The interaction of human lymphoma cells with high endothelial venules (HEVs) on sections of lymphatic tissues was studied in 44 cases of non-Hodgkin's lymphoma (NHL) with the in vitro HEV binding assay. The relative adherence ratio (RAR) of lymphoma cells to HEVs as related to that of reactive lymphocytes was 0.29 to 4.64 in 38 cases of B chronic lymphocytic leukemia (CLL), 1.15 and 1.54 in two cases of immunocytoma NHL, 1.12 and 0.70 in two cases of centrocytic NHL, 1.98 in one case of a peripheral T-NHL, whereas plasma cell leukemia cells adhered very weakly (RAR 0.1). Among the patients suffering from CLL a pronounced HEV binding ability of tumor cells correlated significantly with the more unfavorable Binet stages B and C (median 1.32) as well as with a widespread lymphatic dissemination, which strongly indicates a hematogenous, HEV-mediated spread (median 1.34). In contrast, weak adherence to HEVs was associated with Binet stage A (median 0.85; P < .05) and with a lacking or only localized clinical involvement of lymph nodes (median 0.84; P < .01). Thus, specific HEV recognition processes even operate in lymphoid neoplasms and via this mechanism seem to influence the dissemination of tumors.

**MATERIALS AND METHODS**

**Classification of NHLs and clinical data of patients.** The classification of NHLs was performed according to the criteria of the Kiel classification by applying conventional histologic and immunohistologic criteria. In vitro binding results were correlated to clinical data, particularly the pattern of lymphatic spread. The degree of lymph node involvement was defined meeting the criteria mentioned previously. The staging procedure routinely included physical examination, chest x-rays, abdominal/pelvic/thoracic computerized tomography, ultrasound examination, and bone marrow (BM) smears and biopsies.

Patients were staged according to the systems of Rai (RAI 0, lymphocytosis in blood and bone marrow only; I, lymphocytosis and enlarged lymph nodes; II, lymphocytosis plus hepatomegaly, or splenomegaly, or both [nodes may or may not be enlarged]; III, lymphocytosis and anemia [Hb < 11 g/dL]; IV, lymphocytosis and thrombocytopenia [platelets < 100 X 10^9/L] [anemia and organomegaly may or may not be present]) and Binet (Binet stage A: less than three areas involved [the three areas include the cervical, axillary, and inguinal lymph nodes, whether unilateral or bilateral, the spleen, and the liver]; B: Hb > 10 g/dL, and platelets > 100 X 10^9/L; stage C: Hb < 10 or platelets < 100 X 10^9/L or both [independently of the areas involved]).

Furthermore, the physiologic lymphatic circulation routes were taken into account because homing receptor-mediated tumor spread must be hematogenous via HEVs. A hematogenous spread...
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Identification of the adhering cells deserves special attention. With regard to their identification in the HEV-binding assay, they were stained after HEV adherence and after fixation similar to the procedure mentioned by Butcher et al.27 We were aware of the possibility that application of MoAbs for cell identification before HEV adherence might influence adherence behavior. The following MoAbs were applied in direct and indirect immunofluorescence assays: for T cells MoAb anti-Leu-4 (CD3; Becton Dickinson); for B-lymphoma cells MoAb anti-Leu-12 (CD19; Becton Dickinson), To15 (CD22; Dakopatts), VIB-C5 (CD24; W. Knapp, Institute for Immunology, Vienna, Austria), and Tfil (CD23; Biotest Diagnostics, Vienna, Austria) in the case of CD23 reactive lymphomas. Plasma cell leukemia cells were recognized by their typical morphologic appearance as well as by staining with MoAb OKT10 (CD38) (Ortho Pharmaceutical, Vienna, Austria). These MoAbs were applied either directly, phycoerythrin-labeled, or rhodamine-conjugated rabbit Ig (Dakopatts) was used as fluorochrome in a second step.

Statistical evaluation. The standard error of all adherence ratio estimates as well as comparisons of organ-specific differences of the adherence ratios between tonsil and lymph node were estimated using the delta method.24 HEV binding and the extent of lymphatic dissemination of CLL were compared by means of the Mann-Whitney U test (one-sided).

RESULTS

Adhesion to HEVs—Comparison of PBLs from different healthy individuals. PBLs from various healthy individuals displayed similar adherence to HEVs, which indicates that these cells are a useful reference population (Table 1). These reactive PBLs were thus always included as the internal standard population.

HEV Binding of various human lymphomas. All patients with lymphoma were either untreated or off treatment for at least 3 months before evaluation. Tumor cells were obtained from peripheral blood from leukemic patients and were identified with the help of relevant MoAbs in direct and indirect immunofluorescence techniques (Fig 1). The adherence of human lymphoma cells and of PBLs from healthy donors to HEVs was compared on sections of lymph node and tonsil. As organ-specific differences between lymph node and tonsil were observed in only 3 of 44 cases of lymphoma (in 2 cases of chronic lymphocytic leukemia [CLL], a significantly stronger adherence to lymph node than to tonsil HEV; in one case of CLL, a significantly

Table 1. Comparison of the Binding of PBLs From Different Individuals to HEV

<table>
<thead>
<tr>
<th>Individual</th>
<th>Relative Adherence Ratio (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.S.*</td>
<td>1</td>
</tr>
<tr>
<td>F.O.</td>
<td>0.95 ± 0.08</td>
</tr>
<tr>
<td>S.H.</td>
<td>1.14 ± 0.15</td>
</tr>
<tr>
<td>H.S.</td>
<td>1.06 ± 0.14</td>
</tr>
<tr>
<td>N.B.</td>
<td>1.09 ± 0.15</td>
</tr>
</tbody>
</table>

The relative adherence ratio (RAR) (±SE) is equivalent to the number of sample cells adhering to HEVs compared with reactive reference PBLs under the same conditions.

* PBLs from R.S. were selected as the reference population.
HEV-binding ability clearly differed when patients of Binet stage A (median of the adherence ratios 0.85) were compared with those of Binet stages B and C (median 1.32) \( (P < 0.05) \) (Fig 3). Furthermore, the anatomic pattern of dissemination (lymphatic vs hematogenous) was taken into consideration as described in the Materials and Methods section. Patients lacking or only showing a localized clinical lymph node involvement (which is consistent with lymphatic, non–HEV-mediated spread) revealed significantly lower adherence ratios (median for stages A, B, C 0.84) than those with a widespread, generalized pattern of disease (which is strongly suggestive of a hematogenous dissemination mechanism mediated via HEVs) (median for stages D and E 1.34) \( (P < 0.01) \) (Fig. 4). No significant difference in HEV adherence was observed among the various prognostic groups of the Rai staging system \( (Rai 0 \text{ v Rai I to IV: median } 0.94 \text{ v 1.24}; P < 0.2; \text{ Rai } 0, I \text{ v II, III, IV: median } 0.95 \text{ v 1.19}; P < 0.9; \text{ Rai } 0, I, II \text{ v III, IV: median } 0.95 \text{ v 1.40}; P < 0.8) \). No significant difference in HEV-binding ability was observed when patients of Binet stage A (median of the adherence ratios 0.85) were compared with those of Binet stages B and C (median 1.32) \( (P < 0.05) \) (Fig 3).

**DISCUSSION**

Although neoplastic cells are postulated to spread by mechanisms related to normal lymphocyte circulation, little data concerning the role of tumor-HEV interaction are available. The results presented here are the first documentation of the adhesive properties of a large panel of human lymphoma cells to human HEVs in vitro. We demonstrate that neoplastic leukocytes are able to bind selectively to HEVs, with various non-Hodgkin’s lymphomas displaying differential HEV-binding capacities even within the same histologic entity (Fig 2). The relative adherence ratio (RAR) of lymphoma cells related to that of reactive PBLs was 0.29 to 4.64 in 38 cases of B-CLL and was 1.15 and 1.54 in two cases of immunocytic NHL (ic). Two cases of centrocytic NHL (cc) exhibited an RAR of 1.12 and 0.70. In one case of a peripheral T-cell NHL, an RAR of 1.98 was measured, whereas plasma cell leukemia cells from one patient exhibited a very weak binding ability \( (RAR = 0.1) \).

**Comparison of HEV adherence and clinical data in CLL.** HEV-binding properties were compared with the clinical stage, particularly the extent and pattern of lymphatic dissemination in 38 patients suffering from CLL.
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Fig 2. Adherence of various human lymphomas to HEVs. RAR (+ SE) of lymphoma cells related to reference PBLs (RE), IC (immunocytic NHL), CC (centrocytic NHL), PI (plasma cell leukemia), T-NHL (peripheral T-NHL).

In contrast to the investigation of Kalasz et al., comparing neoplastic cells from peripheral blood and BM, lymphoma cells were obtained from peripheral blood from leukemic patients in all of the cases evaluated in this study. In agreement with observations in murine lymphomas, the involvement of lymph nodes thus reflects the capacity of cells having successfully entered the blood stream to migrate into HEV-bearing lymphatic tissues rather than the capacity of lymphomas to enter the peripheral blood. Thus, the lack of lymph node involvement in patients might be explained by an intrinsic deficiency or low HEV-binding capacity of tumor cells. To further define the underlying molecular mechanisms, the presence of structures involved in the interaction of leukocytes with HEVs (CD44, LFA-1 (CD11a/CD18), L-selectin, VLA-α4, and with endothelial cells (CD11b, CD11c, and ICAM-1) was investigated. In concurrence with recent observations, we failed to define a causative role of these adhesion molecules for differential HEV-binding capacities, as most lymphoma...
cells revealed a homogeneous expression pattern. However, the presence of an adhesion structure per se does not prove its functional significance; ie, several structures are not constitutively active but must be activated.\(^{35,36}\) Adhesive processes might be mediated by combinations of molecules\(^{13}\) or by alternatively spliced or glycosylated isoforms,\(^{39,40}\) which cannot be differentiated by the antibody employed.

In conclusion, these results confirm previous hypotheses that HEV-mediated homing mechanisms seem to direct the propagation of human lymphomas into HEV-bearing organs. Further HEV-binding experiments, particularly propagation of human lymphomas into HEV-bearing or-}

<table>
<thead>
<tr>
<th>Molecules</th>
<th>Positive/No. of Samples</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFA-1 alpha</td>
<td>4/37</td>
<td>11</td>
</tr>
<tr>
<td>LFA-1 beta</td>
<td>4/37</td>
<td>11</td>
</tr>
<tr>
<td>CD11b</td>
<td>1/37</td>
<td>3</td>
</tr>
<tr>
<td>CD11c</td>
<td>4/37</td>
<td>11</td>
</tr>
<tr>
<td>VLA-4</td>
<td>5/36</td>
<td>14</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0/37</td>
<td>0</td>
</tr>
<tr>
<td>CD44</td>
<td>36/36</td>
<td>100</td>
</tr>
<tr>
<td>L-selectin</td>
<td>24/36</td>
<td>67</td>
</tr>
</tbody>
</table>

Comparison of the HEV binding capacity and the receptor profile of CLL cells showed no significant correlation to any of these adhesion structures (data not shown).

**ACKNOWLEDGMENT**

The kind gifts of antibodies anti-ICAM-1 (CD54), 7F7 (M.P. Dierich; Institute for Hygiene, Innsbruck, Austria); anti-CD44, F 10-44-2 (R. Dalechau; Blind McIndoe Centre for Medical Research, East Grinstead, Sussex, UK); anti-VLA-alpha4-chain (CDw49d), BSG10 (M.E. Hemler; Dana Farber Cancer Institute, Harvard Medical School, Boston, MA), and VIB-C5 (CD24; W. Knapp, Institute for Immunology, Vienna, Austria) are gratefully acknowledged. We express our gratitude to U. Zilian for help with the statistical analysis and to F. Oberwasserlechner for excellent technical assistance.

This work is dedicated to Prof Herbert Braunsteiner on the occasion of his 70th birthday.

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Adhesion to high endothelial venules: a model for dissemination mechanisms in non-Hodgkin's lymphoma

R Stauder, S Hamader, B Fasching, G Kemmler, J Thaler and H Huber