The Role of Hyaluronan and Interleukin 8 in the Migration of Chronic Lymphocytic Leukemia Cells within Lymphoreticular Tissues

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ABSTRACT

Malignant lymphocyte migration into and within lymphoreticular tissue is an important aspect of chronic lymphocytic leukemia (CLL), yet little is known about the processes involved. Our previous studies of integrin expression and function in CLL have shown that the abnormal cells are relatively nonadhesive and nonmotile on the protein ligands of these receptors. Here we show that CLL cells adhere to a non-protein ligand, hyaluronan (HA), and become motile (as assessed by both Boyden chamber migration and time-lapse video microscopy) on this ligand when stimulated with interleukin (IL) 8. The combined presence of HA and IL-8 was essential for this motility because IL-8 did not stimulate movement on other surfaces. Blocking antibodies showed that this motility is mediated by the receptor for HA-mediated motility (RHAMM), without the involvement of CD44. Moreover, confocal microscopy showed a polarized distribution of RHAMM and F-actin, but not CD44, in cells which had become motile on HA in the presence of IL-8. Immunohistochemical studies of nodes and spleen demonstrated an abundant reticular network of HA-containing fibers throughout diseased nodes and in splenic white pulp. The splenic red pulp and the luminal surface of high endothelial venules lacked HA. IL-8 was ubiquitously present in these tissues. CLL cells were shown to move spontaneously on fibroblast monolayers derived from lymphoid tissue; this movement was largely blocked by hyaluronidase or anti-RHAMM or anti-IL-8 antibodies. These studies indicate that IL-8-induced motility on HA is likely to be important for CLL cell migration through lymphoid tissue.

INTRODUCTION

Malignant cell migration is a central aspect of the pathophysiology of chronic lymphoid leukemias. For example, this process determines the patterns and extent of organ involvement, which is known to be prognostically important (1), and also delivers lymphocytes to sites (e.g., proliferation centers in CLL) favoring growth and survival (2). Therefore, there is even the prospect that interfering with cell migration might have therapeutic potential by influencing the survival of malignant cells at such sites. This study presents data that are likely to be relevant to the migration of CLL cells within lymph nodes and the white pulp of the spleen, organs that are frequently infiltrated in this chronic but incurable B-cell leukemia.

We have already studied in some detail the receptors and functional responses potentially involved in the tissue migration of the malignant B cells of CLL and compared the findings with those in HC leukemia, in which the tissue distribution of malignant cells is very different (3–5). The most prominent finding of these studies was that whereas the HCs of HC leukemia show pronounced motility on ligands such as vitronectin (6), CLL cells were nonmotile on all surfaces tested (7). We related this difference in cell behavior to the markedly lower expression of different integrin receptors on CLL cells as compared with HCs. It therefore became clear that non-integrin receptors and adhesive ligands other than ECM proteins have to be considered in studies of the mechanisms of CLL cell tissue migration. Moreover, a second pronounced difference between CLL cells and HCs was that HCs moved spontaneously and/or became stimulated to move on certain surfaces by integrin engagement. In contrast, the stimuli required for inducing CLL cell motility remains unknown.

Regarding non-integrin adhesion receptors and their ligands, we concentrated on HA and its cellular receptors because this GAG is widely distributed in tissues (8) and because preplasma cells become motile on adhesion to a surface coated with this ligand (9).

In general, cell motility is regulated by the coordinated actions of adhesion receptors and cytokines, especially chemokines. Here we concentrate on IL-8 because: (a) CLL cells constitutively produce this chemokine (but not a range of other chemokines; Ref, 10), (b) IL-8 induces the motility of a range of other cell types (11, 12), and (c) previous studies of IL-8 in CLL have been confined to its ant apoptotic effect (13). This member of the C-X-C chemokine family (14) therefore seemed to be a potential mediator of CLL cell motility, especially because IL-8 has recently been shown to induce movement in some nonmalignant B cells (15, 16). In the present study, we therefore focused on IL-8 and compared its effects with those of MIP-1α, a C-C family chemokine reported to stimulate normal B-cell motility (17).

We show here that in the presence of IL-8 but not MIP-1α, CLL cells become motile on HA and that this motility is mediated by the RHAMM. Furthermore, we present tissue staining and functional data suggesting that this HA/IL-8-induced motility may be of pathophysiological importance in CLL.

MATERIALS AND METHODS

Patients. Peripheral blood from six cases of CLL was studied with informed consent. All of the malignant lymphocytes were morphologically typical and expressed low-density, light chain-restricted, surface immunoglobulin, together with CD5 and CD23.

Three of the cases had prominent lymph node enlargement (two of these cases also had splenomegaly), whereas the other three cases had no organomegaly. The numbers (n) quoted in Results refer to the number of cases studied.

Cell Preparation and Culture

CLL Cells. CLL cells were isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation. Highly pure CLL B cells (CD19 > 95%, CD3 and CD14 < 1%) were obtained by removing T lymphocytes and monocytes with magnetic beads coated with CD3 or CD14 (Miltenyi Biotec, Camberley, United Kingdom).

Fibroblasts and Endothelial Cells. Lymphoreticular fibroblasts were isolated from reactive tonsils (tonsillectomacy material) or normal lymph nodes (see below); cultured in Iscove’s modified MEM (Life Technologies, Inc., Paisley, United Kingdom) containing 20% FCS (Advanced Protein Products, Brierley Hill, United Kingdom), 10 ng/ml fibroblast growth factor (Fred Baker, Run- corn, United Kingdom), 100 units of penicillin/streptomycin, and 100 μg/ml l-glutamine (all from Life Technologies, Inc.); and incubated at 37°C with 5%
CO, in air. A microvascular endothelial cell line (HMEC-1) was also used (18). Cells were cultured at 37°C (5% CO2 and air) in endothelial basal medium (MCDB-131; Life Technologies, Inc.) containing 10 ng/ml endothelial cell growth factor (Fred Baker), 1 μg/ml hydrocortisone (Life Technologies, Inc.), 10% FCS (Advanced Protein Products), 100 units of penicillin/streptomycin, and 100 μg/ml l-glutamine (all from Life Technologies, Inc.).

**Tissues.** CLL nodes (n = 4) were diagnostic samples; splenectomy material (n = 2) had been removed for treatment of autoimmune hemolytic anemia.

Normal nodes (n = 3) were obtained from axillary clearances for breast cancer and were macroscopically and microscopically normal. “Normal” spleen was tissue removed for the treatment of thrombocytopenic purpura cancer and were macroscopically and microscopically normal. “Normal” splenic (n = 2) or because of surgical trauma during laparotomy (n = 2).

Most tissues were formalin-fixed and paraffin-embedded. CLL spleen (n = 1) and normal node (n = 1) tissues were also fixed in acid-formalin/ethanol, a method reported to allow optimal visualization of HA (19). Similar staining was observed with both fixation methods.

**mAbs and Their Detection**

**Antibodies.** The mAbs described below were used against the following potential HA receptors: (a) RHAMM [3T3.5; a blocking antibody that was a gift from Dr. E. Turley (Hospital for Sick Children, Toronto, Canada)]; (b) CD44 [50B4 (a blocking mAb that was a gift from Dr. M. Letarte; Hospital for Sick Children, Toronto, Canada) and Leu-44 (a nonblocking reagent from Becton Dickinson, Oxford, United Kingdom)]; (c) CD38 [a blocking antibody from R&D Systems (Abingdon, United Kingdom) and Leu-54 (a nonblocking mAb from Becton Dickinson)]; and (d) CD38 (Leu-17, a nonblocking mAb from Becton Dickinson). mAbs against chemokine receptors IL-8RA, IL-8RB, and MIP-1α (all from Pharmingen, Oxford, United Kingdom) and a blocking mAb to IL-8 (R&D Systems) were also used. All of these antibodies are of the IgG1 isotype; therefore, nonimmune mouse IgG1 was used to control for nonspecific effects.

**FACS.** The mAb staining of cells in suspension was detected by an indirect technique using GAM immunoglobulin-FITC (Becton Dickinson) as a second layer. Both first and second layer antibodies were used at saturating concentrations. Cells were analyzed on a FACSscan using Lysis II software (Becton Dickinson) to generate histograms giving the percentage of positive cells as compared with class-specific controls, together with the mean fluorescence intensity.

**Tissue Staining**

HA was detected in lymphoid tissue sections with HRP-conjugated HABP (a kind gift from Chugai Biopharmaceuticals, San Diego, CA) using the method of Ichiada et al. (20). After clearing with xylene, slides were treated with 3% H2O2 and blocked with 10 mg/ml BSA. Slides were incubated with HABP-HRP (2–20 μg/ml; maximal staining with >10 μg/ml) for 30 min and then incubated with diaminobenzidine substrate (0.5 mg/ml diaminobenzidine and 1 μg/ml H2O2; Sigma, Poole, United Kingdom) for 20 min. Slides were finally counterstained with hematoxylin. In addition, to ensure the specificity of staining, some sections were digested for 30 min at 37°C with Streptomyces hyaluronidase (50 turbidity-reducing units/ml in PBS; Calbiochem, Nottingham, United Kingdom) before staining for HA.

IL-8 was detected by the method of Brew et al. (21). Briefly, after clearing and rehydration, slides were boiled in 10 mM sodium citrate buffer (pH 6) for 10 min and blocked with 10 ng/ml BSA before overnight incubation with an anti-IL-8 mAb (25 mg/ml). Sections were incubated with GAM-biotin (Zymed, San Francisco, CA) and then incubated with ExtrAvidin alkaline phosphatase (Sigma) before exposure to substrate (Fast Red/Naphthol AS MX phosphate levamisole; Sigma). Slides were counterstained with hematoxylin.

**Chemotaxis/Chemokinesis**

A modified Boyden chamber method was used. Briefly, nitrocellulose filters were soaked overnight in either 20 μg/ml FN (Sigma) or 100 μg/ml HA (Pharmacia, Uppsala, Sweden) and washed. IL-8 at 0, 0.5, 5, and 50 ng/ml or MIP-1α at 0, 10, 100, and 1000 pg/ml (both chemokines were from R&D Systems) was added to the bottom wells. Purified CLL cells were then added to the top wells, together with IL-8 or MIP-1α (using the concentrations described above) to form a checkerboard of concentrations; each combination of concentrations was set up in triplicate wells. The chamber was then incubated for 2 h. The filter was removed, fixed with formaldehyde, and stained with Mayer’s hematoxylin before clearing with xylene. The movement of the leading front cells was then measured using a calibrated microscope. Six different fields were examined for each well, so that each figure given in the “Results” represents the mean ± SE of 18 readings. To ensure reproducibility of the method in a given case, the checkerboard analysis with IL-8- and HA-coated filters was performed on two separate occasions in three patients, and very similar results were obtained.

Checkerboard analysis allows differentiation between chemotaxis and chemokinesis; chemotaxis is indicated by movement toward a higher concentration of chemokine in the lower well; other movement indicates that chemokinesis has taken place. Statistical analysis of the effects of IL-8 on CLL cell movement was performed with Wilcoxon ranks analysis.

To establish the receptors responsible for cell motility, CLL cells were incubated with mAbs to RHAMM (10 μg/ml), CD44 (20 μg/ml), and CD54 (40 μg/ml) and an IgG1 control (40 μg/ml) for 30 min on ice. Cells were then added to HA-coated filters, and the chemokinesis assay was performed as described before. In addition, to ensure that the observed effect was due to HA, HA-coated filters were preincubated with hyaluronidase (50 turbidity-reducing units) for 30 min at 37°C before performing the chemotaxis assay as above.

**Time-Lapse Video Microscopy**

Petri dishes were coated overnight with either HA (100 μg/ml) or FN (20 μg/ml) and washed. Purified CLL cells were added to these dishes and allowed to adhere for 5 min before adding either IL-8 (5 ng/ml, a concentration that usually produced maximal movement in the Boyden chambers) or MIP-1α (100 pg/ml; Ref. 17). The cells were then placed on a heated (37°C) microscope stage and filmed for 2 h using time-lapse video. The movement of cells over 30 min was determined by sequential tracing of cell outlines on the video screen. A cell was considered to have moved when its position had changed by more than one cell diameter. This methodology allows the percentage and velocity of motile cells to be calculated. For the velocity calculations, the CLL cells were assumed to be 8 μm in diameter. To test the reproducibility of the method, three cases were studied on two separate occasions for movement on HA ± IL-8, and very similar results were obtained.

The effect of the HA receptor blockade was tested with relevant mAbs as described in the chemotaxis assay. HA was again removed with hyaluronidase as described above.

**Confocal Microscopy**

Briefly, glass coverslips were coated overnight with HA (100 μg/ml) and then placed in the wells of a 24-well plate. Purified CLL cells were added to these coverslips and then incubated in the presence or absence of IL-8 for 1, 5, and 10 min. The cells were then fixed with glutaraldehyde, and the coverslips were washed thoroughly and allowed to dry. Cells were then stained with anti-RHAMM and anti-CD44 mAbs, followed by GAM-rhodamine (Molecular Probes, Leiden, Holland) as a second layer. For F-actin, cells were stained with rhodamine-phalloidin (Molecular Probes). The location of HA receptors and polymerized actin was then analyzed using a Microvidrace confocal microscope (Bio-Rad, Hemel Hempstead, United Kingdom).

**Transendothelial Migration**

A method measuring cell migration from the upper chamber to the lower chamber of transwell plates (Costar, Cambridge, MA) was used. In brief, HMEC-1 endothelial cells were grown to confluence on the polycarbonate membranes (pore size, 5 μm) separating the upper and lower chambers. Purified CLL cells (2 × 106) were added to the upper chamber, whereas IL-8 (5 ng/ml) was added to the lower chamber, and the plates were incubated for 4 h. In some experiments, IL-8 was added above and below the membrane to form a checkerboard of concentrations, as described for the chemotaxis assay. Lymphocytes were harvested from above and below the inserts using 0.2% EDTA, counted, and expressed as the percentage of transmigrating cells.

Staining of the confluent HMEC-1 cells with HABP indicated the presence of only weak reactivity. Because TNF-α is known to enhance HA production by endothelial cells (22), in some experiments the HMEC-1 cells were pre-treated with TNF-α (10 ng/ml, 24 h) before performing the migration assay.
HABP staining demonstrated markedly enhanced levels of HA after such stimulation. In other experiments, exogenous HA (20 μg/ml, 24 h) was added to the HMEC-1 cells before performing the migration experiments; again, HABP staining showed greatly increased HA in association with the HMEC-1 cells.

**Motility on a Fibroblast Monolayer**

Purified CLL cells were placed on fibroblast monolayers derived from tonsil or lymph node, and motility was analyzed by time-lapse video microscopy in the same way described for movement on HA. The effect of blocking antibodies and hyaluronidase digestion was also analyzed as described before.

**RESULTS**

**IL-8 Induces CLL Cell Chemotaxis and Chemokinesis into HA-coated Filters**

In the absence of cytokine, CLL cells moved to only a minor extent into the filters, regardless of whether they had been coated with HA or FN or were untreated [range of means: HA = 16–36 μm (n = 6); FN = 16–36 μm (n = 3); untreated = 20–36 μm (n = 3)]. In contrast, IL-8, either above or below the filter, induced significant migration when the surface was coated with HA (Table 1A). Identical patterns of movement were observed in patients with and without lymph node enlargement.

In experiments with untreated (n = 3) or FN-coated (n = 3) filters, IL-8 had no effect (data not shown). Moreover, digestion of HA with hyaluronidase abolished the IL-8-induced motility (n = 3; Table 1B). In similar experiments, MIP-1α (at 10, 100, and 1000 pg/ml) did not induce CLL cell movement, regardless of whether the filters were coated with HA (Table 1A; n = 3) or FN (data not shown; n = 3).

It was therefore concluded that IL-8, but not MIP-1α, induces both chemokinesis and chemotaxis of CLL cells on HA-coated filters, but not on either FN-coated or uncoated filters. We next extended these observations by time-lapse video analysis.

**Video Analysis Confirms that IL-8 Induces CLL Cell Motility on HA**

HA-adherent CLL cells were exposed to IL-8 (5 ng/ml) and observed by time-lapse video microscopy for up to 2 h. In the absence of chemokine, no movement was observed. However, within 5 min of the addition of IL-8, 16–27% of the CLL cells became motile, with an average velocity of 11 ± 4 μm/min (n = 6; Table 2). Most of the remaining cells were seen to be actively deforming but were considered nonmotile because minimal positional change (less than one cell diameter) was observed. Motile cells exhibited polarized membrane ruffling. Motility continued for around 90 min and then gradually ceased. Digestion of HA with hyaluronidase abolished the IL-8-induced movement (n = 3; data not shown), as did pretreatment of the cells with pertussis toxin (1 μg/ml; Sigma; n = 3; data not shown).

Movement was similar in patients with and without lymphadenopathy [for patients with lymphadenopathy, 17–27% of cells were motile with an average velocity of 12 ± 4 μm/min (n = 3); for patients without lymphadenopathy, 18–26% of cells were motile with an average velocity of 12 ± 6 μm/min (n = 3)]. Little or no cell movement was observed on untreated or FN-coated plates in the presence or absence of IL-8. MIP-1α (100 pg/ml) did not induce CLL cell movement on HA- or FN-coated plates or on uncoated plates (n = 3; data not shown).

Having established that CLL cells become motile in response to the combination of adhesion to HA and stimulation with IL-8, but not in response to either agent alone, we next analyzed the receptors and ligands involved.

**CLL Cells Express a Number of Potential Receptors for HA and IL-8**

HA can bind to a number of cell surface receptors including CD44, RHAMM, CD54 (ICAM-1), and CD38 (23). We therefore used specific mAbs to analyze the expression of these molecules by FACS.

The two principal cell receptors for HA, CD44 and RHAMM, were both present on all CLL cells, whereas CD54 was detected on only a minority (~30%) population, and CD38 was absent (Table 3). Expression of all four receptors was similar in patients with and without organomegaly. Although RHAMM was present on the entire leukemic cell population, its expression was weak, as indicated by a low mean fluorescence intensity compared with CD44.

Because the expression of HA receptors might have been altered by exposure to IL-8 during the course of our motility experiments, FACS was also performed after incubation of CLL cells with IL-8 for 30, 60, and 90 min. IL-8 had no effect on any of the four HA receptors studied (n = 3; data not shown).

We next looked for the presence of receptors for the two chemokines used in the present study. Receptors for IL-8 (both IL-8RA and...
ROLE OF HA AND IL-8 IN THE MIGRATION OF CLL CELLS

Table 2  CLL cell movement as assessed by time-lapse video microscopy
The data show the percentage of motile cells on HA and their average velocity in the presence of IL-8 in six CLL patients with or without lymphadenopathy. No cell movement was seen on HA in the absence of IL-8 or on uncoated plates in the presence of IL-8.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Lymphadenopathy</th>
<th>% Motile</th>
<th>Average velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Present</td>
<td>16</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>Present</td>
<td>25</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>Present</td>
<td>27</td>
<td>11 ± 7</td>
</tr>
<tr>
<td>4</td>
<td>Absent</td>
<td>18</td>
<td>18 ± 9</td>
</tr>
<tr>
<td>5</td>
<td>Absent</td>
<td>26</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>Absent</td>
<td>24</td>
<td>10 ± 9</td>
</tr>
</tbody>
</table>

Table 3  HA and chemokine receptors on CLL cells
The results show the percentage of positive cells and their mean fluorescence intensity (MFI) as determined by FACS.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Cases with organomegaly (n = 3)</th>
<th>Cases without organomegaly (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>93 ± 5</td>
<td>124 ± 32</td>
</tr>
<tr>
<td>RHAMM</td>
<td>100*</td>
<td>21 ± 25</td>
</tr>
<tr>
<td>CD54</td>
<td>25 ± 19</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>CD38</td>
<td>5 ± 7</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>IL-8RA</td>
<td>59 ± 18</td>
<td>82 ± 72</td>
</tr>
<tr>
<td>IL-8RB</td>
<td>42 ± 34</td>
<td>46 ± 40</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>63 ± 24</td>
<td>56 ± 42</td>
</tr>
</tbody>
</table>

* Because in these instances the reactivity of all the CLL cells was shifted to the right relative to the isotypic control, they were considered to be 100% positive.

IL-8RB and MIP-1α were constitutively present on the majority of cells (Table 3). Again, there was no difference in receptor expression between patients with and without organomegaly.

IL-8-induced Movement on HA Is Mediated by RHAMM

We next used blocking mAbs to determine which of the potential HA receptors present on CLL cells are involved in the IL-8-induced movement.

The anti-RHAMM mAb completely inhibited the IL-8-induced chemotaxis/chemokinesis into HA-coated filters (Fig. 1). In contrast, anti-CD44, anti-CD54, and nonimmune IgG1 (20 µg/ml) had no effect on such movement (Fig. 1).

In similar experiments involving video analysis of CLL cells on a HA-coated surface, anti-RHAMM abrogated movement, whereas the other antibodies and nonimmune IgG1 had no effect (Fig. 1).

IL-8-induced Movement of CLL Cells on HA Involves Actin Polymerization and Redistribution of RHAMM

Because RHAMM and F-actin are known to be redistributed in motile cells (24), we next used confocal microscopy to analyze the distribution of these two molecules in CLL cells on HA.

In the absence of IL-8, CLL cells on HA displayed a weak diffuse staining for F-actin (Fig. 2a); the addition of IL-8 induced rapid (within 1 min) formation of a F-actin cap at one pole of a proportion of the cells (Fig. 2b). In the absence of IL-8, both RHAMM and CD44 were localized to the perimeter of the cells (Fig. 2c and e). After the addition of IL-8, RHAMM (Fig. 2d), but not CD44 (Fig. 2f), became redistributed in a polar fashion resembling that of the polymerized F-actin noted above.

These confocal findings therefore showed that on HA, IL-8 induces in CLL cells the type of polarized morphology associated with cell motility and that this motility is mediated by RHAMM.

We next used a number of approaches to relate these findings to the behavior of CLL cells within tissues.

Fig. 1. Effect of blocking mAb on CLL cell movement on HA + IL-8. The first three groups of histograms refer to movement into HA-coated filters, whereas the group at the bottom involves movement on HA + IL-8 as measured by time-lapse video microscopy. Chemokinesis was the distance moved into the HA-coated filter in 2 h in the presence of IL-8 (5 ng/ml) above the filter. Chemotaxis was the distance moved in 2 h with IL-8 (5 ng/ml) below the filter. Anti-RHAMM, but not anti-CD44, anti-CD54 (data not shown), or the IgG1 control, significantly (P < 0.01) inhibited movement. Error bars, 1 SE. The data for movement into filters are a representative example of the three cases studied. The time-lapse video microscopy results refer to three experiments in three patients.  

CLL Cells Do Not Migrate Across Endothelial Layers in the Presence of HA ± IL-8

We next measured the movement of CLL cells across a monolayer of microvascular endothelial cells (HMEC-1) grown to confluence on polycarbonate inserts in a transwell system.

The CLL cells did not migrate through the HMEC-1 monolayer (migrating cells, <2%; n = 6) whether or not IL-8 (5 ng/ml) was present above or below the filter; stimulation of the HMEC-1 cells with TNF-α or the addition of exogenous HA had no effect (migrating cells, <2%; n = 3). In contrast, >90% neutrophils migrated through the HMEC-1 monolayer in the presence of a chemotactic gradient of IL-8 (5 ng/ml below the filter), but not in the absence of cytokine.

We therefore concluded that it is unlikely that HA and IL-8 mediate the endothelial transmigration of CLL cells, a conclusion supported by our demonstration below that the luminal surface of microvascular endothelium (including HEVs) of lymphoreticular tissues lacks HA and IL-8.

CLL Lymphoreticular Tissues Contain Abundant HA and IL-8 in a Distinctive Distribution Resembling That of the Interfollicular Areas of Normal Nodes

We next examined the distribution of HA and IL-8 in CLL lymph nodes and spleen as compared with normal tissue.

Lymph Nodes. In CLL nodes, the normal architecture is completely replaced by closely packed malignant lymphocytes among which a variety of vascular and lymphatic channels are discernible (25).

HA, as detected by HRP-labeled HABP, was present throughout the...
abnormal node. Hyaluronidase treatment completely abolished staining. The HA was frequently distributed as a reticular network and was particularly prominent around the abundant HEVs, where the network was often seen to extend to nearby sinuses (Fig. 3a). The luminal surface of HEVs and other vessels was completely unreactive for HA (Fig. 3a).

IL-8, as detected by a triple-layer avidin-biotin method, was observed throughout the nodal tissue, with staining within and between the CLL cells (Fig. 3b). Vessels were made prominent by their lack of staining; in particular, HEVs were negative (Fig. 3b).

In normal nodes (data not shown), HA staining in the interfollicular cortical areas broadly resembled that seen in CLL nodes. The HA network was again particularly obvious around the HEVs and extended to adjacent sinus regions; follicles contained little or no staining except in their germinal centers, which were variably reactive. As in CLL nodes, the luminal surfaces of HEVs and other vessels completely lacked HA. Some HA staining was seen to be associated with fibroblastic reticular cells, particularly those close to HEVs.

With regard to IL-8 in normal nodes, the cytokine was detectable throughout. Reactivity was stronger in the interfollicular zones; some of this was associated with fibroblastic reticular cells; however, HEVs were completely unreactive.

Thus, apart from the absence of follicles in CLL, the distribution of HA and IL-8 was similar in CLL and normal nodes.

Spleen. CLL spleen, in contrast to the nodes, retains some normal architecture. Thus, red and white pulp can still be distinguished, but the white pulp is greatly expanded by malignant cells, and the red pulp contains CLL lymphocytes both as islands and as diffusely scattered cells (2).

Apart from staining around blood vessels, the red pulp was completely unreactive for HA. In contrast, the expanded white pulp contained a network of HA staining (Fig. 3c) resembling that observed in the nodes. In both the red and white pulps, the endothelial cells of vessels lacked HA.

Staining for IL-8 was observed in both the red and white pulp but

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Fig. 2. Confocal microscopy of CLL cells on HA ± IL-8 (5 ng/ml) and redistribution of F-actin and RHAMM, but not CD44, on stimulation with IL-8. The images represent the appearances 1 min after the addition of IL-8 (ng/ml) or medium (control) and show the plane of section immediately adjacent to the HA substratum. Only a proportion of cells became motile, and these cells show polarized staining of F-actin and RHAMM (examples marked ↑). Similar but less marked appearances were seen after 5 and 10 min.
was stronger in the white pulp areas (Fig. 3d). The cytokine was again detected both extracellularly and within the CLL lymphocytes, and the endothelial cells of vessels were negative.

As in CLL, in normal spleen (data not shown) HA was completely absent from the red pulp but was present in variable amounts within the different parts of the white pulp that were not readily discernible in CLL. Thus, in normal spleen, staining was particularly prominent around the central arterioles (presumed T-cell areas) where the HA formed a reticular network resembling that of the interfollicular zones of the normal node. Little staining was observed in the follicular zones (B-cell areas), except in follicle centers where variable amounts of HA were present. As in CLL, IL-8 staining was present throughout normal spleen (data not shown), although fewer lymphocytes containing IL-8 were observed in the red pulp. In the white pulp, reactivity was stronger in the outer B-cell zones than in the periarteriolar T-cell areas. As in CLL spleen, vascular endothelial cells lacked IL-8.

In conclusion, these tissue studies, taken together with our in vitro observations presented earlier in this study, suggest that IL-8-induced motility along HA may be important in the migration of CLL and possibly also normal B lymphocytes within, but not into, specific areas of lymphoreticular tissue. In particular, the prominent HA staining around HEVs and the reticular network of this GAG in the area between HEVs and sinuses suggest that movement along HA may be important in lymphocyte migration from perivenular areas to adjacent sinuses. Because HA is known to be produced mainly by fibroblasts (26) and was seen to be associated with those immediately surrounding HEVs, and because migrating lymphocytes must pass between these perivenular cells (27), we next examined CLL cell motility on fibroblasts derived from normal lymphoreticular tissue.

**CLL Cells Are Motile on Fibroblast Layers, and This Motility Involves RHAMM and IL-8**

Time-lapse video microscopy showed that CLL cells were motile on fibroblasts derived from tonsil or nodes. A high proportion of the CLL B cells moved spontaneously (Fig. 4). The number of motile cells was greatly reduced by blocking anti-RHAMM (10 μg/ml) and anti-IL-8 antibodies (10 μg/ml; Fig. 4), but not by an anti-CD44 antibody (20 μg/ml). Similar levels of inhibition were observed in the combined presence of anti-IL-8 and anti-RHAMM mAb (Fig. 4).

Immunohistochemical staining of the fibroblast layer demonstrated the presence of both HA and IL-8. In addition, complete digestion of HA with hyaluronidase markedly reduced motility (only 13% and 20% of cells were motile; n = 2) without affecting the viability of the fibroblast layer as assessed by trypan blue exclusion.

**DISCUSSION**

The present study is concerned with the ill-defined processes governing the movement of CLL cells into and within lymphoid tissues.
Using two different techniques (a Boyden-type chemotactic assay and time-lapse video microscopy), we clearly show that B-CLL cells become motile on HA (but not on FN or uncoated plastic) in the presence of IL-8. No motility was observed in the presence of HA alone, and a C-C chemokine (MIP-1α) had no effect. Hyaluronidase treatment of the HA substratum and blocking of IL-8 receptor signaling with pertussis toxin abrogated the IL-8-induced motility.

We next examined the receptors involved in this motility. The identity of HA receptors is currently undergoing controversial re-evaluation (28, 29). Because CLL cells do not express CD38, we have nothing to add concerning the role of this molecule in HA-mediated motility. Although CLL cells express ICAM-1 and CD44, we show here that neither of these molecules is involved in IL-8-induced CLL cell motility on HA. The most relevant controversy for the present work concerns the molecule known as RHAMM. The gene for RHAMM has been independently cloned and sequenced by two groups (30, 31), and there is still on-going debate about the cell surface expression of the protein. By using an anti-RHAMM antibody (3T3.5) extensively used in previous functional studies of RHAMM (9, 23, 29), we clearly demonstrate that the protein recognized by this antibody is present in the surface of CLL cells. This is in contrast with a previous study of RHAMM in CLL where most cases were reported to be negative (32). Because the same antibody was used in the present study, we suggest that this apparent discrepancy is the result of the relatively small, but definite, peak shift observed here being interpreted as a negative result.

We then went on to show that blocking RHAMM with this 3T3.5 mAb inhibited IL-8-induced CLL cell movement on HA, whether such movement was assessed by Boyden-type assay or by time-lapse video microscopy. Furthermore, confocal microscopy of cells on HA exposed to IL-8 showed that RHAMM and F-actin, but not CD44, were rearranged in a polar fashion.

We next investigated how these findings might relate to the behavior of CLL cells within lymphoid tissues. We first analyzed the role of HA and IL-8 in transendothelial migration and demonstrated that such movement was assessed by Boyden-type assay or by time-lapse video microscopy. Furthermore, confocal microscopy of cells on HA exposed to IL-8 showed that RHAMM and F-actin, but not CD44, were rearranged in a polar fashion.

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weak reactivity on the luminal surface of HEVs. However, our unequivocal demonstration of the absence of HA on endothelial cells of lymphoid tissue suggests that the labeled CD44 was detecting an alternative ligand on HEVs.

Clark et al. (34) have already implicated HA in directing the movement of lymphocytes within normal lymphoid tissues. The cellular receptor for HA involved was found to be CD44, and the receptor-ligand interaction was of a type that would allow movement. However, the study was concerned with measurement of the strength of cell adhesion rather than directly with stimulated cell movement. Thus, our demonstration of IL-8-stimulated and RHAMM-dependent movement of CLL cells on HA is an entirely novel finding.

Our conclusion that the combined presence of HA and IL-8 may direct the movement of CLL cells within lymph nodes and the white pulp of spleen is very much in line with the emerging concept that specific cytokine receptors and their ligands [e.g., TNF (37), stromal cell-derived factor α (38), and BLR-1 (39, 40)] directly the migration of different lymphoid cell types to particular areas of lymphoid tissue.

Although the present study was concerned with CLL, HA and IL-8 may be important in the trafficking of certain other normal and malignant B-cell types. Work is now in progress in this laboratory to examine this proposition.

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The Role of Hyaluronan and Interleukin 8 in the Migration of Chronic Lymphocytic Leukemia Cells within Lymphoreticular Tissues

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