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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 was 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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DIREC TED MIGRATION of lymphocytes from the bloodstream (“homing”) is initiated by selective binding to specialized endothelial cells, the high endothelial venules (HEVs). A system of organ-specific lymphocyte homing receptors (LHRs) mediates this selective lymphocyte-HEV recognition and thus presumably controls extravasation to lymphoid tissues and inflammation sites.1,3 Recent interest has focused on the role of tumor-HEV interactions in metastatic spread. Evidence supporting an HEV-mediated distribution of lymphomas was given by the observations of Bargatze et al.4 A pronounced in vitro HEV binding capacity of passaged murine lymphomas was associated with a wide dissemination after transplantation into syngeneic recipients. In contrast, in nonbinding lymphomas often only the regional lymph nodes were involved, although both had access to the blood stream. It was concluded that the ability to bind to HEVs regulates the hematogenous dissemination of neoplastic lymphocytes.

Clinical observations concerning the characteristic and individually different patterns of organ involvement and tumor recurrence in non-Hodgkin’s lymphoma (NHL) suggest that these features might reflect different homing properties of neoplastic cells. In support of this concept, recent examinations have shown that the presence of the adhesion receptors CD44 and LFA-1 (CD11a/CD18), which are both relevant for lymphocyte traffic,5-7 on tumor cells correlates with a generalized lymph node involvement4 and an unfavorable clinical prognosis.8-11 Furthermore, lymphomas from different locations showed different expression patterns of L-selectin (also known as LECAM-1, LAM-1, or Leu-8), which is the human equivalent of the mouse peripheral lymph node homing receptor MEL-14.12 Nodal NHLs expressed L-selectin, whereas gastrointestinal-localized tumors generally lacked this molecule.13-15 These results support the concept that LHRs are major determinants of lymphoma localization and propagation and hence of the clinical outcome of patients.

To further investigate the contribution of HEV-mediated spread for tumor metastasis, we analyzed the HEV-binding properties of various human lymphoma cells from leukemic patients in an in vitro system. This assay in which viable lymphocytes adhere specifically to HEVs during incubation on frozen tissue sections allows qualitative and quantitative analysis of the HEV-binding capacity of leukocytes.15,16 Adhesion of lymphoma cells was related to that of reactive lymphocytes from healthy donors and compared with clinical data. These results demonstrate that homing processes via HEVs even exist in neoplastic lymphocytes and seem to determine the traffic and dissemination of tumor cells.

MATERIALS AND METHODS

Classification of NHLs and clinical data of patients. The classification of NHLs was performed according to the criteria of the Kiel classification17 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.18 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 Rai19 (RAI 0, lymphocytosis in blood and bone marrow only; I, lymphocytosis and enlarged lymph nodes; II, lymphocytosis plus hepatosplenomegaly, 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 Binet20 (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.21 A hematogenous spread

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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 (Nycodenz; Oslo, Norway). In patients with B-NHL with large numbers of contaminating normal T lymphocytes, these cells were depleted by sheep 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) anti-Leu-12 (CD19; Becton Dickinson), anti-Leu-14 (CD20), anti-Leu-16 (CD21), anti-Leu-22 (CD45), and anti-Leu-17 (CD5) (Becton Dickinson). Reactive peripheral blood lymphocytes (PBLs) from R.S. were selected as the reference population.

Immunophenotyping of lymphomas. The presence of relevant adhesion structures was investigated in an indirect immunoperoxidase staining technique on cytospin preparations. Staining intensity was scored semiquantitatively on a scale of 0 to + (0, negative; +, faint, not clearly above background; +, weak positive; 2+, moderate 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-CD11c, LeuM5 (Becton Dickinson, Mountain View, CA); anti-Leu-8, (L-selectin) Becton Dickinson; anti-ICAM-1 (CD54), 7F12 (gift of M. P. Dierich); anti-CD44, F 10-44-22 (gift of R. Dalechau); anti-VLA-a4 chain (CD49d), 4B5G10 (gift of M.E. Hemler).

In vitro assay of lymphocyte binding to HEVs. The HEV-binding assay was performed by the method of Butcher et al., a modification of the assay originally described by Stamper and Woodruff. In brief, 100 μL of 2 × 10⁵ cells suspended in HEPES-buffered RPMI 1640 were incubated on 8 μL fresh-frozen sections of lymph nodes or tonsils in a wax pen circle. These lymphatic tissues were taken from surgical or biopsy specimens pathologically determined to be normal. The sections were rotated on a shaker for 30 minutes at 7°C during agitation. Medium and nonadherent cells were removed by rapping the edge of the slide against an absorbent towel. Subsequently, slides were fixed overnight in 1% paraformaldehyde in phosphate-buffered saline (PBS) and examined microscopically under phase-contrast illumination.

To facilitate quantitative comparison, an internal standard population of fluorescein isothiocyanate (FITC)-labeled lymphocytes (from healthy donors) was mixed with each sample population. Reference samples of unlabeled peripheral blood lymphocytes (PBLs) were included in each experiment to permit comparison of experiments. The relative adherence ratio (RAR) was determined as described by Butcher et al. The RAR is equivalent to the calculated number of sample cells bound to HEVs in comparison with reactive reference PBLs bound under the same conditions.

Identification of the adhering cells deserves special attention. With regard to their identification in the HEV-binding assay, we stained cells after HEV adherence and after fixation similar to the procedure mentioned by Butcher et al. 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 Tul (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. 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 |
|-----------------------------|-----------|
| Individual | Relative Adherence Ratio (±SE) |
| R.S.* | 1.09 ± 0.15 |
| F.O. | 1.06 ± 0.14 |
| S.H. | 1.14 ± 0.15 |
| H.S. | 0.95 ± 0.08 |

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 III, IV: median 0.95 v 1.19; \(P < .9\); Rai 0, I, II v III, 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-\(\alpha 4\) 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 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 properties. Conclusions from electron microscopy29 and from adhesion studies of neoplastic plasma cells to rat HEVs,30 as well as from distribution experiments of labeled lymphoma cells in humans31,32 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 cells33 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.

**Fig 1.** (A) Phase-contrast illumination showing a large HEV of a human lymph node. The HEV is identified by the typical nuclei (arrow) and the characteristic contour, which is outlined by the basement membrane (original magnification \(\times 62.5\)). (B) Phase-contrast illumination showing an HEV with numerous bound lymphocytes that are lying in focus above the plane of the section. (C) Fluorescent staining of the same HEV showing FITC-labeled lymphocytes from healthy donors as internal standard population; CLL cells also reveal a distinct HEV binding and are identified by MoAb VIB-C5 (CD24) and TRITC conjugate (original magnification \(\times 62.5\)).
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, the better the extent of lymph node involvement is integrated. 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 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 (CDllb/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.\textsuperscript{35,36} Adhesive processes might be mediated by combinations of molecules\textsuperscript{13} or by alternatively spliced or glycosylated isoforms,\textsuperscript{37,38} 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 (data not shown).

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**REFERENCES**


38. Lesley J, Hyman R: CD44 can be activated to function as a hyaluronic acid receptor in normal murine T cells. Eur J Immunol 22:2719, 1992
Adhesion to high endothelial venules: a model for dissemination mechanisms in non-Hodgkin's lymphoma

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