A significant number of patients with non-Hodgkin's lymphoma have peripheral blood involvement during the course of their disease. Because the expression of receptor for the lectin peanut agglutinin PNA by normal lymphocytes is associated with noncirculating (stationary phase) cells, we studied the relationship between PNA binding by lymphoma cells and the presence of clonal B cells in the blood of 38 patients with B-cell lymphoma. The binding of PNA by cells in tissues was determined by the immunoperoxidase method and by two-color flow cytometry. Circulating lymphoma cells (clonal B cells) were identified by a sensitive flow-cytometric technique (~ = 0.05 analysis) and were also studied for PNA binding in some cases. In all, 16 of 38 (42%) of lymphomas were PNA+, indicating a spectrum of histologic types. Circulating lymphoma cells were demonstrated in 17 of 22 PNA-lymphomas, whereas only 3 of 16 of PNA+ lymphomas had such circulating cells. Thus, there is a significant association between PNA binding and peripheral blood involvement by lymphoma (P < .005 by chi-square analysis). In 12 cases, the circulating and tissue lymphoma cells had similar expression of PNA receptor (2 PNA+ and 10 PNA- cases), indicating that modulation of the PNA binding sites did not occur. In three patients who presented with lymphosarcoma cell leukemia, the circulating malignant cells were PNA-. These findings suggest that for both normal and malignant lymphocytes the absence of binding sites for PNA is associated with the capacity of these cells to circulate freely.

SOME CASES of non-Hodgkin's lymphoma are associated with an overt leukemic phase, based on morphological observation. The incidence of leukemic involvement in nodular and diffuse forms of poorly differentiated lymphocytic lymphoma was 9% and 13%, respectively, in one large series. However, immunologic studies have shown that a much greater proportion of lymphoma patients have peripheral blood involvement than is suspected by morphology alone. With a sensitive flow-cytometric technique, we were able to show that 50% to 80% of patients with non-Hodgkin's lymphoma have circulating clonal B cells during the course of their disease, regardless of histologic type. We also showed that, after treatment, patients with histologic subtypes of lymphoma most susceptible to relapse (low grade) are far more likely to retain circulating clonal B cells despite apparent clinical remission. Thus, circulating lymphoma cells are a prominent feature of the biology of non-Hodgkin's lymphomas and may be important in the frequent early dissemination and high relapse rate that are characteristic of these malignancies.

The features that control the dissemination of malignant lymphoma are poorly understood. Malignant lymphoid cells, like their benign counterparts, may exhibit extensive recirculation between tissue and peripheral blood, thus leading to early systemic spread. This idea led us to examine malignant lymphomas for features that may be associated with the tendency of the malignant cells to enter the peripheral circulation. One such feature associated with localization of normal lymphocytes is the expression of surface binding sites for the lectin peanut agglutinin (PNA). Lymphocyte populations that remain localized to tissue-specific sites, such as thymic cortical lymphocytes and lymph node germinal center cells, strongly bind PNA, whereas freely circulating lymphocytes bind the lectin only weakly. Thus, "stationary-phase" and "circulating-phase" lymphocytes may be distinguished on the basis of PNA binding avidity. By studying a series of non-Hodgkin's lymphomas for their ability to bind PNA, we sought to determine whether a lack of expression of PNA binding sites on the malignant cells in tissues is associated with the presence of circulating lymphoma cells and whether modulation of receptor sites might be involved in entry of lymphoma cells into the migratory phase. We used flow-cytometric techniques that allow identification and phenotypic characterization of even small numbers of clonal B cells in tissues and blood. We found a strong association between lectin binding and the appearance of lymphoma cells in the peripheral blood, suggesting that malignant lymphoid cells spread systemically by mechanisms related to normal lymphocyte migration.

MATERIALS AND METHODS

Tissues and blood. Tissues (mostly lymph nodes) from patients with non-Hodgkin's lymphoma were fixed in B5 and 10% buffered formalin, and a portion was snap-frozen in isopentane and dry ice. If sufficient tissue was available, a portion was used to prepare single-cell suspensions by teasing the tissue with forceps in Hanks' balanced salt solution (HBSS, GIBCO, Grand Island, NY) containing 0.2% bovine serum albumin (BSA, Sigma, St Louis). Viable cells were obtained after centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), suspended in tissue culture medium (RPMI 1640, 10% fetal calf serum (FCS), HEPES buffer, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 50 U/mL streptomycin (GIBCO) at 2 x 10^6 cells/mL] and subjected to two 1-hour incubations in tissue culture flasks (75 cm^2; Corning Glass Works, Corning, NY) at 37°C in an atmosphere of 5% CO₂, with nonadherent cells removed after each step. The nonadherent cells were kept at 4°C until further processing. Peripheral blood from patients was...
obtained by venipuncture and subjected to isolation of nonadherent mononuclear cells in a manner identical to that described above. Fixed tissues were processed for routine histologic sections, stained with hematoxylin and eosin, and the lymphomas were classified according to the Rappaport system. The phenotype of the lymphoma was confirmed using standard immunoperoxidase methods on cryostat sections (described below). Sections and blood were obtained with the approval of the Human Subjects Committee of the Brigham and Women's Hospital.

Immunoperoxidase staining. Cryostat sections of tissues were prepared for immunoperoxidase studies by fixation in acetone at 4°C for 4 minutes and washed in Tris buffer (0.05 mol/L, pH 7.6) containing 2% swine serum (GIBCO). Sections of paraffin-embedded tissue were deparaffinized by standard methods. Staining for PNA binding was performed in all cases on paraffin-embedded tissues and on cryostat sections (if available) as well. Sections were incubated with affinity-purified PNA (25 ng/mL) (Vector Laboratories, Burlingame, CA) in HBSS for 30 minutes at room temperature. The slides were then washed with buffer and incubated sequentially with rabbit anti-PNA antibody (E-Y Laboratories, San Mateo, CA) at 1:200 dilution, swine anti-rabbit immunoglobulin antibody (Dako, Santa Barbara, CA) at 1:100 dilution, and horseradish peroxidase–rabbit anti-horseradish peroxidase immunoperoxidase complex (Dako) at 1:150 dilution. Staining was achieved by using 3,3'-diaminobenzidine tetrahydrochloride (Aldrich Chemical, Milwaukee) as chromagen. Sections were counterstained with methyl green or light hematoxylin and mounted. Controls consisted of sections incubated with HBSS instead of PNA or with a mixture containing PNA and either 0.1 mol/L galacose or 0.1 mol/L mannose. The specificity of lectin binding was verified by inhibition of staining by galactose but not mannose. Equivalent results were obtained on frozen and paraffin-embedded tissues in cases for which frozen tissue was available. Phenotyping of the lymphoma, containing two cases of nodular lymphomas that bound PNA (Fig 1). In general, nodular lymphoma cells were detected by examination of the red fluorescence of light chain–positive cells (stained with fluoresceinated antibody to the appropriate light chain as determined by k-λ analysis and PNA-biotin), as compared with the red fluorescence of cells labeled with antibody to light chain and red fluorescent secondary staining reagent alone.

RESULTS

PNA binding by non-Hodgkin’s lymphomas. Seventy-one cases of non-Hodgkin’s lymphoma, including a wide variety of histologic types, were studied for PNA binding on tissue sections (Table 1). In all, 39% of the cases studied showed binding of PNA. The staining of the malignant lymphoid cells appeared to be peripheral and surface-membrane associated, as distinguished from the cytoplasmic staining observed for tissue histiocytes and dendritic cells (Fig 1). In general, nodular lymphomas that bound PNA showed staining of most lymphoid cells within nodules and of variable numbers of cells between nodules. Diffuse lymphomas, when positive, showed variable numbers of PNA-binding cells among negative background cells but always contained >50% PNA+ cells. The cells in PNA− cases uniformly failed to bind the lectin. Differences in PNA binding were also evident among cases of non-Hodgkin’s lymphoma of similar histologic type. Indeed, histologically identical cases sometimes showed striking differences with regard to lectin binding. Figure 1 shows two cases of nodular...
Table 1. PNA Binding by non-Hodgkin’s Lymphomas

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>PNA +/Total (%+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDL, N</td>
<td>6/8 (75)</td>
</tr>
<tr>
<td>PDL, N + D</td>
<td>6/10 (60)</td>
</tr>
<tr>
<td>PDL, D</td>
<td>2/11 (18)</td>
</tr>
<tr>
<td>MLH, N</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>MLH, N + D</td>
<td>6/9 (66)</td>
</tr>
<tr>
<td>HL, N</td>
<td>2/4 (50)</td>
</tr>
<tr>
<td>HL, N + D</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>HL, D</td>
<td>1/6 (17)</td>
</tr>
<tr>
<td>DUL</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>ILL</td>
<td>1/6 (17)</td>
</tr>
<tr>
<td>WDLL</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>CLL</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>True HL</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>HCL</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Plasmacytoma</td>
<td>1/2 (50)</td>
</tr>
<tr>
<td>Total</td>
<td>28/71 (39)</td>
</tr>
</tbody>
</table>

PDL, poorly differentiated lymphocytic lymphoma; MLH, mixed lymphocytic-histiocytic lymphoma; HL, histiocytic (large cell) lymphoma; DUL, diffuse undifferentiated lymphocytic lymphoma; ILL, intermediate lymphocytic lymphoma; WDLL, well-differentiated lymphocytic lymphoma; CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia.

The lectin-binding properties of the malignant lymphoid cells could also be studied and verified using two-color flow cytometry. Once the light chain type of a B cell lymphoma was determined using k-λ analysis (later confirmed by tissue immunoperoxidase studies), cells were colabeled with the appropriate anti-light chain antibody and biotinylated PNA. Binding of anti-light chain antibody was detected by fluoresceinated anti-rabbit immunoglobulin, and binding of the lectin was revealed with avidin-phycerothrin. Then, with two-color flow cytometry, the binding of lectin by malignant lymphoid cells could be specifically determined. With this method, the results of tissue immunoperoxidase studies for PNA binding were confirmed by flow cytometry in 32 cases (15 PNA-, 17 PNA+). Examples of three such cases are shown in Fig 2. This same method was used to examine circulating malignant lymphoma cells for PNA binding activity (described below).

Overall, nodular lymphomas showed a higher incidence of PNA binding than did diffuse lesions. Large cell ("histiocytic") lymphomas also showed heterogeneity with regard to PNA binding. Other forms of lymphoproliferative disease, such as intermediate lymphocytic lymphoma and well-differen-

![Fig 1](https://www.bloodjournal.org/)

Fig 1. Binding of PNA by two cases of nodular poorly differentiated lymphocytic lymphoma. In one case (A,B), the malignant lymphoid cells bind PNA, with reactivity localized predominantly in the nodules (A). At higher magnification (B), the staining of lymphocytes is circumferential, consistent with binding of the lectin to the plasma membrane. Staining of scattered lymphocytes between the nodules is also observed. Another histologically identical case (C,D) fails to bind PNA, although cytoplasmic binding by tissue histiocytes is apparent between the nodules (D). (A, C × 20; B, D × 400; light hematoxylin counterstain.)
cases of tissue involvement by CLL were removed from the PNA− group.

Table 3 shows the results according to histologic type, with the proportion of cases having blood involvement shown for PNA+ and PNA− cases of each type. Although the numbers of cases in each group were small, some interesting features emerged. Histologic types known to be associated with a high incidence of blood involvement, such as CLL and WDLL, were entirely PNA−. Also included are four cases in which only peripheral blood could be studied, including three cases of lymphosarcoma cell leukemia and a single case of hairy cell leukemia, all of which were PNA−. Among large cell lymphomas, the diffuse types studied were all PNA+ with a high incidence of blood involvement, whereas nodular lesions were PNA+ and showed no circulating cells. In the mixed type, only the PNA− cases showed peripheral blood involvement. The three PNA+ cases with circulating clonal B cells were restricted to the PDL group.

**PNA binding by circulating lymphoma cells.** With two-color analysis, we were able to examine circulating lymphoma cells for PNA binding capacity. In tissues, the light chain type of lymphoma cells was first identified; peripheral blood mononuclear cells were then stained simultaneously with PNA and antibody to the appropriate light chain type. An example of a case studied in this manner is shown in Fig 2.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Peripheral Blood Involvement (+/total)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDL, N</td>
<td>2/4</td>
</tr>
<tr>
<td>PDL, N + D</td>
<td>1/3</td>
</tr>
<tr>
<td>PDL, D</td>
<td>0/2</td>
</tr>
<tr>
<td>MLH, N</td>
<td>0/1</td>
</tr>
<tr>
<td>MLH, N + D</td>
<td>0/3</td>
</tr>
<tr>
<td>HL, N</td>
<td>0/2</td>
</tr>
<tr>
<td>HL, N + D</td>
<td>0/1</td>
</tr>
<tr>
<td>HL, D</td>
<td>0/3</td>
</tr>
<tr>
<td>DUL</td>
<td>0/1</td>
</tr>
<tr>
<td>ILL</td>
<td>0/2</td>
</tr>
<tr>
<td>WDLL</td>
<td>0/3</td>
</tr>
<tr>
<td>CLL</td>
<td>2/2</td>
</tr>
<tr>
<td>HCL†</td>
<td>2/2</td>
</tr>
<tr>
<td>LSCL‡</td>
<td>3/3</td>
</tr>
</tbody>
</table>

LSCL, lymphosarcoma cell leukemia.

*Proportion of PNA+ and PNA− cases with peripheral blood involvement for each histologic type.

†Includes one case in which only peripheral blood was studied.

‡Only peripheral blood available for study.
3. Peripheral blood from 12 cases of B-cell lymphoma was examined, including 10 PNA− and 2 PNA+ cases. In each individual case, tissue and peripheral blood lymphoma cells showed similar binding of PNA. Flow-cytometric analysis of both peripheral blood and tissue from a case of PNA+ nodular PDL is shown in Fig 4. Although in this case the degree of lectin binding by the λ-positive cells in peripheral blood was not as great as in tissue, the level of binding was greater than that of the background cells (predominantly T cells). Thus, although most cases of PNA+ lymphoma did not have peripheral blood involvement, modulation of PNA binding sites could not explain the presence of circulating malignant cells in some cases of PNA+ lymphoma.

DISCUSSION

Occasional cases of non-Hodgkin's lymphoma are associated with frank peripheral blood involvement, most often in poorly differentiated lymphocytic lymphoma.1 More sensitive flow-cytometric methods applied to detection of clonal B cells in blood2 showed that many more lymphoma patients have circulating tumor cells than can be appreciated by morphology alone.3 In previous studies, we showed that ~50% to 80% of patients with non-Hodgkin's lymphoma have circulating clonal B cells during the course of their disease, regardless of histologic type.4 Using Southern blot techniques to detect clonal immunoglobulin gene rearrangements, we confirmed the ability of the flow-cytometric method (κ-λ analysis) to detect clonal B cells and showed that the circulating cells are clonally identical to the malignant cells in involved tissues.4 Moreover, we showed that application of the more sensitive Southern blot method did not reveal additional cases of blood involvement not detected by flow cytometry. These findings indicate that peripheral blood involvement is not an intrinsic property of all lymphomas and that cases may differ in the functional capacity of malignant cells to enter the blood.

The frequent presence of malignant cells in the peripheral blood of lymphoma patients may reflect the migratory behavior of normal lymphocytes. Lymphoid cells recirculate extensively in the lymphatic tissues, a process believed to play a role in immune surveillance and in providing systemic distribution of antigen-primed cells.7 Although the mechanism of entry of circulating lymphocytes into lymphoid tissues appears to involve receptor-mediated binding to HEV,8,9 the mechanism of exit of cells into the peripheral blood is largely unknown. One feature that appears to distinguish freely circulating lymphocytes from cells that remain localized in specific tissue microenvironments is the presence of high-avidity binding sites for the lectin PNA.8 PNA strongly binds to immature T cells in the thymic...
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cortex,9 lymph node germinal center cells,8,10,19 and bone marrow pre-B cells20—cell types not usually present in the peripheral blood. Peripheral blood lymphocytes, on the other hand, bind the lectin only weakly.21 PNA has greatest affinity for the disaccharide Gal-β (1 → 3) GalNAc9 and binds to terminal galactose residues on glycoproteins and glycolipids. Because cells that bind PNA eventually differentiate to cells that circulate freely and do not bind PNA, the lectin binding site is presumed to be modified as the cell matures, possibly by addition of terminal sialic acid residues.8 Because the capacity for lymphocytes to circulate freely appears to be related to loss of PNA binding sites, we investigated whether a similar relationship exists in non-Hodgkin's lymphoma.

Our initial studies revealed that non-Hodgkin's lymphomas are heterogeneous with regard to PNA binding, in agreement with previous studies by other researchers.22-24 In many cases, otherwise histologically and phenotypically identical cases exhibited markedly different abilities to bind PNA. Although normal follicular center cells bind PNA, cells in neoplastic follicles (nodules) in some cases fail to bind the lectin. This phenomenon may indicate either altered expression of glyconjugates, frequently seen in neoplastic cells,25-26 or differentiation of the malignant cells to the extent that binding sites for PNA are sialylated and no longer available. Flow-cytometric studies confirmed the lectin binding status of tissues in all cases examined by both flow and immunoperoxidase methods, indicating that PNA binding sites are not altered by tissue processing. Therefore, peripheral blood cells studied by flow cytometry alone could be justifiably compared with cells in tissues with regard to PNA binding.

In this study, we demonstrated a significantly greater incidence of peripheral blood involvement in PNA− lymphomas than in PNA+ lymphomas. Excluding the nodular poorly differentiated lymphocytic (NPDL) group, only PNA− cases had detectable circulating lymphoma cells. Lymphoproliferative disorders characterized by frank peripheral blood involvement, such as CLL and lymphosarcoma cell leukemia, were PNA−. The two PNA+ cases with circulating clonal B cells were in the NPDL group. The presence of such circulating cells in PNA+ cases could not be explained by modulation of PNA binding sites, because dual-color flow-cytometric studies confirmed the binding of PNA to the peripheral blood clonal B cells. Although the degree of binding of PNA by the circulating cells appeared to be somewhat less than by the tissue cells in two such cases, direct comparison of separately performed studies with regard to the degree of fluorescence intensity is probably not valid. The occurrence of occasional cases with PNA+ circulating cells, however, indicates that the absence of PNA binding sites is certainly not the sole determinant of peripheral blood involvement by lymphoma.

Galton and Wilthshaw27 proposed that lymphoma cells escape into the peripheral blood by two possible mechanisms: (a) by means similar to that of normal lymphocyte recirculation and migration, and (b) by direct tumor invasion through lymphatic and capillary vessels. Because extensive recirculation is a major biologic function of lymphocytes, we propose that in most cases peripheral blood involvement in lymphoma reflects the migratory behavior that occurs in normal lymphocytes. In such cases, cell surface glycosylation may play an important role in peripheral blood involvement. In other cases, “pathologic” mechanisms may play a role, and cells with phenotypes not normally seen in the peripheral blood (eg, PNA+) can appear.

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Circulating malignant cells in non-Hodgkin’s lymphoma: correlation with binding by peanut agglutinin

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