Follicular Non-Hodgkin’s Lymphoma Cell Adhesion to Normal Germinal Centers and Neoplastic Follicles Involves Very Late Antigen-4 and Vascular Cell Adhesion Molecule-1

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Follicular lymphomas recapitulate the architecture of germinal centers (GCs) of normal secondary lymphoid follicles. Using an in vitro binding assay, it has recently been demonstrated that the normal B lymphocytes bind to GCs. This interaction is mediated by a receptor-ligand pair consisting of the β, integrin very late antigen 4 (VLA-4) on the B cell, and the vascular cell adhesion molecule-1 (VCAM-1) expressed on follicular dendritic cells (FDC). Considering the similarities between follicular lymphomas and normal GCs, the adhesive interaction of follicular non-Hodgkin’s lymphoma (NHL) cells and GCs was examined. Cells isolated from 16 of 24 cases of follicular NHL bound to normal GCs. Neoplastic follicles could similarly support the binding of follicular NHL cells. This adhesion was inhibited by monoclonal antibodies (MoAbs) directed against VLA-4 and VCAM-1. This supports the hypothesis that the neoplastic follicles used the identical adhesive interactions responsible, at least in part, for the localization of normal B cells to GCs. Adhesion receptors have an important role in the regulation of normal lymphoid cell proliferation, differentiation, and localization. Therefore, an understanding of the adhesive interaction of follicular NHL cells with GCs may provide insight into the clinical and biologic behavior of these diseases.

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THE GERMINAL CENTER (GC) of the secondary lymphoid follicle is a specialized microenvironment that plays a central role in the regulation of B-cell proliferation and differentiation, as well as the generation of secondary immune responses. Interactions between B cells and the other cellular components present in the GC are critical to GC function. Experimental studies in murine systems support the hypothesis that following entry into secondary lymphoid tissues, B cells localize in GCs. To examine this mechanism, we have recently developed an in vitro frozen-section binding assay, which demonstrated that activated human B cells preferentially adhered to GCs. By antibody-blocking studies, this interaction was shown to be mediated by the receptor-ligand pair very late antigen-4 (VLA-4):vascular cell adhesion molecule-1 (VCAM-1), with the integrin VLA-4 on the lymphoid cell and VCAM-1 on the follicular dendritic cell (FDC). Other adhesion receptors, including lymphocyte function-associated antigen-1 (LFA-1), ICAM-1, CD44, and lymphocyte adhesion molecule-1 (LAM-1), were not involved in GC binding in studies using this binding assay. However, it has been suggested that clustering of isolated FDCs and B cells involves an LFA-1/ICAM-1 interaction.

Cell surface molecules, which mediate adhesion of lymphoid cells to other cells or extracellular matrix components, appear to be important for the normal function and differentiation of lymphoid cells. Receptor-ligand pairs, such as VLA-4:VCAM-1 on B cells and FDCs, respectively, are therefore likely to be important in normal GC function.

Follicular non-Hodgkin’s lymphomas (NHLs) are thought to be neoplastic counterparts of normal GC B cells. This is based on both morphologic and phenotypic analysis. The architecture of these tumors recapitulates the structure and cellular composition of normal secondary follicles, as evidenced by the presence of FDCs and CD4+ T cells. Moreover, the neoplastic cells have a cell surface phenotype similar to normal follicular center B cells by their expression of receptors for complement cleavage fragments, CD10, and adhesion molecules such as LFA-1 (CD11a/18), with variable expression of surface immunoglobulin (Ig). Aside from these structural similarities, it also has been suggested that these tumors cells functionally resemble normal GC B cells. Follicular NHLs are “monoclonal,” which is analogous to the B cells in normal GCs, which can be monoclonal or oligoclonal. Follicular NHL cells have been shown to undergo somatic mutation in rearranged Ig genes, a process hypothesized to occur during the differentiation of normal GC B cells.

It is presently unknown what drives follicular NHLs to recapitulate the structure of normal GCs. One possible explanation would be that neoplastic B cells use a similar mechanism of localization and adhesion as normal B cells in their binding to GCs. In the present study, we have examined the mechanism of binding of follicular NHL cells to normal and neoplastic GCs. An understanding of those molecules that mediate neoplastic GC adhesion may provide insight into both the biologic and clinical behavior of these diseases.

MATERIALS AND METHODS

Monoclonal antibodies. The 4B4 (IgG1) and L25 (IgG1) monoclonal antibodies (MoAbs) reactive with the VLA-4 (CD29) and VLA-4a (CD49d) chains, respectively, and MoAbs directed against CD4 (IgG2a, 19Thy5D7), CD8 (IgG2a, 7Pt3F9), CD14 (IgG1, 4B4), CD11a (IgG1, 440D), CD11b (IgG1, 480D), CD14 (IgG1, 13-2), CD19 (IgG1, 302), CD20 (IgG1, 2H7), and CD21 (IgG1, 8B11) were obtained from the American Type Culture Collection. The 4B4 (IgG1) and L25 (IgG1) monoclonal antibodies (MoAbs) reactive with the VLA-4 (CD29) and VLA-4a (CD49d) chains, respectively, and MoAbs directed against CD4 (IgG2a, 19Thy5D7), CD8 (IgG2a, 7Pt3F9), CD14 (IgG1, 4B4), CD11a (IgG1, 440D), CD11b (IgG1, 480D), CD14 (IgG1, 13-2), CD19 (IgG1, 302), CD20 (IgG1, 2H7), and CD21 (IgG1, 8B11) were obtained from the American Type Culture Collection.
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(IgG1, MY4), CD19 (IgG1, B4), CD20 (IgG2a, B1), CD56 (IgG1, NKH1), and human Ig-k and -a light chains have been previously described. The anti–VCAM-1 MoAb 4B9 (IgG1) was provided by Drs Tim Carlos and John Harlan (University of Washington, Seattle). All MoAbs were used as ascites preparations at saturating binding concentrations.

Patient and cell samples. Mononuclear cells were isolated from single-cell suspensions of lymph nodes from patients with follicular small cleaved cell and follicular mixed small cleaved and large cell NHL (Working Formulation A and B), as determined by conventional histology. All tumors were of B-cell origin by their expression of CD19, CD20, HLA-DR, and monoclonal Ig light chain. Cells were cryopreserved and maintained in the vapor phase of liquid nitrogen until use. Before use in binding studies, cells were treated with anti-CD4, -CD8, -CD14, and -CD56 MoAbs and goat anti-mouse Ig coupled to magnetic beads (Dynal, Gaithersburg, MD), to deplete T cells, natural killer (NK) cells, and monocytes as previously described. Tonsils were obtained from routine elective tonsillectomies. Splenic B cells were prepared and cultured for 3 days with Staphylococcus aureus Cowan strain I (SAC; Calbiochem, La Jolla, CA) as previously described. All tissue was obtained according to appropriate Human Protection Committee validation and informed consent.

Frozen-section binding assay. The in vitro frozen-section binding assay was performed using tissue sections from normal human tonsil and specimens of lymph nodes from patients with histologically and immunohistochemical diagnosis of follicular small cleaved cell and follicular mixed small cleaved and large cell NHL. Nalm-6 cells, RH-L4 cells, normal SAC-stimulated splenic B cells, or cells isolated from patients with follicular small cleaved cell or follicular mixed small cleaved and large cell NHL were cultured for 30 minutes at 37°C in a 1:200 dilution of sulfofluorescein diacetate (SFDA; Molecular Probes, Eugene, OR) (stock solution 5 mg/mL in dimethylsulfoxide) in phosphate-buffered saline (PBS). Cells were washed twice with RPMI-1640 containing 25 mmol/L HEPES buffer and 5% fetal calf serum (FCS), and resuspended at 3 × 10^7/mL. Immediately before the assay, clumps of cells were removed by passing the cell suspension through nylon mesh. One hundred microliters of the cell suspension was placed onto two 8-μm frozen sections of tonsil within a 2.2-cm diameter circle delineated by a ring of 12 mol/L dimethylpolysiloxane, rotating at 70 rpm for 25 minutes at 25°C. After incubation, slides were fixed in 3% glutaraldehyde (Polysciences, Warrington, PA) in PBS overnight at 4°C. Sections were rinsed and stained with hematoxylin. Slides were then independently examined blindly under brightfield and fluorescent illumination. GC binding of cells was qualitatively scored: 3+, extensive binding to GCs (Fig 1A); 2+, intermediate binding to GCs (Fig 1B); 1+, limited binding to GCs (Fig 1C); 0, rare cells or no bound to GCs (Fig 1D). Effect of antibody treatment of Nalm-6 or NHL cells and tissue sections on GC binding was performed as previously described. Cells were incubated in media alone or media containing MoAb L25, or the negative control MoAb B4 at saturating binding concentrations for 20 minutes at 25°C. Cells were then washed and resuspended in fresh media before application to tissue sections. Tissue sections were similarly treated with MoAbs 4B9 or the negative control MoAbB4 in PBS for 20 minutes at 25°C; then washed in PBS before the application of Nalm-6 cells.

Indirect immunofluorescence and flow cytometric analysis. Cells were examined for the expression of CD20 antigen, Ig-k or -a light chain isotype, and VLA-4 α and β chains by incubation with MoAb, as well as isotype-identical nonreactive monoclonal mouse IgG or IgM followed by fluoresceinlabeled (FITC) goat anti-mouse IgG and IgM (Tago, Burlingame, CA) as previously described. Cells were then analyzed on an EPICS C (Coulter Electronics, Hialeah, FL) flow cytometer.

**RESULTS**

Follicular NHL cells bind to normal GCs. Follicular lymphoma cells were in vitro frozen-section binding assay. As seen in Table 1, cells isolated from 24 cases of follicular NHL were studied. These cases all consisted of a monoclonal population of B cells, with greater than 90% of cells expressing a single Ig light chain isotype. As a positive control, the pre-B cell line Nalm-6 demonstrated a high level of GC binding (Fig 1A). The binding of Nalm-6 was more to the light zone of the GCs (unpublished observations) where more FDCs are present. As previously shown, normal stimulated B cells also demonstrated a high level of GC binding. The low-grade B cell NHL line RH-L4 gave an intermediate level of binding. Heterogeneous binding of the 14 follicular lymphomas to normal GCs was observed. In six cases, an intermediate level of GC binding was seen (Fig 1B), and 10 cases demonstrated a low level of GC binding (Fig 1C). In contrast, the remaining eight cases demonstrated essentially no cells adhering to GCs (Fig 1D). We also examined eight cases of diffuse lymphomas (diffuse small cleaved and diffuse large cell) and observed that five cases bound to GCs, two at an intermediate level and three at a low level.

Since previous studies support the notion that VLA-4 on the lymphoid cells and VCAM-1 on FDCs are a receptor-ligand pair involved in B cell-GC adhesion, the effect of anti–VLA-4α MoAb on binding of follicular NHL cells to normal GCs was examined. As seen in Table 1, GC adhesion of follicular NHL cells preincubated with anti-VLA-4α MoAb was significantly inhibited, with only rare cells binding to GCs. This was to a similar degree to that seen in the six cases of follicular NHL (Fig 1D). Conversely, treatment of the tonsil sections with anti–VCAM-1 MoAb gave near complete inhibition of binding of follicular NHL cells.
Lymphocyte binding assay on serial sections of a single human tonsillar lymphoid follicle. The GC occupies most of each panel. Nalm-6 cells and cells isolated from four representative cases of follicular NHL were internally labeled with the fluorochrome SFDA then used in a frozen-section binding assay. Slides were then examined under fluorescent illumination. (A) 3+, extensive GC binding of Nalm-6 cells; (B) 2+, intermediate GC binding of NHL cells; (C) 1+, low level GC binding of NHL cells; (D) 0, rare GC binding of NHL cells.

Cells to normal GCs. To serve as controls in these studies, the binding of the cell lines Nalm-6 and RH-L4, as well as normal stimulated B cell, was inhibited by anti-VLA-4α and anti-VCAM-1 MoAbs.

To investigate whether the variability in binding of follicular NHL cells to GCs from different patients was related to VLA-4 expression, cells were examined by indirect immunofluorescence and flow cytometric analysis. As shown in Fig 2, three representative cases of follicular lymphoma (Fig 2A to C) demonstrated low levels of VLA-4 expression and Nalm-6 is presented as a positive control (Fig 2D). Although the expression of VLA-4 on these three lymphomas was nearly identical, their GC binding significantly differed, since case A exhibited intermediate binding, case B had a low level of binding, and case C did not bind to any significant degree. These observations were seen in the remaining 11 cases. Therefore, for the follicular NHLs, there was no relationship between the extent of GC binding and intensity of VLA-4 expression. This is reminiscent of the previous observation that VLA-4 expression on B cell lines did not correlate with degree of GC binding.41

**Follicular NHL cells bind to neoplastic GCs.** Considering that GCs in normal lymphoid tissues support the binding of normal B cells and follicular lymphoma cells, neoplastic GCs were next examined to determine whether they could also support this adhesion. As a positive control, the binding of Nalm-6 to lymph node specimens from 16 patients with follicular NHL was studied. Immunohisto-
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Fig 2. Fluorescence-activated cell sorter histograms of the expression of VLA-4b (CD29) and VLA-4a (CD49d) on cells from three representative cases of follicular NHL (A, B, C) and Nalm-6 cells. Negative control was obtained using an isotype-identical irrelevant MoAb, developed with goat anti-mouse Ig FITC.

chemistry using DRC-1 demonstrated the presence of FDCs in all cases, and these cells expressed VCAM-1 as shown by staining with MoAb 4B9. Of the 16 lymphoma specimens examined, Nalm-6 binding to follicles was seen in all but three cases, generally of an intermediate level (Table 2). Similar to the observed inhibition of binding of Nalm-6 cells to normal tonsil, MoAbs to VLA-4a and VCAM-1 blocked the adhesion of Nalm-6 to neoplastic follicles.

Eight cases of follicular NHL, which had been previously shown to bind to normal tonsil GC, were examined for binding to tissue sections of follicular NHL. Table 3 depicts the binding of eight cases of follicular lymphoma to a panel of tissue sections obtained from four patients with follicular lymphoma. Two cases (no. 1 and 2) demonstrated an intermediate level of binding, whereas a low level was seen in four cases (no. 3 through 6). Rare binding was noted in the remaining two cases (no. 7 and 8). When blocking studies were performed, with anti–VLA-4a and anti–VCAM-1 MoAbs, the binding of follicular lymphoma cells to malignant follicles was inhibited in all cases.

**DISCUSSION**

In the present study, we have observed that follicular lymphoma cells bind to normal and neoplastic GCs in a frozen-section tissue binding assay. We have previously shown that binding of normal B cells and B-cell lines to GCs is mediated by the receptor-ligand pair VLA-4:VCAM-1.

**Table 2. Binding of Nalm-6 to Neoplastic GCs**

<table>
<thead>
<tr>
<th>No. of Cases</th>
<th>Relative GC Binding Following Cell/Tissue Treatment*</th>
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<tr>
<td>Case No.</td>
<td>Control*</td>
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Abbreviation: ND, not determined.

*Treatment of cells and tissue sections with media containing isotype-identical irrelevant MoAb.

Table 3. Binding of Follicular NHLs to Neoplastic GCs

<table>
<thead>
<tr>
<th>NHL Case No.</th>
<th>No of Tissue Specimens</th>
<th>GC Binding Following Cell/Tissue Treatment*</th>
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<tbody>
<tr>
<td></td>
<td>Control (no. of tissue specimens with cells bound)</td>
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<tr>
<td></td>
<td>Anti-VLA-4a</td>
<td>Anti–VCAM-1</td>
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Abbreviation: ND, not determined.

*Relative GC binding.

We now demonstrate that neoplastic lymphoid cells use the identical adhesive interaction with FDCs within normal and neoplastic GCs. Heterogeneity of GC binding among the NHLs was observed, although this did not correlate with the intensity of VLA-4 expression. These results may provide a mechanism which may explain how follicular lymphomas recapitulate the architecture of normal secondary lymphoid follicles.

Cell surface molecules, which mediate the adhesion of lymphoid cells with other cells or extracellular matrix components, have been shown to be central to the generation of the immune response. These adhesion receptors are involved in the transduction of signals to normal lymphoid cells, which influence their activation and proliferation. Specifically, the interaction of VLA-4 on T lymphocytes with its ligands upregulates proliferation. To date, the function of VLA-4 on normal B cells is unknown, although B-cell activation may be similarly upregulated via VLA-4. Within the GC, VLA-4:VCAM-1 mediates at least one of the important interactions between normal B cells and FDCs. It is likely that this receptor-ligand pair plays a role in the growth and differentiation of normal GC B cells. Therefore, a deregulated or dysfunctional VLA-4:VCAM-1 interaction in follicular NHL may be similarly important to the proliferation of the neoplastic cells.

VLA-4, other β integrins, and the β integrin LFA-1 have been shown to have increased affinity for their respective ligands following cellular activation, without a change in antigen density. Previous studies demonstrated that activated, but not unstimulated, B cells preferentially bind to GCs, and preliminary studies suggest that activation of B-cell lines with several mitogens can dramatically increase GC binding without changing the expression or structure of VLA-4 (unpublished observations). In addition, B-cell lines with similar amounts of cell surface VLA-4 have different in vitro GC binding characteristics. In the present study, the level of expression of VLA-4 and the degree of GC binding of follicular NHLs was less than the Nalm-6 cell line. The lower affinity of follicular NHL cells for FDCs may be related to a low-level expression of VLA-4 on the tumor cells, but may also be due to a difference in its affinity for VCAM-1. The differences in GC adhesion in vitro may also reflect the heterogeneity of the population of NHLs.
malignant cells isolated from individual nodes. The lack of binding of some cases may be due to the likelihood that tissue processing, cryopreservation, and other technical factors may not maintain cells exactly as they are in vivo. It is also possible that, although in vivo a VLA-4:VCAM-1 interaction may be present in the lymphomas, the in vitro frozen-section assay itself may only select for a certain level of intensity of the interaction.

Follicular NHLs are characteristically diseases that are widely disseminated at diagnosis with infiltration of peripheral and mesenteric lymphoid tissue, and bone marrow, as well as involvement of peripheral blood. The hypothesis that follicular NHLs are the neoplastic counterparts of normal GC B cells may also explain the patterns of spread seen clinically. A subset of normal GC B cells leave the GC microenvironment to become terminally differentiated B cells in bone marrow, circulating memory B cells, or mucosal-associated plasma cells. Follicular lymphomas reflect the migration patterns of normal GC cells, with some neoplastic cells remaining in follicles and others having the capacity to migrate to the bone marrow, gut-associated lymphoid tissue, and mesenteric nodes, the commonly seen sites of involvement with these diseases.

Neoplastic and normal GCs are composed of similar types of nonneoplastic cells. In particular, the FDCs in follicular NHL are reported to be phenotypically similar to normal GC FDCs. The finding that Nalm-6 cells did not bind to all cases of follicular NHL tissue and generally bound less intensely than to normal GC suggests that the FDCs in neoplastic GCs may be functionally different or less numerous. Although VCAM-1 was expressed in the neoplastic GCs, the ability of it to function as a ligand for VLA-4 may be abnormal. VCAM-1 is present on FDCs. The alternative forms of VCAM-1 may account for differences in binding to normal and neoplastic follicles. Alternatively, analogous to CD2 where activation-associated epitopes are associated with different functions, an altered conformation of VCAM-1 may explain the binding properties of normal and neoplastic GCs.

Adhesion molecules are hypothesized to be important in tumor invasion and metastasis. Alterations in integrin expression and structure occur in neoplastic transformation and have been associated with increased as well as decreased adhesion to their ligands. The disseminated nature of follicular NHLs may reflect the possibility that the adhesive interactions, including VLA-4 with VCAM-1, are aberrant, and therefore a lower adhesive capacity may explain their tendency to disseminate. In addition, the wide expression of VCAM-1 on FDCs in multiple secondary lymphoid tissues including spleen and gut-associated lymphoid tissue may contribute to the tropism for these sites seen in follicular NHL. The observation that some diffuse NHL cells bound to GCs may indicate that the FDCs, rather than the follicular lymphoma cells, are determining and directing the architecture of the tumor. Moreover, de novo appearance of numerous FDCs are reported to be present in the paratrabecular bone marrow infiltrates seen in follicular NHL. This suggests that cells of two different lineages, the lymphoid neoplastic B cells and the nonlymphoid FDCs, are proliferating in this neoplasm. The cellular interactions between follicular NHL cells and their microenvironment may not only be of biologic significance, but may also be of prognostic importance. The localization of follicular NHL B cells and their ability to transform to a more aggressive diffuse NHL may be affected by these cellular interactions. Future studies may provide novel approaches with which to consider the regulation of growth and dissemination of these cells in vivo and thereby suggest new therapeutic approaches.

ACKNOWLEDGMENT

The authors thank John Daley for technical assistance and Kerstin Hildebrandt for preparation of the manuscript. We also thank Drs Carl O'Hara, Geraldine Pinkus, and Nancy Harris for frozen specimens of lymphoma tissue.

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