Circulating Malignant Cells in non-Hodgkin's Lymphoma: Correlation With Binding by Peanut Agglutinin

By David S. Weinberg, Kenneth A. Ault, and Geraldine S. Pinkus

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 (k-λ analysis) and were also studied for PNA binding in some cases. In all, 16 of 38 (42%) of lymphomas were PNA+, including 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.

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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.11 The phenotype of the lymphoma
was confirmed using standard immunoperoxidase methods on
cryostat sections (described below). Tissues 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-embed-
ded 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 µg/mL) (Vector Labora-
tories, Burlingame, CA) in HBSS for 30 minutes at room tempera-
ture. 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 1:30 dilution, and horserad-
ish peroxidase–rabbit anti-horseradish peroxidase immunoelectro
complex (Dako) at 1:150 dilution.12 Staining was achieved by using 3-
3’diaminobenzidine tetrahydrochloride (Aldrich Chemical, Mil-
waukee) 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 galacrose 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.13 In cases for
which frozen tissue was available, phenotyping of the lymphoma,
including light-chain determination, was performed by standard
immunoperoxidase methods, as described previously.14

**κ-λ analysis.** Nonadherent mononuclear cells were prepared
from tissues and blood as described above. The cells were separated
into two aliquots, each labeled with F(ab’)2-fragments of rabbit
heteroantiserum to either κ or λ immunoglobulin light chains. The
staining procedure has been described in detail previously.2 Cells (1
to 2 x 10⁶) from each sample were analyzed for fluorescence on a
flow cytometer (FACS Analyzer, Becton Dickinson Immunocytom-
etry Systems, Mountain View, CA), and histograms were obtained
by plotting relative cell number v log fluorescence intensity. The two
curves thus obtained were analyzed for evidence of a monoclonal
population, revealed as a relative shift between the κ and λ curves
with regard to mean fluorescence intensity and/or differences in the
shapes of the fluorescence distribution curves. The analysis of the
fluorescence curves was aided by computer, with Kolmogorov-
Smirnov statistics applied to detect significant differences between
the two curves. This technique for detecting clonal excess has been
described in detail previously,14 and we showed this method to be
routinely capable of detecting 5% to 10% clonal B cells in tissues and
in blood.24 Furthermore, sensitivity and accuracy of κ-λ analysis was
confirmed by Southern blot analysis.4

**Two-color flow-cytometric analysis.** Once the light chain type
of the lymphoma cells was determined by κ-λ analysis, two-color
flow-cytometric analysis was used to measure the binding of PNA
to the malignant lymphoid cells, using biotinylated PNA in combina-
tion with antiserum to either κ or λ light chain, as previously
described.13 Staining was performed by adding to a pellet of 0.5 to
1.0 x 10⁷ cells 5/µL biotin-conjugated PNA (Vector) and 5 µL
heteroantiserum to either κ or λ light chain. The optimum concentra-
tion for each lot of PNA, generally in the range of 60 to 125 µg/mL,
was determined by dilution against peripheral blood monocytes, which
avidly bind PNA.17 Cells were then washed three times in ice-cold
phosphate-buffered saline (PBS) and incubated with 5 µL of each
secondary staining reagent, consisting of either avidin-conjugated
Texas Red (Molecular Probes, Junction City, OR) or phycoerythrin-
avidin (Becton Dickinson) and fluoresceinated goat antibody to
rabbit IgG (Cappel Laboratories, West Chester, PA). All reagents
were used at saturating concentrations, and staining was performed
at 4°C. The stained cells were washed in PBS, fixed in 1% para-
formaldehyde, and stored at 4°C before analysis. Cells stained with
Texas Red-avidin or phycoerythrin-avidin alone comprised the red
fluorescence control. Binding of PNA could be blocked by adding
d-galactose to the staining reagents, as previously demonstrated.13
Positive red fluorescent staining of B cells could be demonstrated
with phycoerythrin-conjugated antibody to HLA-DR (Becton
Dickinson).

Two-color flow-cytometric analysis was performed on either a
FACS analyzer or FACS 440 (Becton Dickinson). The FACS
analyzer, which uses a mercury arc lamp as a light source, was used
with appropriate combinations of filters to measure emission from
fluorescein and phycoerythrin simultaneously. The FACS 440,
equipped with argon and krypton lasers, could also be used with
appropriate combinations of filters to detect fluorescein and Texas
Red. Gates were selected by using either forward-angle light scatter
on the FACS 440 or electronic volume on the FACS analyzer; data
from 10,000 to 20,000 cells were included in each analysis. Elec-
tronic compensation was used to remove background green fluores-
cence from the red fluorescence channel when fluorescein and phycoerythrin were used in combination. Data analysis was per-
formed with Consort 30 and Consort 40 hardware and software
(Becton Dickinson) for the FACS analyzer and FACS 440, respec-
tively. Data were usually collected from the two fluorescence chan-
nels as dual-parameter correlated data. Two-color fluorescence data
were displayed as log green fluorescence in the x axis, log red fluores-
cence (phycoerythrin or Texas Red) in the y axis, and cell
number in the z axis, with contour lines drawn to depict regions of
equal cell number. PNA binding to lymphoma cells could be
detected by examination of the red fluorescence of light chain-
positive cells (stained with fluoresceinated antibody to the appropri-
ate light chain as determined by κ-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 lympho-
mas, 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

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Table 1. PNA Binding by non-Hodgkin's Lymphomas

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>PNA+/Total (%+)</th>
</tr>
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<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.

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.).
CIRCULATING MALIGNANT CELLS IN LYMPHOMA

Table 2. Peripheral Blood Involvement in PNA+ and PNA− B Cell Lymphomas

<table>
<thead>
<tr>
<th>Tissue PNA</th>
<th>Circulating Lymphoma Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+ (%)</td>
</tr>
<tr>
<td>+</td>
<td>3 (18.8%)</td>
</tr>
<tr>
<td>−</td>
<td>17 (77.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>20 (52.6%)</td>
</tr>
</tbody>
</table>

* Determined by κ-λ analysis.
† Groups significantly different by chi-square analysis (P < .005).

Table 3. Peripheral Blood Involvement in PNA+ and PNA− Lymphomas According to Histologic Type

<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>0/2</td>
</tr>
<tr>
<td>DUL</td>
<td>0/1</td>
</tr>
<tr>
<td>ILL</td>
<td>0/1</td>
</tr>
<tr>
<td>WDLL</td>
<td>0/1</td>
</tr>
<tr>
<td>CLL</td>
<td>0/1</td>
</tr>
<tr>
<td>LSCL †</td>
<td>0/1</td>
</tr>
<tr>
<td>HCL ‡</td>
<td>0/1</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.

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 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

Peripheral blood involvement. Thirty-eight cases of B-cell lymphoma (confirmed by phenotyping) were studied for peripheral blood involvement, including 16 PNA+ cases and 22 PNA− cases. Most of these cases were studied at the time of initial diagnosis in untreated patients. The overall incidence of circulating clonal B cells, determined by κ-λ analysis, was 52%, consistent with the incidence we have seen in previous studies. Only 3 of 16 PNA+ cases had detectable circulating lymphoma cells, whereas 17 of 22 PNA− cases had peripheral blood involvement based on κ-λ analysis (Table 2). The difference between the PNA+ and PNA− groups was significant by chi-square analysis even if the two
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. More sensitive flow-cytometric methods applied to detection of clonal B cells in blood showed that many more lymphoma patients have circulating tumor cells than can be appreciated by morphology alone. 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. Using Southern blot techniques to detect clonal immunoglobulin gene rearrangements, we confirmed the ability of the flow-cytometric method (λ-λ analysis) to detect clonal B cells and showed that the circulating cells are clonally identical to the malignant cells in involved tissues. 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. Although the mechanism of entry of circulating lymphocytes into lymphoid tissues appears to involve receptor-mediated binding to HEV, 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. PNA strongly binds to immature T cells in the thymic...
circumstance, lymph node germinal center cells, and bone marrow pre-B cells—cell types not usually present in the peripheral blood. Peripheral blood lymphocytes, on the other hand, bind the lectin only weakly. PNA has greatest affinity for the disaccharide Galβ (1→3) GalNAcα 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. 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. 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, 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 Wilshaw 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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