surface marker studies are to be performed.

The E-Rosette Phenomenon: Spontaneous Nonimmune Rosettes With Sheep Red Cells. The E-rosette method (following Jondal et al [28] and Bach [29]) has become an established parameter for the identification of normal human T lymphocytes. Extended to neoplastic lymphocytes, a positive E-rosette test has been taken as evidence for the T-cell origin of certain lymphomas and leukemias. However, the great variation in reported scores of normal peripheral blood T cells (range, 10 to 80%) suggests that cautious interpretation is required.

It has become increasingly obvious that diverse factors may influence the values obtained in an E-rosette test, not the least of which is the experience of the investigator or technician performing the test. Variations in the practical details of the performance of the test are of great importance, eg, the proportion of lymphoid cells to sheep red cells, the ultimate total concentration of cells, the force of centrifugation or pelleting applied to bring the cells together, the temperature and time of incubation allowed for the rosettes to form, the technique of resuspension, and the method of preparation for visual counting. E rosettes are notoriously unstable and fragile: rough handling may reduce the score significantly [30] and gentle resuspension may raise it.[31]

A high proportion of sheep red cells to viable lymphoid cells results in an apparent elevation of the E-rosette score or, alternatively, in a preparation in which red cells are so numerous that identification of negative cells is difficult. The ratio of sheep red cells to lymphoid cells varies widely in different reports, and likewise there is no uniformity of procedure. Our results suggest that the E-rosette score reaches a plateau when 2 or more hours are allowed for rosette formation at 4 C. Shorter incubation periods may detect a subset of strongly reacting cells.[32] At 37 C, normal peripheral blood lymphocytes show little evidence of rosette formation, but this may not be true of neoplastic T cells.[33]

Rosette formation may also be influenced by various experimental manipulations. Some

degree of enhancement of rosette formation by performing the test in group AB serum, in the presence of Ficoll, or after incubation overnight in fetal calf serum has been reported. For example, in our series Ficoll produces an average elevation of the E-rosette score by 3 to 5 percentage points. Pretreatment of lymphocytes with neuraminidase [34] produces a consistently higher score, apparently by recruitment of lymphocytes normally bearing surface Ig and having the capacity for binding aggregated Ig (Fc receptor). The augmentation of E-rosette scores following these procedures may be related to the removal of inhibitory serum factors such as those reported by Browne et al [35] in a variety of neoplasms and by Bobrove et al [36] and Fuks et al [37] in Hodgkin disease.

Although the usual spontaneous E-rosette test in humans employs sheep red cells, several investigators have shown that human T cells will form rosettes with both autologous and allogeneic human red cells.[38-41] However, such rosettes are unstable and require temperatures of less 4 C for optimum formation. Dog and goat red cells also rosette with a proportion of human lymphocytes but score consistently lower than sheep red cells.[42] Murine red cells are remarkable in that they consistently form rosettes with human B lymphocytes rather than T lymphocytes.[43-46]

Finally, in animal studies the percentage of T cells has been shown to vary greatly with physiologic function, age, and organ site. Similar variation has been reported in human lymph node, spleen, and peripheral blood in healthy and diseased individuals (reduced E rosettes in a variety of nonlymphoid malignancies).[35,47,48] Chemotherapy also may influence the E-rosette score, tending to produce an overall lowering of values.[49] Drugs other than the usual cytotoxic agents must also be considered. For example, marijuana, contrary to its much publicized effects on the whole organism, appears to "turn off" a proportion of rosetting cells.[50] Even manual work, as defined by bicycle ergometer testing, produces a diminution in E-rosette scores.[51] Hiai [52] reported that in mouse lymphomas rosette formation could occur by two other mechanisms: a) expression of IgM heterophile antibody by lymphoma cells and b) expression of viral hemadsorption activity by lymphoma cells infected with Rauscher leukemia virus. Presumably analogous situations might occur in human lymphoma.

Thus, in the face of so many variables, it is important that each laboratory establish its own optimal conditions for the performance of this test, with reference to standardized populations of lymphocytes, eg, pooled frozen lymphocytes, which serve as regular controls to be included when abnormal populations are examined.

Surface Immunoglobulin as a Marker for B Lymphocytes. At first, the methodology employed in the detection of cell surface immunoglobulin (SIg) might appear more uniform than that described for the E-rosette method. However, the reliability of immunofluorescence methods for the determination of SIg is vitally dependent on the sensitivity and specificity of the antiserums available. The use of common commercial antiserums does allow some direct comparison between the work of different centers, although the specificity of commercial reagents often leaves much to be desired.[53] This means that each batch of antiserum obtained commercially must be subjected to rigorous assessment of sensitivity and specificity, with reference to positive and negative controls, and also preferably by blocking studies and by immunodiffusion against purified light and heavy chain components from sources other than those used in the production of the antiserum in question. Finally, the dilution of antiserum for use in the test system should be determined by titration to the "plateau end point," beyond which the percentage of SIg-positive cells falls progressively with dilution.[53,54]

Cell identification in immunofluorescent preparations may also present a real problem.[53,54] Mature segmented granulocytes are often distinguishable by phase contrast and by a patchy pattern of SIg staining, but the differentiation of monocytes may be much more difficult. Immunofluorescence methods are generally not compatible with orthodox staining methods or cytochemical techniques, and it is here that immunoperoxidase [54] or immunomicrosphere methods [55,56] have a distinct advantage, in that, performed in parallel

with fluorescence studies, they permit identification of SIg-positive cell types according to cytologic criteria.

EA and EAC Rosettes. The problems of performance and interpretation of EA (Fc receptor) and EAC (C₃ receptor) rosette procedures are also manifold. If sheep red cells are used as the indicator cell, then the formation of spontaneous E rosettes may lead to erroneous results (some abnormal T cells form E rosettes at 37 C,[33] and these might be confused with sheep red cell EAC rosettes forming at 37 C, particularly if pelleting is employed). The use of red cells of other species, eg, pigeons,[57] circumvents this problem, but the use of a wide range of sensitizing antiserums [58] and different complement sources (human and mouse complement differ in their content of C3b and C3d components) introduces a wide range of variability into the results.[59] Also, Fc receptor activity may be measured by use of labeled aggregated immunoglobulin, and again the values obtained vary from those of the EA-rosette method. Contrary to previous beliefs, Fc receptor does not appear to be confined to B lymphocytes (null or L cells;[60,61] T cells [62]).

Finally, it might seem that methods utilizing specific antiserums to lymphocyte surface antigens should offer the most specific approach to lymphocyte identification. However, numerous investigators have reported the use of anti-T cell and anti-B cell serums raised against varying antigen sources (fetal thymocytes, peripheral T cells, human brain, T-cell-lines–anti-T-cell; CLL cells, splenic lymphocyte membranes, B-cell lines, pregnancy anti-B cell serums, B cells [54]) in different animal species, and results again differ quantitatively and qualitatively both from results obtained using other antiserums and from results using separate techniques (E rosettes, SIg). Thus, much remains to be explained. We should continue to be cautious in interpretation, particularly in view of the facts that many of these methods were developed using normal lymphocyte populations and neoplastic
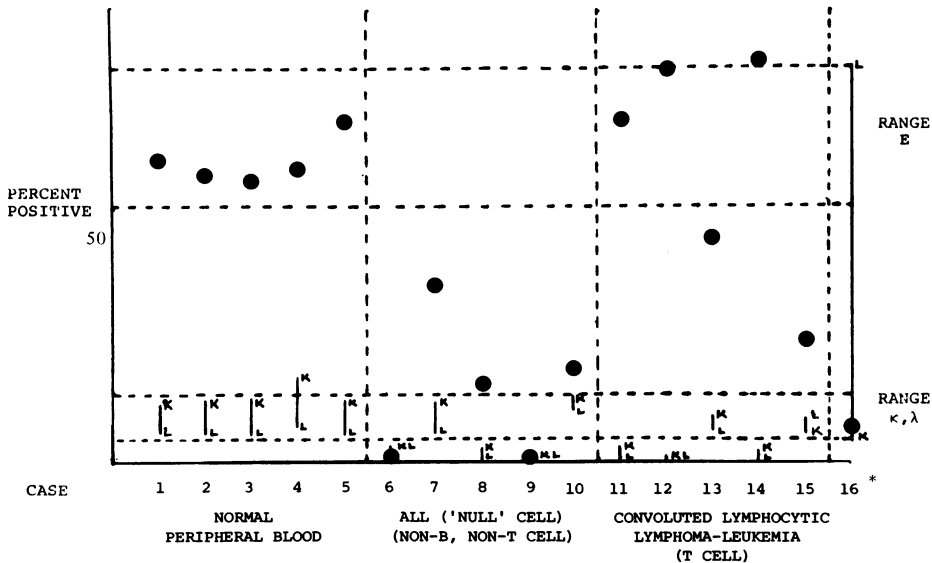
TEXT-FIGURE 1—Scattergram of E rosette scores (*solid circles*) and surface immunoglobulin scores (anti-κ , anti-λ) for peripheral blood from normal individuals and from patients with ALL, convoluted lymphocytic lymphoma–leukemia, and small noncleaved follicular center cell lymphoma–leukemia (Burkitt-like). Percentage scores for κ and λ are joined by a line, the length of which is a reflection of the observed differences between the percentage of κ -positive and λ-positive cells. Thus, a long κ –λ line indicates a marked disturbance in the proportion of κ - and λ -bearing cells and suggests a monoclonal (monotypic, presumably neoplastic) B-cell population. *Asterisk* (Case 16) involves small noncleaved (Burkitt-like) lymphoma–leukemia.

dividing lymphocytes may diverge significantly from their normal counterparts. Overall, it is perhaps remarkable that the correspondence is so close.

Interpretation of Results

The interpretation of the E-rosette procedure and SIg method may best be reviewed by reference to selected case results from our series.

E-Rosette Test in Lymphoma–Leukemia. In addition to the use of a "controlled" E-rosette method, it is necessary to determine the morphology of those cells forming E rosettes, not simply the percentage score. For example, convoluted cell lymphoma-leukemia characteristically shows E-rosette formation by the circulating leukemic cells (eg, Cases 11 through 15 in Text-figure 1). However, on a simple percentage basis, Cases 11 and 12 are not distinguishable from the normal population (Cases 1 through 5 in Text-figure 1) by E-rosette score alone, and in Cases 13 and 15 sufficient residual lymphocytes are present to give an SIg score in the normal range. Clearly in each case not all the neoplastic cells form rosettes, and in all cases a proportion of the total rosetting cells may be residual normal T lymphocytes. In these instances the diagnosis is made by the observation of definite rosette formation by a proportion of the neoplastic cells recognizable in cytocentrifuge E-rosette preparations.

Similarly, Cases 13 and 15 are only distinguishable from cases of null cell ALL (Cases 6 through 10 in Text-figure 1) by attention to the cytologic detail of the rosetting cells, because in many instances sufficient residual normal lymphocytes may be present, coexisting with cells of a nonmarking ALL (Case 7 in Text-figure 1) to produce intermediate E and SIg values. It is important to note that depression of the SIg score may be the feature alerting one to the presence of a leukemic cell population, be it T cell or null cell. This principle, requiring the cytologic identification of rosette formation in relation to morphologically recognizable neoplastic cells is also shown in Text-figure 2, in which all but two
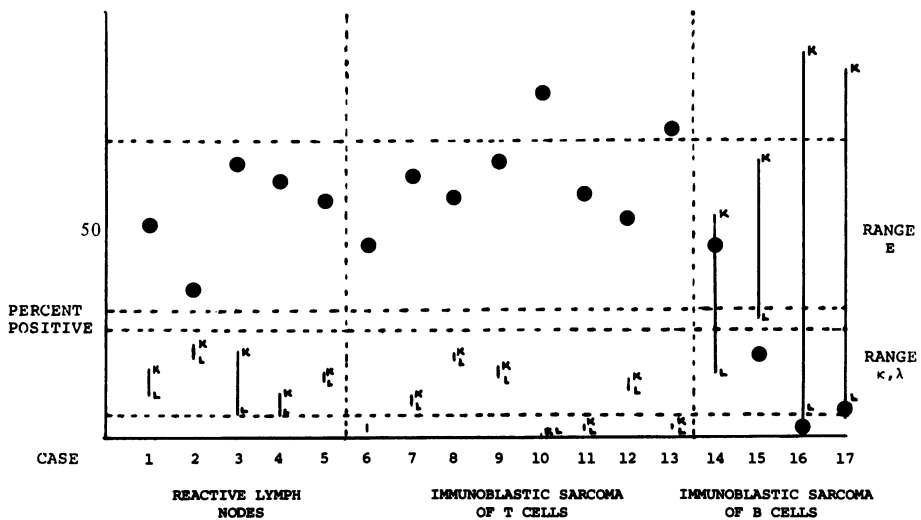
TEXT-FIGURE 2—Scattergram of E rosette scores (*solid circles*) and surface immunoglobulin scores (anti-κ, anti-λ) for cell suspensions from reactive lymph nodes and lymph nodes with immunoblastic sarcoma of T- and B-cell types. Percentage scores for κ and λ are joined by a line, the length of which is a reflection of the observed differences between the percentage of κ-positive and λ-positive cells. Thus, a long κ–λ line indicates a marked disturbance in the proportion of κ- and λ-bearing cells and suggests a monoclonal (monotypic, presumably neoplastic) B-cell population. (Compare Text-figure 2 with Text-figure 3.)

examples of T-cell immunoblastic sarcoma (IBS) fall within the normal range, indicative of the presence of an admixture of normal T and B lymphocytes within the tumor cell population.

SIg Measurement in Lymphoma and Leukemia. The problems inherent in interpretation of SIg scores can be illustrated by reference to cases of CLL. Twelve cases are represented in Text-figure 3. In making an interpretation it is important to recognize that cells may bear SIg by a variety of mechanisms, as illustrated in Text-figure 4. Only the demonstration of synthesized SIg is indicative of a B-cell origin for the cell under study. Ultimate proof of synthesis is dependent on the observation of SIg resynthesis following its removal by enzymatic stripping (eg, trypsin [63]), by incubation at 37 C,[60,61,64] or by combination with anti-Ig antibody and shedding.[65] Generally, however, the demonstration of monoclonality of a cell population (exclusively one light chain and one heavy chain) is regarded as sufficient proof of SIg synthesis by a neoplastic cell population. Thus, CLL Cases 6 through 11 in Text-figure 3 would be regarded as showing a typical monoclonal distribution for light chain. Usually monoclonal populations are also restricted to a single heavy chain class. However, observations of a single chain with more than one class of heavy chain (usually μ or δ) are regarded as still compatible with a monoclonal cell population, following reports that such cells ($\mu\delta$) are present in the normal lymphocyte population in the mouse [66] and that normal lymphocytes undergo sequential changes in expression of heavy chain class during maturation.[67-69] Evidence for the synthesis and expression of both light chains simultaneously is limited to certain experimental studies [70,71] and to anecdotal, although well-documented, case reports.[72-76]

The means by which false-positive SIg populations may arise through adsorption of serum immunoglobulin by any of several mechanisms is illustrated in Text-figure 4. In addition, adsorption of polyclonal serum immunoglobulin by a neoplastic B-cell population, via any of these mechanisms, may conceal or mask an underlying monoclonal population (as has possibly occurred in Cases 12 and 13 in Text-figure 3). The possibility

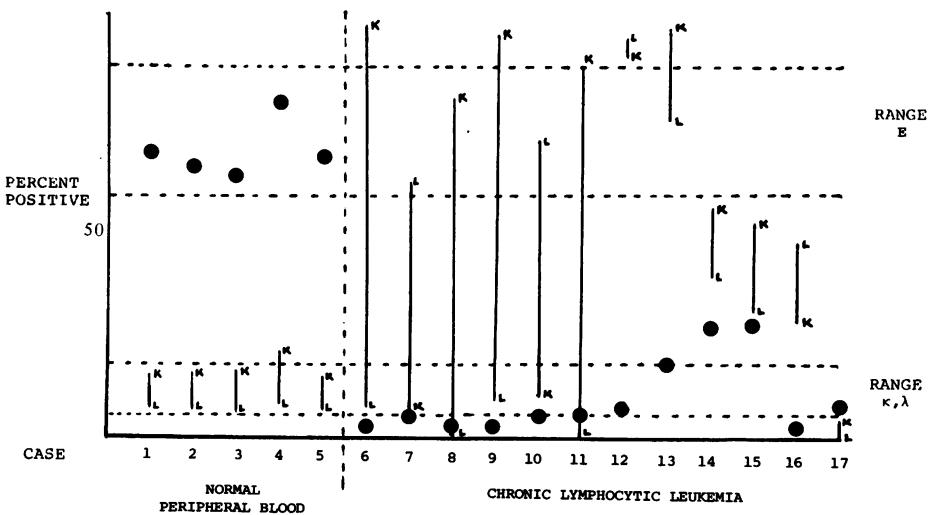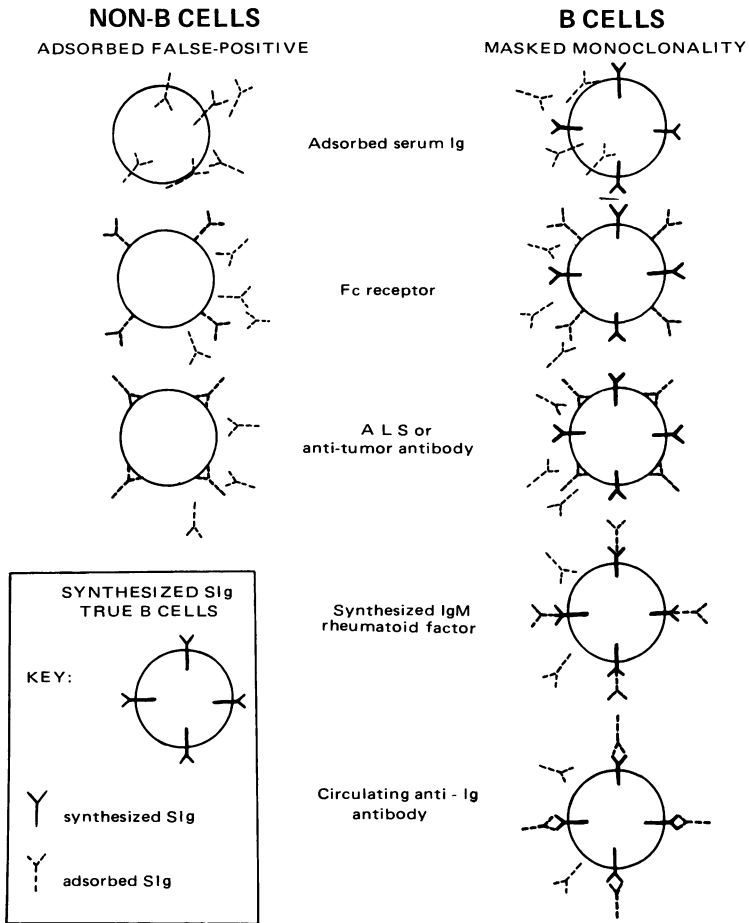
TEXT-FIGURE 3—Scattergram of E rosette scores (*solid circles*) and surface immunoglobulin scores (anti-κ, anti-λ) for peripheral blood from normal individuals and from patients with CLL. Percentage scores for κ and λ are joined by a line, the length of which is a reflection of the observed differences between the percentage of κ-positive and λ-positive cells. Thus, a long κ–λ line indicates a marked disturbance in the proportion of κ- and λ-bearing cells and suggests a monoclonal (monotypic, presumably neoplastic) B-cell population. (Compare Text-figure 3 with Text-figure 2.)

NON-B CELLS

ADSORBED FALSE-POSITIVE

B CELLS

MASKED MONOCLONALITY

Adsorbed serum Ig

Fc receptor

A L S or
anti-tumor antibody

SYNTHESIZED SIg
TRUE B CELLS

KEY:

Υ synthesized SIg

$\dot{\Upsilon}$ adsorbed SIg

Synthesized IgM
rheumatoid factor

Circulating anti - Ig
antibody

TEXT-FIGURE 4—Synthesized cell surface immunoglobulin (ie, one light and heavy chain class per cell) is represented by the solid-line Y. Immunoglobulin adsorbed to the cell surface by any of the mechanisms described (multiple light and heavy chain classes per cell) is represented by the broken-line Y.

Adsorption of serum immunoglobulin may occur nonspecifically and may be detected if washing is inadequate. This may occur particularly in hypergammaglobulinemia and in hyperviscosity states.

Adsorption of IgG via Fc receptor activity may result in false postivity of non-SIg synthesizing lymphocytes or false postivity of other cell types such as granulocytes or monocytes. In addition, Fc adsorption of IgG ($\kappa\gamma$, $\lambda\gamma$) by SIg synthetic positive lymphocytes may mask an underlying monoclonal population, eg, $\lambda\alpha$ cells, would then mark as $\kappa\lambda\gamma\alpha$. Fc receptors for IgM and IgA may also exist and, if present, might produce similar complications.

Similarly, adsorption of antitumor or antilymphocyte antibodies to a cell population might result either in false positivity or in the masking of a monoclonal population by adsorption of $\kappa\lambda\gamma$.

Rarely, a neoplastic clone synthesizes SIg or IgM type having rheumatoid factor type activity (anti-IgG). Under these circumstances the IgM rheumatoid factor may bind with, and thus adsorb, circulating IgG ($\kappa\gamma$, $\lambda\gamma$) *in vivo*, masking the underlying monoclonicity of the cell population which would then mark as $\kappa\lambda\gamma\mu$. Alternatively, there might be adsorption of IgG aggregates in the labeled antiserums used to study the cell population.

Finally, the presence of any circulating antibody against immunoglobulin (including a circulating rheumatoid factor) might result in binding to the surface immunoglobulin of SIg bearing lymphocytes, again masking the true pattern of the synthesized SIg.

Possibly the most certain way of demonstrating synthetic SIg is by trypsin stripping and resynthesis.

remains that rare cases may produce a cross-reacting light chain or both light chains simultaneously, but proof of this requires stripping and resynthesis and exclusion of adsorption by the mechanisms depicted in Text-figure 4.

In Cases 14 and 15 (Text-figure 3) a persisting E-rosette score of more then 20% shows that involvement of the peripheral blood by leukemic cells is only partial. In these circumstances absolute T- and B-cell numbers should be calculated because percentage figures are meaningless if the total lymphocyte count is significantly abnormal. In fact, for scientific accuracy this principle should be applied to all determinations of B- and T-cell number. By such means it appears that some cases of B-cell CLL are actually associated with an increased number, although decreased percentages, of circulating T cells, and such patients may have a more favorable prognosis.[77,78]

Finally, in some putative B-cell neoplasms, such as CLL Case 17 (Text-figure 3), both the E-rosette score and the SIg score may be greatly reduced. Such cases may represent B cells in a nonsecretory phase of the cell cycle,[59,79-81] B cells with levels of SIg below the threshold for detection by the technique employed, T cells, or any other cell type.[13,66] In Case 17 it was possible to show that 90% of the neoplastic small lymphocytes carried μ chain, although a corresponding light chain was not detectable.

In cases of partial involvement of blood or tissues (CLL Cases 14 and 15 in Text-figure 3 and IBS Cases 9 and 10 in Text-figure 2) it may be important to examine the morphologic features of the SIg-bearing cells to assess whether they represent neoplastic lymphocytes or residual normal lymphocytes. This can be achieved by immunoperoxidase methods or by immunomicrosphere methods.[54-56] Again the principles described for the assessment of peripheral blood cells apply to tissue-derived lymphocyte populations. In fact, the determination of the nature of the SIg-positive (or E-rosette–positive) cells is of particular importance in the study of lymphoma cell populations because of the tendency for such populations to contain a high proportion of residual normal lymphocytes in which the characteristics of the neoplastic cells may be lost (B-cell IBS Case 5 in Text-figure 2 and T-cell IBS Cases 2, 3, 4, and 7, in which the simple raw scores do not differ from those of reactive lymph nodes).

Results

The findings in this study demonstrate that, for the most part, cases in each of the cytologic types mark in a predictable manner according to the B- and T-cell subtypes. The results thus provide support for the proposal that the cytologic types of the Lukes–Collins classification principally involve B and T subtypes and rarely the histiocytic type. Admittedly, there are problems of technique and interpretation, and the cells in a small proportion of the cases in each of the cytologic types fail to mark.

The distribution of the 299 cases of non-Hodgkin lymphoma and related leukemias according to major cytologic groups are shown in Table 2.

Table 2—Distribution of Cases of Malignant Lymphomas by Major Cytologic Group

	No. of cases	%
B cell	190	64
T cell	63	21
U cell (undefined)	45	15
Histiocytes	1	0.3
Total	299	100

Table 3—Distribution of Cases of Malignant Lymphomas by Cytologic Types of Lukes and Collins

	No. of cases	%
B cell		
Small lymphocyte (B)	23	7.7
Plasmacytoid lymphocyte	23	7.7
Follicular center cell (FCC)	(123)	(41.2)
Small cleaved	70	23.4
Large cleaved	19	6.4
Small noncleaved	23	7.7
Large noncleaved	11	3.7
Immunoblastic sarcoma (B)	9	3.0
Hairy cell leukemia	12	4.0
T cell		
Small lymphocyte (T)	5	1.7
Convoluted lymphocyte	33	11.0
Cerebriform lymphocyte (Sézary-Mycosis fungoides)	6	2.0
Immunoblastic sarcoma (T)	15	5.0
Lymphoepithelioid cell	4	1.3
Histiocytes	1	0.3
U Cell	45	15.1
Total	299	100.0

The B-cell group is the largest, with 190 cases (64%); the histiocytic group is the least common, with only 1 case (0.3%). There are 63 cases (21%) in the T-cell group and 45 cases (15%) in the U-cell group, which is composed entirely of patients with acute lymphocytic leukemia primarily of the childhood age group.

In Table 3 the cases of malignant lymphoma are listed according to the cytologic types of the classification of Lukes and Collins.[2-4] Of the 190

Table 4—Immunoglobulin Surface Marker Studies: B-Cell Lymphomas

Lymphoma, cytologic type	No. of cases	Cases with sufficient cells	Pattern of SIg staining			
			Mono-clonal	Bitypic*	Poly-clonal*	Low
Small lymphocyte	23	23	52	9	26	13
Plasmacytoid lymphocyte	23	18	28	5	28	39
Small cleaved FCC	70	60	47	20	18	15
Large cleaved FCC	19	8	37	0	25	37
Small noncleaved FCC	23	20	65	5	20	10
Large noncleaved FCC	11	8	25	0	63	12
Immunoblastic sarcoma	9	5	60	0	20	20
Hairy cell leukemia	12	11	18	36	28	18
Total	190	153 (81%)				

FCC = follicular center cell.
* Anomalous patterns of staining: bitypic, presence of both light chains and one heavy chain; polyclonal, both light chains and more than one heavy chain.

cases in the B-cell group, 123 cases involved the features of follicular center cell (FCC) types. The small cleaved FCC lymphoma comprising 70 cases is the most common of the FCC types and of the whole B-cell group. Of the 63 cases in the T-cell group, convoluted lymphocytic lymphoma and IBS of T cells are the most common, comprising 33 and 15 cases, respectively.

B-Cell Types

The results of the SIg studies are recorded in Table 4 according to the cytologic types of B cells of Lukes and Collins.[2-4] Of the 190 cases in the B-cell group, 153 (81%) had sufficient cells for study. There was a high rate of cell loss in collection, and, as a result, there commonly was an insufficient number of cells for study in the IBS-case group and the large cleaved FCC type. The former has a high turnover rate proliferation and the latter commonly exhibits a prominent degree of sclerosis, both of which seem to be associated with difficulty of cell separation, increased cell fragility, and limitation in cell viability. By contrast, there is minimal case loss in the small B lymphocyte and hairy cell leukemia groups, even when cells are obtained by separation from tissue rather than peripheral blood or bone marrow.

Examination of the clonal character of the SIg does not reveal a strikingly high frequency of cases with monoclonal SIg in any of the cytologic types. A proportion of the cases in each cytologic type exhibit monoclonality, and it ranges from a low in hairy cell leukemia of 18% to a high of 65% of the cases in the small noncleaved FCC type. A number of the patients with several cytologic types have a bitypic clonal anomaly, with a large proportion of cells having a single heavy Ig chain, with an equally high frequency of cells exhibiting both κ and λ light chains. These cases essentially all mark as IgM, $\kappa\lambda$. It seems possible that this anomaly is a result of a nonspecific antibody crossover and the cells actually have monoclonal SIg. A second anomaly of polyclonal type involving multiple heavy and both light Ig chains was also encountered and seems to have obscured additional monoclonal cases, possibly by nonspecific adsorption of Ig and/or the presence of Fc receptors binding Ig. This problem was considered in some detail in the previous section. This anomaly of polyclonal type was observed in every cytologic type and ranged from a low of 18% in the small cleaved FCC to a high of 63% in the large noncleaved FCC types. A small number of cases having a low percentage of cells exhibiting SIg, but without evidence of monoclonicity, also were observed in every cytologic type and were most common in the plasmacytoid, lymphocytic type (39%) and the large cleaved FCC type (37%). The

Table 5—Immunologic Surface Marker Studies in the Small Cleaved FCC Type

No. of cases	70
Cases with sufficient cells	60
Surface immunoglobulin	
Monoclonal	28 (47%)
Anomalous patterns (Table 4)	23 (38%)
Low frequency of E rosettes*	9 (15%)

* No evidence of rosette formation by neoplastic cells

limited frequency of cells with SIg in each B-cytologic type would seem to indicate that B-cell lymphomas at times may either fail to produce Ig on their surface or produce it in minimal amounts that are difficult to detect.

In Table 5 the detailed findings in the study of the most common cytologic type of the B-cell lymphomas, the small cleaved FCC with 70 cases, are shown as an illustration of the detailed findings. Of the 70 cases there were sufficient cells in 60 cases (86%) for complete evaluation. Of these, 28 cases (47%) exhibited monoclonal SIg. The anomalous finding of both light Ig chains was observed in 12 cases (20%) and the polyclonal type with both multiple heavy and light chains in 11 cases (18%). A low percentage of lymphoma cells bearing detectable SIg was recorded in 9 cases (15%). Unquestionably, many, if not all, of the cases with anomalous clonality may in reality have monoclonal SIg that is obscured by technical features related possibly either to nonspecificity of the antiserums or Ig adsorption on the cell surface. Nevertheless, a small proportion (9 cases [15%]), although morphologically acceptable, have little Ig on their surfaces.

In all the cytologic types of B-cell lymphomas, a variable proportion of cells forming E rosettes for T cells are usually found in small numbers, but in cytocentrifuge preparations they are distinguishable morphologically from the lymphoma cells. They range in frequency in the small cleaved FCC from 0 to 49%.

Table 6—Convoluted T-Cell Lymphoma-Leukemia

No. of cases	33
Cases with sufficient cells for complete study	24
E-rosette study performed* (range 0-97%;	
median 24%: >50%, 8; <20%, 14)	29
S-Ig studies†	24
Polyconal SIg (range 0-95% positive cells:	24
>30%, 3; 30%, 10; <10%, 18)	
Monoclonal SIg	0

* Neoplastic cells formed E rosettes although the percentage was frequently low.
† Residual normal B cells and possible adsorption of serum Ig by mechanisms illustrated in Text-figure 4. No evidence of synthesized Ig.

T-Cell Lymphomas

The diagnosis and classification of the lymphomas of T-cell type were established on the basis of the morphologic features. In the cases of the T-cell subtypes, lymphoma cells were demonstrated to form E rosettes in cytocentrifuge preparations, although the frequency of E rosettes varied widely and was recorded at times as less than 20%. The determining factor in our view is not the percentage of E rosettes but the demonstration of lymphoma–leukemia cells forming E rosettes in the cytocentrifuge preparations. SIg was observed on a limited proportion of the cells, but in a few cases the percentage of cells bearing SIg with all heavy and both light chains and with an unusual fluorescent character was high. These findings were interpreted as indicative of an autoantibody reaction (Text-figure 4).

The results of the immunologic surface marker studies on the 33 cases interpreted as convoluted lymphocytic lymphoma–leukemia are listed in Table 6. There were sufficient numbers of cells for E-rosette studies in 29 cases (88%) and complete evaluation in 24 cases (74%). There was a wide range in the frequency of E-rosette formation (from 0 to 97%), but the frequency only exceeded 50% in 8 cases and the median was 24%. There were 14 cases in which the overall frequency was less than 20%. The formation of EAC rosettes was 30% or greater in 5 cases, ranging from 30 to 62%, with a median of 51%. In 2 of these cases, the presence of a C_3 receptor was demonstrated using a fluorescein-conjugated anti-C_3 serum. Acid phosphatase stains were performed on tissue imprints or smears of peripheral blood or bone marrow in every case. Lymphoma–leukemia cells with a prominently positive globular structure, as described by Stein et al,[82] however, were found only occasionally and in a small proportion of cases. This discrepancy in results, judging from our experience since completing this study, appears to be due to differences in the timing of fixation. With delay of at least 24 hours in fixation of air-dried specimens rather than immediate fixation as previously practiced, a strongly positive acid phosphatase globular structure has become apparent in convoluted lymphoma–leukemia cases.

A new T-cell lymphoma appears to be emerging from the heterogeneous group of disorders included in the so-called Lennert lesion.[83] The term "lymphoepithelial cell lymphoma" has been proposed by Lennert et al[84] for this lymphoma. The morphologic features suggest that it is a distinctive cell type.[85] The 4 patients included in their study all exhibited a high percentage of E rosettes and demonstrated E-rosette formation about the lymphoma cells in cytocentrifuge preparations.

The results of immunologic surface marker studies in 15 cases of immu-

Table 7—Immunoblastic Sarcoma of T Cells

No. of cases	15
No. with sufficient cells	12
E rosettes*	
Range	30–90%
Median	58%
Surface Ig (low, polyclonal)	
Range†	5–31%
Median	13%

* Cytocentrifuge preparations show lymphoma cells forming rosettes.
† Range given is for polyvalent antiserum. Staining patterns were also determined with specific antiheavy and antilight chain serums.

noblastic sarcoma of T cells are recorded in Table 7 as an illustration of the findings in one of the lymphomas of transformed lymphocytes. There was a sufficient number of cells for study in 12 cases (80%). The range of E rosettes varied from 30 to 90%, with a median of 58%. E rosettes were demonstrated about the lymphoma cells in each case. SIg of polyclonal type was recorded in 5 to 31% of the cells, with a median of 13%. Immunoperoxidase detection of cytoplasmic Ig and muramidase on paraffin sections failed to demonstrate either substances in the lymphoma cells.

Histiocytic Lymphomas

The single case of the series interpreted morphologically as the histiocytic type was readily distinguished from the lymphomas of transformed lymphocytes. It exhibited a component of cells resembling benign histiocytes with abundant cytoplasm and a second component of mesenchymal cells presenting spindle cell features. The lymphoma cells in tissue imprints were positive in the α-naphthylbutyrate stain for histiocytes and monocytes. Subsequently, a pretherapy bone marrow biopsy specimen revealed a diffuse leukemic type involvement by a primitive cellular proliferation that was difficult to classify precisely.

U-Cell Lymphomas

The U-cell type was established for those lymphoma–leukemia processes with primitive cytologic features that are not readily classified and in which the available techniques fail to reveal specific characteristics. In this study there were 45 cases in the U-cell group, primarily ALL of childhood. In this group the tumor cells essentially failed to mark except in 14 cases, in which a low percentage of E rosettes was recorded (<20% and commonly between 5 and 10%). In each instance a few tumor cells were shown to form E rosettes in cytocentrifuge preparations, but they lacked the cytologic features of the convoluted T cells. Whether these cases represent a type of T-cell lymphoma–leukemia with low E-rosette formation or a null

cell must await the results of a combined study on such cases using specific antiserum for T cells in parallel with E rosettes.

Discussion

The results of this study provide substantial support for the hypothesis that malignant lymphomas are neoplasms of the immune system. The Lukes–Collins classification of lymphomas was developed in an attempt to express this concept in morphologic terms, particularly in relation to the newly discovered process of lymphocyte transformation and the realization of the existence of B and T lymphocyte subtypes.[2-4]

By judicious application of this classification, it has proved possible to identify homogeneous cytologic types of lymphoma with consistency. Furthermore, the results of our surface marker studies reveal that the immunologic parameters frequently are in accordance with the initial morphologic impression. Nevertheless, discrepancies do occur between morphologic opinion and the results of surface marker studies, and in many instances these inconsistencies are inexplicable in terms of technique or known biologic variation. For this reason, morphology must remain the basis for identification of the various lymphoma types. Certainly initial morphologic observations, correlated with detailed immunologic studies, have permitted the identification of distinctive clinical–morphologic–immunologic entities such as the convoluted T-cell lymphoma–leukemia[86] and immunoblastic sarcoma of T- and B-cell types.[87]

However, variation that occurs among immunologic findings within single morphologic types does not necessarily imply that immunologic marker methods are without intrinsic value. We may yet learn much from them, although admittedly there are problems of interpretation of some of the immunologic findings. As described in the previous section, many factors, biologic and technical, influence the results of surface marker studies of lymphomas. Considering the many variables involved, it is perhaps surprising that the patterns of SIg staining and rosette formation of various neoplastic lymphocyte populations have been as consistent as has been reported by several investigators and that there is such close resemblance overall to the marker patterns of normal lymphocytes.

Obviously, the reading and interpretation of these tests is crucial. Our approach in assessing immunologic marker methods has been to record and attempt to analyze all observations, whether of obvious relevance or not, to avoid interpretative bias. For example, the patterns of SIg staining are many: variations are produced by adsorbed serum Ig, synthesized Ig, combinations of both, nonspecific adsorption of antiserums, and mixtures of normal and neoplastic cells. Clearly, final interpretation depends on the consideration of all of these factors. Even with the use of appropriate controls, interpretation of results in studying lymphoma–leukemia cells is much more difficult than with normal peripheral blood lymphocytes.

Our results demonstrate that within morphologically homogeneous lymphoma cell types the marker results are not homogeneous. This lack of homogeneity may be due, in part, to the factors discussed above and to our desire not to discard data, although some of it may be artifactual. However, we believe that some of the heterogeneity of surface marker findings within lymphoma types indicates that these groups are functionally heterogeneous, in spite of their homogeneous morphologic appearances. For example, variation in marker patterns may result from sampling of lymphoma cells at different stages in the tumor's development or cell cycle or from fundamental biologic differences which may affect clinical behavior and response to therapy but may not be identifiable by morphologic criteria. The clues we are accumulating from our multiparameter approach continue to suggest that there are ways of identifying clinically meaningful subtypes of lymphoma within the morphologic groups already identified. The definition of biologic subtypes, and the correlation of such subtypes with clinical behavior, continues to be our long-range goal.

The questions remain concerning the general applicability of the tests we and others are using to characterize lymphoma cells and the potential usefulness of new methods reflective of the more recently described subtypes. Carefully controlled E-rosette and SIg studies must continue to provide the baseline for new techniques such as detection of IgM and IgG receptors on T cells (T_μ and T_γ cells);[88,89] B-lymphocyte–specific HLA-related antigens of B cells (HL–B);[14,90,91] Ia-like determinants;[92] B alloantigens;[93,94] functional subgroups of T cells, helper cells, and suppressor cells;[95,96] and subsets of the so-called null cell, or non-B, non-T cell group, eg, antibody-dependent direct cytotoxicity test for κ cells.[97] Further refinements of anti-T and anti-B cell serums seem to offer the best long-term prospect. In immediate practice the specificity of the SIg procedure may be improved by taking countermeasures against the mechanisms depicted in Text-figure 4, principally by prior incubation of test cells at 37 C to remove *in vivo* adsorbed serum Ig and by the use of Fab fragments (F[ab]2, lacking the Fc portion) to obviate false-positive results due to Fc receptor binding of the labeled antiserums.

Conclusions

1. The results of this combined morphologic-immunologic surface marker study of 299 cases of non-Hodgkin lymphomas and associated leukemias provide general support for a functional approach to malignant lymphoma and favor the proposals that a) these disorders involve the B- and T-cell systems; b) lymphomas of large cells morphologically and

functionally resemble transformed lymphocytes rather than histiocytes; and c) nodular lymphomas involve follicular center cells (B cells) and as such are best considered as lymphomatous follicles. Of the 299 cases, 190 (64%) were classified morphologically as B-cell subtypes, 63 cases (21%) as T-cell subtypes, 1 case as a true histiocytic type, and 45 cases (15%) as U (unmarked) cells. The immunologic surface marker studies were in agreement with the great majority of cases assigned to the T-cell subtypes on the basis of morphologic criteria and with a variable proportion of the cases assigned to each of the B-cell subtypes.

2. The morphologic types of the Lukes–Collins classification [2-4] of lymphomas in general are predictive of the immunologic surface marker results and their B- and T-cell nature, although there are technical problems and variations in the surface marker characteristics, particularly in each cytologic type of B-cell lymphoma. There are three principal technical problems: a) excessive cell loss in the collection of specimens, most commonly from increased cell fragility of the large transformed lymphocytic types and from difficulty of separating cells in lymphomas associated with sclerosis; b) difficulty in demonstrating monoclonal SIg in certain B-cell lymphomas because of the possible masking effect of adsorbed Ig on the surface of the cell or as a result of production of limited amounts of SIg by the cells; and c) variability in the degree of E-rosette formation by T-cell lymphomas and leukemias that necessitates the morphologic demonstration of E-rosette formation by the neoplastic cells in stained preparations, rather than simple reliance on an arbitrary percentage of E rosettes for acceptance of the process as of T-cell type. In view of the reported variation in surface marker studies, and the problems of interpretation of the results in a proportion of cases, it is clear that the critical application of the Lukes–Collins classification will best serve the practicing pathologist in distinguishing the various recognized subtypes of lymphoma. Immunologic methods remain of value for confirmation of the cytologic diagnosis, for assisting diagnosis in cases in which the cytologic criteria are difficult to interpret, and for the purposes of continuing research.

3. The establishment of lymphomas as neoplasms of the immune system with involvement principally of the B- and T-cell systems has major implications for diagnosis and therapy. To achieve the required precision in cytologic identification and classification, it is essential for the pathologist to collect fresh biopsy material for ideal fixation and for preparation of tissue imprints for cytochemical techniques, such as the acid phosphatase reaction used to facilitate identification of the convoluted T lymphocyte and the nonspecific esterase stain used for the identification of histiocytes

and monocytes. Immunoperoxidase studies for the detection of cytoplasmic immunoglobulin and lysozyme (muramidase) on paraffin-embedded tissue provide the needed support for the morphologic interpretation of lymphomas of large cell type. With the development and general availability of specific anti-B cell and anti-T cell serums, methods such as the SIg and sheep erythrocyte rosette techniques may become defunct. For clinicians, it is hoped that this dynamic multifaceted approach to understanding the biology and immunology of lymphomas will facilitate a more rational approach to the design of therapy.

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Acknowledgments

[End of Article]