Correlation of Tumor Cell Kinetic Studies with Surface Marker Results in Childhood Non-Hodgkin’s Lymphoma

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ABSTRACT

Tumor cell kinetic observations were made in 40 children with advanced stage, diffuse types of non-Hodgkin’s lymphomas (NHL’s) in order to characterize the patterns of proliferation present and to explain the diversity of clinical behavior exhibited by childhood lymphoproliferative neoplasms. The in vitro $[^3H]$thymidine autoradiographic labeling index (LI) percentage was determined on samples of blood, marrow, effusions, cerebrospinal fluid, and solid tumors. Blasts from 33 of the 40 children also had T- and B-cell surface markers determined. Extensive variability in the LI percentage of tumor cells was related to surface markers, individual patient differences, sample source, and stage of disease progression. The median LI of marrow blasts in the leukemic phase of NHL was 42% (range, 13 to 60%; $n = 17$). This is significantly higher ($p < 0.005$) than childhood acute lymphoblastic leukemia cases studied, either at diagnosis or relapse. Study of surface markers from marrow blasts in NHL and acute lymphoblastic leukemia revealed a hierarchical relationship in proliferative activity: B-cells > T-cells > non-T and non-B. The greater LI of B-cell NHL was also shown for samples of cerebrospinal fluid (median, 30%) and for pleural fluid blasts, particularly at relapse (median, 39.5%). We conclude that the intrinsic state of neoplastic lymphoid cell differentiation (B, T, or ‘null’) conditions the growth fraction of lymphoid neoplasia. Conceivably, the clinical and kinetic behavior of childhood lymphoproliferative disease reflects the patterns of growth characteristic of their normal lymphocyte counterparts. Eleven children had tumor cells sampled simultaneously from more than one site, and the results typically differed markedly. Local environmental or systemic factors apparently regulate tumor growth as well.

INTRODUCTION

The study of patterns of tumor cell proliferation in childhood NHL may contribute to our understanding of the biological diversity of human lymphoid neoplasms. Childhood NHL differs from the more common childhood lymphoproliferative disease, ALL, exhibiting a variable clinical course depending upon the primary site, stage, and histological and immunological classification of the tumor type. Clinically, the course of disease is acute and often fulminant, as children may present with large tumor masses developing over a period as short as 2 to 4 weeks. Histologically, the tumors are typically diffuse, high-grade cancers with numerous mitotic figures in evidence. Study of tumor cell surface markers in lymphomas has revealed evidence for T- or B-cell characteristics, important prognostically and critical to our understanding of proliferating lymphomatous cells, giving some indication of the nature of the target cell hit by the neoplastic event. Therefore, it seemed likely that the study of lymphoma cell kinetics might explain some aspects of the variable clinical behavior of these neoplasms and provide an instructive comparison to blast cell kinetic studies we have reported in childhood leukemias (18). Lymphoid leukemias and lymphomas form a model system for the study of human tumor cell kinetics, allowing comparison of the state of tumor cell differentiation with its kinetic characteristics.

The existing data regarding the cell kinetic characteristics of human lymphomas are sparse, consisting of observations of flash in vitro $[^3H]$thymidine labeling of tumor cells (6, 7, 20), analyses of DNA content of tumor cell populations (6, 7, 20), estimates of cell cycle phase durations and potential doubling times (12, 21), and attempted correlations with histopathological classification and prognosis (4, 26, 27). Reported investigations have been exclusively in adults, except for limited studies of childhood African Burkitt’s tumors (6, 12). No previous studies have detailed lymphoma cell kinetics in children and related them to neoplastic lymphoid cell subpopulations.

MATERIALS AND METHODS

Forty children with NHL were subjects of kinetic studies. The patients ranged in age from 3 to 17 years (median, 9 years). All had diffuse histological subtypes of NHL, either undifferentiated or lymphoblastic. The stage of disease at diagnosis of 38 of the 40 children was advanced, Stage III or IV according to the clinical staging classification described elsewhere (17). The children studied were an unselected group representing all patients with NHL seen over a 3-year period in whom it was possible to sample tumor cells readily in the routine course of diagnosis and management of their NHL. It was often possible to obtain multiple samples for kinetic studies from individual patients, either from separate involved sites or sequentially over time, from diagnosis to relapse. All observations reflect the unperturbed steady state, either at diagnosis prior to initial therapy or in full relapse prior to specific retreatment. Any kinetic samples conceivably influenced by drug effects have been excluded from this analysis.

A subset of NHL patients (33 of 40) had studies of their tumor cells for surface markers. Tumor cells were designated as T-cell in origin, based on spontaneous rosette formation with untreated sheep RBC at 4 and 37°C (3) or with sheep cells pretreated with 2-aminoethyl isothiouronium bromide to enhance the sensitivity of the assay (22). Tumor cells were designated as B-cells upon the demonstration of surface im-

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3 The abbreviations used are: NHL, non-Hodgkin’s lymphoma; ALL, acute lymphoblastic leukemia; LI, labeling index.

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munoglobulin by direct immunofluorescence, using commercially available fluorescein-conjugated polyvalent antimunoglobulin (2). In 11 patients, additional immunological assays of blasts for reactivity with heterologous anti-T and anti-B antisera were performed by indirect immunofluorescence (1, 16), confirming the more conventional surface marker results in each instance. One child with a mediastinal tumor whose marrow and pleural fluid were negative in the sheep erythrocyte rosette assay was designated as T-cell on the basis of blast reactivity with anti-T antisera.

For comparison with the marker and kinetic studies of NHL blasts in the marrow, previously reported studies of bone marrow lymphoblast LI percentage from children with ALL are included. Fifty-four children with ALL had blast LI percentage and sheep erythrocyte rosette assays done at diagnosis (18), and 14 children had blast LI percentage determined at the time of first hematological relapse (23).

For determination of the LI percentage, tumor cells from the marrow, blood, cerebrospinal fluid, or pleural effusions were obtained by percutaneous aspiration and were incubated in vitro for 1 hr at 37° with \(^{3}\text{H}\text{thymidine} (\text{methyl-}^{3}\text{H}; 1.9 \text{Ci/ mmol}; 1 \mu\text{Ci/ml of blood, marrow, or cell suspension}). Lymph node or tumor tissue obtained by surgical excision was finely minced in tissue culture medium first to form a cell suspension, and then was incubated as above. Cells were concentrated by centrifugation and spread on slides for autoradiography, using the dipping technique with Kodak NTB2 emulsion. After an exposure at 4° for 12 to 13 days, slides were developed in Kodak D19 developer and stained with Wright's stain. The percentage of labeled tumor cells was determined after counting 1000 to 3000 tumor cells. Statistical comparisons between 2 distributions of LI percentages were performed by the 2-sample rank test (Mann-Whitney U-test) (10).

RESULTS

The kinetic indexes of NHL blast cells were found to vary over an extremely wide range. The variability related mainly to differences between patients, but also was related to tumor cell surface markers, sample source, and stage of disease.

The Leukemic Phase of NHL

Conversion of childhood NHL to a leukemic phase occurs commonly, and the bone marrow becomes replaced by blasts. Seventeen children had kinetic studies of marrow blasts; 4 at the time of diagnosis when the marrow was partially replaced and 13 at the time of relapse. The range of in vitro tumor cell \(^{3}\text{H}\)thymidine labeling of NHL blasts was broad (13.8 to 60%; median, 42%). These results are shown in Chart 1A. For comparison, studies are shown of bone marrow blasts in children with ALL, determined at first hematological relapse (Chart 1B) and at diagnosis and pretreatment (Chart 1, C and D). A striking difference in these distributions of bone marrow blast LI percentage is apparent.

The median LI of NHL blasts in a leukemic phase (42%) is roughly 10-fold greater than that observed in the common type of childhood ALL at diagnosis (4.8%). As shown in Chart 1, marrow leukemic blast cells from children with ALL studied at first relapse have significantly greater LI's than at diagnosis (25). Since 13 of the 17 cases of NHL in a leukemic phase were studied at relapse, the comparison of their LI's to ALL in relapse was necessary. Yet the median and range observed for NHL marrow blasts is still significantly greater than those for ALL at relapse (p < 0.005).

Comparison of the neoplastic lymphoid cell surface marker results with LI percentage in these cases is revealing. The LI percentage of T-cell ALL (Chart 1C) is significantly greater (p < 0.01) than that for the common type of non-T childhood ALL at diagnosis (Chart 1D). Ten of the 17 cases of NHL are of proven B-cell type (shown as × symbols in Chart 1A), and these samples are significantly greater (p < 0.005) than all the rest of the leukemic samples studied, either at diagnosis or during relapse.

In 6 children with NHL, tumor cells circulating in the blood were examined. The range of LI was from 2.7 to 24%, and the median was 15%. These findings contrast with the usual findings in acute leukemia, in which it has generally been observed that circulating leukemic cells constitute a nonproliferating compartment with absent or low in vitro \(^{3}\text{H}\text{thymidine} labeling (15).

NHL Cells in Effusions

Pleural Effusions. Thirty samples of malignant pleural effusions obtained by percutaneous thoracentesis were examined. Nineteen samples were obtained from children at diagnosis, and 11 samples were studied from children with progressive disease. Tumor cell markers were obtained in all cases but one. The results according to the cell type and stage of disease

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are shown in Chart 2, A and B. The median LI of pleural fluid blasts at diagnosis was 13%. The median at relapse was 3-fold greater, 39.5%, and the ranges differ significantly ($p < 0.01$).

Tumor cell surface markers, stage of disease, and cell density were considered as possible factors influencing the greater LI percentages observed at relapse. Ten of the 11 observations at relapse were in cases of proven B-cell origin. Only one patient with B-cell disease was studied both at diagnosis and at relapse; his pleural fluid blast cell LI increased 10-fold with disease progression, from 5 to 56%. Cell density seems an unlikely sole determinant of tumor cell proliferation in effusions, as there was no correlation between the blast count per cu mm in pleural fluids at diagnosis and the LI percentage.

**Ascitic Effusions.** The LI of blasts from malignant ascites from 6 children at diagnosis, all of whom were of B-cell type, are shown in Chart 2C. The individual patient variability is typically broad, with the values ranging from 14 to 48% (median, 24%).

**Cerebrospinal Fluid Blasts**

The LI percentage of NHL blasts in cerebrospinal fluid obtained by lumbar puncture was determined from 13 children at the time of the first documentation of malignant pleocytosis and/or symptoms of central nervous system involvement by lymphoma. The results are shown in Chart 3. The values ranged from 5 to 45% (median, 24%). Ten of these 13 children had surface marker determinations done on their blasts. The B-cell cases examined all had a significantly greater LI ($n = 7$; range, 14 to 45; median, 30%) than did the 3 T-cell cases studied (LI, 5.1, 8.8, and 9%). As in the pleural fluids examined, there was no correlation between the spinal fluid blast cell counts and the LI percentages. Three children had sequential determinations of LI percentage performed during multiple bouts of central nervous system disease over a period of 1 to 6 months, and the values observed remained remarkably consistent and independent of the cell count. For example, in one child with an intraabdominal B-cell tumor, the initial LI of cerebrospinal fluid blasts was 35% (380 blasts/cu mm); 9 days later, it was 38% (35 blasts/cu mm); and, 6 weeks later, it was 30% (760 blasts/cu mm).

**Specimens of Solid Tumor**

The results of LI percentage and surface marker determinations from 7 samples of solid tumor from various sources are listed in Table 1. All samples were obtained at diagnosis, except the testicular biopsy, which was obtained 18 months following diagnosis in a boy with a primary mediastinal NHL in relapse. The 3 characteristically high LI percentages were found in the proven cases of B-cell disease, the intermediate were found in T-cell tumors, and the lowest were found in the non-T and non-B sample.

**Variability in Simultaneous Samples from Different Sites**

In 11 patients with generalized disease, tumor cells were obtained from 2 or 3 different sites on the same day, and LI percentages were determined simultaneously. The results are shown in Table 2. Rather marked variability was typically encountered, exceeding the difference expected from in vitro experimental variation.

**DISCUSSION**

The data presented here strongly support an intrinsic difference in lymphoid tumor cell proliferation due to the state of tumor cell differentiation. Though there exists a considerable
variation within each type of neoplastic lymphoid cell, the distribution of values of LI percentage for B-cell NHL samples from marrow, spinal fluid, pleural fluid, and solid tumor specimens is significantly higher than for T-cell tumor samples. Both B- and T-cell marrow blasts have significantly higher LI percentages than the common, non-T, non-B, or null cell type of lymphoblast.

An explanation for these results may be found by considering the normal pathways of lymphocyte differentiation and by hypothesizing that the kinetic features of these lymphoid neoplasms are a reflection of the usual growth fraction of their normal counterpart lymphoid cell subpopulation. The B-cell series illustrates this best. Briefly, B-cell lymphomas and leukemias arise from the B-cell series of lymphoid cells. Arising from a stem cell, the first readily identified B-lymphocyte is the B1-cell. After antigen triggers a B1-cell, an immunoblast (B2) is formed. After division, the immunoblast may differentiate in the direction of the plasma cell series (B4 and B5) or may become a memory B-lymphocyte (B3) (24). The kinetic features of these childhood B-cell lymphomas mirror those of the normal germinal center cell, the B2-immunoblast (13). Histologically, these tumors are composed of basophilic and pyroninophilic cells larger than typical lymphoblasts and having the same appearance as lymphocytes observed after antigen or mitogen stimulation, the noncleaved cells of lymphoid germinal centers. In an elegant study of morphology, histochemistry, and immunological surface markers, Mann et al. (14) investigated a large series of nonendemic Burkitt’s lymphoma cases, parallel to the childhood B-cell lymphomas reported here, and concluded that these tumors were related to germinal centers. Ishii and co-workers (11) observed LI’s from 50 to 90% in autoradiographs of the proliferating germinal centers of guinea pig lymph nodes following antigenic stimulation. Based on morphological studies of human germinal center cells, Lukes and Collins (13) recognized cleaved and noncleaved cells and proposed that the noncleaved cell is the dividing cell of the follicular center while the cleaved cell is essentially nondividing. Our kinetic study of these noncleaved, B-cell lymphomas of childhood is entirely consistent with that hypothesis and suggests, furthermore, that these tumor cells maintain their characteristic high growth rate no matter what their milieu. Similarly, high percentages of labeled tumor cells (30 to 50%) have been reported for samples of Burkitt’s lymphoma from African patients (6, 12).

The degree of cell proliferation apparently parallels the degree of cell differentiation in other B-cell lymphoid neoplasms observed in adults. For example, chronic lymphocytic leukemia and “well-differentiated” lymphocytic lymphoma are low-grade neoplasms generally considered to be expressions of diseases of the small, B1-immunoblast (24). In kinetic studies of adults with these low-grade, good-prognosis lymphoid neoplasms, a very low percentage of tumor cells, generally from 0.5 to 2%, were in the S phase (4, 26, 27, 29). At the other extreme end of B-cell differentiation, B5, are plasma cells. Consistent with the low rate of cell division of end-stage, differentiated cells, myeloma cells typically have a quite low LI (median, 2%) (8). Thus, a coherent thesis is to suppose that lymphoid neoplasms share the kinetic characteristics of their normal lymphocyte counterparts as well as other morphological, biochemical, and functional characteristics, like surface markers, cytochemistries, and secretory products.

The null cell of childhood ALL is considered to be a stem cell, uncommitted to differentiation and expressing neither T- nor B-cell markers. Our studies, as shown in Chart 1, have documented a low LI (median, 4.8%) for null cell ALL (18). Tsukimoto et al. (30) also reported that null lymphoblasts from childhood ALL cases had low LI’s, generally less than 10%.

| Table 1 |
| NHL solid tumor specimens |

These are the results of autoradiographic determinations of tumor cell LI percentages following in vitro [3H]thymidine exposure of cell suspensions of solid tumor specimens from 7 children with NHL. The tumor cell type determined from surface marker studies of blast cells is given in the second column.

<table>
<thead>
<tr>
<th>Specimen source</th>
<th>In vitro [3H]thymidine LI (%)</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node</td>
<td>10</td>
<td>Non-T, non-B</td>
</tr>
<tr>
<td>Lymph node</td>
<td>22</td>
<td>T</td>
</tr>
<tr>
<td>Testicle</td>
<td>22</td>
<td>T</td>
</tr>
<tr>
<td>Abdominal tumor, omental</td>
<td>43</td>
<td>B</td>
</tr>
<tr>
<td>Anterior chest wall tumor, s.c.</td>
<td>41</td>
<td>B</td>
</tr>
<tr>
<td>Abdominal tumor, omental</td>
<td>37</td>
<td>B</td>
</tr>
<tr>
<td>Abdominal tumor, retroperitoneal</td>
<td>9</td>
<td>Not done</td>
</tr>
</tbody>
</table>

| Table 2 |
| Variability in tumor cell LI percentages within an individual at any one time |

These tumor cell LI percentages are from samples obtained at the same time from 2 or 3 different body compartments in 11 children with NHL. The tumor cell type as determined from surface marker assays is listed in the second column. Considerable variation is evident, suggesting nonuniform rates of tumor cell proliferation within an individual.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cell marker</th>
<th>Bone marrow</th>
<th>Blood</th>
<th>Cerebrospinal fluid</th>
<th>Pleural fluid</th>
<th>Ascitic fluid</th>
<th>Solid tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T</td>
<td>42</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>T</td>
<td>30</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>T</td>
<td>18</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Non-T, non-B</td>
<td>18</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>40</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>29</td>
<td>29</td>
<td></td>
<td></td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>50</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>B</td>
<td>29</td>
<td>38</td>
<td></td>
<td></td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>40</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>B</td>
<td>48</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>B</td>
<td>43</td>
<td>22</td>
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Those workers also observed somewhat higher LI percentages in 7 cases of childhood T-cell lymphoblastic leukemia, in agreement with our own findings. That the common type of childhood ALL, stem cell leukemia, should have a low proliferative activity is perhaps not surprising considering the resting, out-of-cycle status of most stem cells. The LI of marrow blast cells in the usual case of acute myelogenous leukemia at diagnosis is likewise low, 5 to 8% (18), in contrast to the more differentiated form of myeloid leukemia, chronic myelocytic, in which the mean LI of myeloblasts in the chronic phase is 20 to 50% (28), more nearly approximating the kinetic characteristics of normal myeloblasts.

These observations relating the proliferative activity of tumor cells to their state of differentiation prompt a reexamination of the relative importance of the factors regulating tumor growth. The growth fraction, i.e., the proportion of cells actively in cycle, is apparently set largely by an intrinsic cellular mechanism, according to cell type. Additional regional and systemic influences must then explain the variable proportions of proliferating and nonproliferating tumor cells one sees from patient to patient and with change in time. The variability of tumor cell LI percentage observed in different body compartments (Table 2) may be the result of the interplay of host and tumor factors. Host factors, like vascularity, oxygen, and nutrient supply, undoubtedly play a role in growth promotion. Additional selective factors operating on the heterogeneity of tumor cell populations may promote the emergence of variants with the capacity for growth in a particular environment, like ascitic or pleural fluid or the central nervous system. Fidler (9) has demonstrated such heterogeneity in a murine tumor system containing highly metastatic variant subpopulations of tumor cells.

The kinetic results reported here have prognostic implications. The high LI percentage and large growth fraction of childhood B-cell lymphomas contribute to their extraordinary initial sensitivity to chemotherapy and to their notable tendency for early relapse due to rapid tumor regrowth. B-cell disease has the shortest survival time and lowest overall cure expectancy of childhood lymphoid neoplasms (5, 30). By contrast, null cell ALL, characterized by the lowest LI percentage, has the best prognosis, longest survival, and greatest chance for cure. T-cell disease, intermediate in its kinetic characteristics, has an intermediate prognosis. Since the surface markers are correlated with the kinetic characteristics of these childhood leukemias and lymphomas, further study will be necessary to determine whether the tumor cell kinetics exert independent prognostic influence.

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