
By R. Stauder, S. Hamadener, B. Fasching, G. Kemmler, J. Thaler, and H. Huber

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 immunocytic 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.

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From the Division of Immunohaematology and Oncology, the Department of Internal Medicine and Institute of Biostatistics, University of Innsbruck, Austria.

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Address reprint requests to R. Stauder, MD, Department of Internal Medicine, Anichstr. 35, 6020 Innsbruck, Austria.

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of lymphomas was considered present when lymph node involvement could not be explained by a contiguous spread along pathways of lymphatic drainage. Based on these criteria, patients were divided into a group with contiguous lymph node infiltration (stages A, B, and C) versus a group with involvement of noncontiguous sites (stages D and E) as suggested recently. Stage A, lymphoma involvement documented in one extranodal site or one lymph node group; stage B, involvement of two or more sites contiguous along pathways of lymphatic drainage and limited to one side of the diaphragm; stage C, contiguous disease on both sides of the diaphragm; stage D, two or more noncontiguous sites of involvement; stage E, generalized lymph node dissemination (BM, spleen, and liver involvement were not considered).

Preparation of cells. Neoplastic and reactive lymphocytes were prepared from peripheral blood of leukemic patients and from healthy donors. Cells were isolated by passage through nylon wool followed by density centrifugation with Lymphoprep (Nycomed; Oslo, Norway). In patients with B-NHL with large numbers of contaminating normal T lymphocytes, these cells were depleted by sheep R E rosettes. This procedure yielded a population consisting of <5% T lymphocytes and >95% lymphocytes expressing B-cell markers as assessed by using monoclonal antibody (MoAb) BMA030 (CD3; Behring, Marburg, Germany) and MoAb To15 (CD22; Dakopatts, Glostrup, Denmark), respectively, in an indirect immunoperoxidase staining technique on cytosin preparations.

Immunophenotyping of lymphomas. The presence of relevant adhesion structures was investigated in an indirect immunoperoxidase staining technique on cytosin preparations. Staining intensity was scored semiquantitatively on a scale of 0 to 3+ (0, negative; ±, faint, not clearly above background; +, weakly positive; 2+, moderately positive; 3+, strong positive). Lymphoma cells were termed positive when greater than 20% of the tumor cells revealed a 1, 2+, or 3+ reactivity with the relevant antibody.

MoAbs used were anti-LFA-1 alpha-chain (CD11a), MHM24 (Dakopatts); anti-LFA-1 beta-chain (CD18), MHM23 (Dakopatts); anti-CD11b, Mac-1 ( Coulter Immunology, Hialeah, FL); anti-Leu-1c, LeuM5 (Becton Dickinson, Mountain View, CA); anti-Leu-8, -L-selectin (Becton Dickinson); anti-ICAM-1 (CD54), 7F724 (gift of M. P. Dierich); anti-CD44, F10-44-ZZ5 (gift of R. Dalchau); anti-CD23, VIB-C5 (CD24; W. Knapp, Institute for Immunology, 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. 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>
<thead>
<tr>
<th>Individual</th>
<th>Relative Adherence Ratio (±SE)</th>
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<tbody>
<tr>
<td>R.S.*</td>
<td>1</td>
</tr>
<tr>
<td>F.O.</td>
<td>0.95 ± 0.09</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>
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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) \( (P < .05) \) (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) \( (P < .01) \) (Fig. 4). No significant difference in HEV adherence was observed among the various prognostic groups of the Rai staging system \( (Rai \ 0 \ v \ Rai \ I \ to \ IV: \ median \ 0.94 \ v \ 1.24; \ P < 0.2; \ Rai \ 0, I \ v \ II, III, IV: \ median \ 0.95 \ v \ 1.19; \ P < .9; \ Rai \ 0, I, I1 \ v \ I1, IV: \ median \ 0.95 \ v \ 1.40; \ P < .08). \)

No correlation was observed between the leukocyte count and the adhesive capacity of these cells to HEVs (data not shown).

**Adhesion molecules and HEV Adhesion in CLL.** To address the molecular mechanisms accounting for the observed differences in lymphoma binding to HEVs, the presence of potentially relevant structures in CLL cells was analyzed in parallel (Table 2). Most cases revealed a strong expression of CD44 as well as of L-selectin but lacked CD11b, CD11c, ICAM-1, and VLA-\alpha4 in the majority of cases. Because of this relatively uniform immunophenotype, the HEV-binding capacity did not significantly correlate to any of these structures (data not shown) and not even to L-selectin, which was expressed more heterogeneously in lymphoma cells.

**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 in vitro. We demonstrate that neoplastic leukocytes are able to bind selectively to HEVs, with various non-Hodgkin’s lymphomas displaying differential HEV-binding properties. Conclusions from electron microscopy and from adhesion studies of neoplastic plasma cells to rat HEVs as well as from distribution experiments of labeled lymphoma cells in humans postulating specific interactions between tumor cells and HEVs are thus corroborated and further elaborated. Most cases of lymphoma revealed a distinct adherence to HEVs when directly compared with peripheral blood lymphocytes. Even in comparison with other lymphocyte subpopulations, the pronounced HEV-binding ability of several NHLs is remarkable. In the majority of cases, the B cell–derived neoplasias studied revealed a binding behavior similar to that of reactive B cells without clear preference for tonsil or lymph node HEV.

The principal finding of this study is the significant correlation between the capacity to bind to HEVs and the pattern stronger binding to tonsil than to lymph node HEV, the results from tonsil and lymph node were pooled.

Various lymphomas displayed 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. The principal finding of this study is the significant correlation between the capacity to bind to HEVs and the pattern...
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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).

and extent of lymph node involvement in CLL. In detail, HEV adherence was not associated with the different Rai stages but with the various prognostic groups of the Binet system. These classification schemes were established for the assessment of prognosis and design of therapy in CLL, with the involvement of lymph nodes representing one of the main criteria. Within the Rai system, the presence of lymphadenopathy at any site is evaluated irrespective of the extent of lymph node involvement. In contrast, the Binet system is a better discriminator of lymphatic dissemination because the number of involved lymph nodes is evaluated and contributes to final segregation of the different prognostic groups. Assuming that attachment to HEVs directly influences lymphatic dissemination, it is plausible that HEV binding correlates better with a staging system than with the extent of lymph node involvement. In support of this concept, the foremost correlation of HEV adherence was observed when patients were grouped on the basis of a hematogenous, HEV-mediated spread versus a continuous lymphatic pattern of metastasis as recently suggested. 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 bloodstream 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. Adhesive processes might be mediated by combinations of molecules or by alternatively spliced or glycosylated isoforms, 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 blocking experiments with MoAbs directed to homing molecules and their putative ligands, will offer an important clue to the functional significance; ie, several structures are not constitutively active but must be activated. Adhesive processes might be mediated by combinations of molecules or by alternatively spliced or glycosylated isoforms, which cannot be differentiated by the antibody employed.

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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