Monoclonal antibody HBME-1 reacts with a minor subset of B cells with villous surface and can be useful in the diagnosis of hairy cell leukemia and other indolent lymphoproliferations of villous B lymphocytes

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Abstract The Hector Battifora mesothelial epitope-1 (HBME-1) monoclonal antibody has been generated against human mesothelioma cells and recognizes a biochemically unknown membrane epitope. We have accidentally found that the HBME-1 reacts with scattered lymphocytes showing villous surface in hyperplastic lymphoid tissue. To evaluate its reactivity pattern, we have performed a consecutive immunohistochemical study in nonneoplastic bone marrow and lymphoid samples ($n=40$), as well as in malignant lymphoproliferations ($n=427$), including hairy cell leukemia (HCL) ($n=72$), HCL variant (HCL-v) ($n=13$), splenic diffuse red pulp small B cell lymphoma (SDRPL) ($n=8$), splenic B cell marginal zone lymphoma (SMZL) ($n=59$), and splenic B cell lymphoma/leukemia, not further classifiable on bone marrow morphology (SBCL) ($n=37$) cases. The staining pattern of HBME-1 was compared to DBA.44. HBME-1$^+$ villous lymphocytes were constantly detected in low number in nonneoplastic lymphoid tissues. With multicolor immunofluorescence staining, HBME-1$^+$ lymphocytes showed a CD20$^+$/CD79a$^+$/IgM$^+$ B cell phenotype. In B cell lymphoproliferations of villous lymphocytes, HBME-1 reactivity was demonstrated in 96 % of HCL, 39 % of HCL-v, 50 % of SDRPL, 12 % of SMZL, and 19 % of SBCL cases. Nodal and extranodal marginal zone lymphoma cases were positive in 12 % of the cases. A small minority (4 %) of the other B cell lymphomas and no T cell lymphoma revealed tumor cell reactivity with HBME-1. In conclusion, our study has established that HBME-1 reacts with a minor subset of B lymphocytes and a small proportion of B cell lymphomas, which has not been described previously. We suggest that HBME-1 can be a useful marker in the diagnosis of HCL and other indolent lymphoproliferations of villous B lymphocytes.

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Introduction

The monoclonal antibody to Hector Battifora mesothelial epitope-1 (HBME-1) has been generated against a biochemically unknown epitope presented in the membrane of the mesothelial cells, outlining their microvillous surface in a so-called thick membrane pattern [2, 19, 34]. HBME-1 is a mouse monoclonal antibody of IgM-class, which suggests that it detects a carbohydrate determinant on a glycoprotein or a polysaccharide antigen [10].

Table 1 Cases and specimens studied

	Bone marrow	Spleen	Lymph node	Other	Total
Nonneoplastic	15	13	12	10	40
B cell lymphomas	198	66	90	50	404
HCL	70	2	0	0	72
HCL-v	8	5	0	0	13
SDRPL	0	8	0	0	8
SMZL	15	44	0	0	59
SBCL	37	0	0	0	37
MZL	0	0	30	21	51
MCL	17	4	9	3	33
B-CLL	34	0	15	0	49
WM	7	0	0	0	7
PC/MM	7	0	0	3	10
FL	1	2	14	4	21
DLBCL	1	1	22	17	41
BL	1	0	0	2	3
T cell lymphomas	2	1	9	6	18
T-LBL	2	0	0	0	2
ALCL	0	0	7	2	9
EATL	0	0	0	3	3
PTCL NOS	0	1	2	1	4
Hodgkin's lymphoma	0	0	4	1	5

ALCL anaplastic large cell lymphoma, *B-CLL* B cell chronic lymphocytic leukemia, *BL* Burkitt lymphoma, *EATL* enteropathy-associated T cell lymphoma, *FL* follicular lymphoma, *HCL* hairy cell leukemia, *HCL-v* hairy cell leukemia variant, *MCL* mantle cell lymphoma, *MZL* marginal zone lymphoma, *PC/MM* plasmacytoma and multiple myeloma, *PTCL NOS* peripheral T cell lymphoma, not otherwise specified, *SBCL* splenic B cell lymphoma/leukemia, unclassifiable, *SDRPL* splenic diffuse red pulp small B cell lymphoma, *SMZL* splenic B cell marginal zone lymphoma, *T-LBL* T lymphoblastic lymphoma/leukemia, *WM* Waldenström's macroglobulinemia

HBME-1 shows reactivity in a rather broad range of epithelial and mesenchymal tumors and represents a multipurpose immunohistochemical marker in surgical pathology. It can be utilized to differentiate epithelial mesothelioma from lung adenocarcinoma [2, 19] as well as to distinguish malignant thyroid tumors from benign thyroid lesions [5, 20]. Peritoneal and ovarian serous carcinomas also display HBME-1 reactivity [26]. Of the mesenchymal tumors, chordoma, synovial sarcoma, and chondrosarcoma demonstrate HBME-1 reactivity [21, 25], which can be employed in the diagnostic work-up of these neoplasms. Interestingly, each of these HBME-1-positive tumors also displays microvillous membrane projections [6, 24, 27, 30].

We have accidentally found that the HBME-1 reacts with scattered lymphocytes showing villous surface in hyperplastic lymphoid tissue. This observation has prompted us to evaluate this antibody in a wide variety of nonneoplastic and neoplastic bone marrow and lymphoid tissue samples, including hairy cell leukemia (HCL) and its mimics.

Materials and methods

Patients and tissue samples

All cases were retrieved from the files of the Laboratory of Tumor Pathology and Molecular Diagnostics, Szeged, Hungary.

The study included nonneoplastic bone marrow ($n=15$), lymph node ($n=12$), splenectomy ($n=13$), tonsil ($n=7$), and gastric biopsy ($n=3$) samples, in addition to a series of malignant lymphoproliferations ($n=427$), including HCL ($n=72$), HCL variant (HCL-v) ($n=13$), splenic diffuse red pulp small B cell lymphoma (SDRPL) ($n=8$), splenic B cell marginal zone lymphoma (SMZL) ($n=59$), indolent splenic B cell lymphoma, not further classifiable on bone marrow morphology (SBCL) ($n=37$), nodal ($n=30$) and extranodal ($n=21$) marginal zone lymphomas (MZL), other B cell lymphomas ($n=164$), classical Hodgkin's lymphoma (cHL) ($n=5$), and T cell lymphomas (TCL) ($n=18$). The cases and specimens studied are summarized in Table 1.

Each case had been previously diagnosed on the basis of clinical information, histomorphology, flow cytometry, and immunophenotypic characteristics. Diagnosis of SMZL was made on splenectomy specimens showing the characteristic involvement [13], or on bone marrow trephine biopsies showing the typical interstitial/intrasinusoidal infiltration with larger nodules containing a follicular dendritic cell meshwork [13, 28]. The diagnosis of SDRPL was restricted to cases of which splenectomy material was available, showing a characteristic diffuse pattern of involvement of the red pulp [15, 28]. We created an SBCL category for those small B cell lymphoma cases with intrasinusoidal bone marrow involvement for which the differential diagnosis was narrowed down to indolent splenic B cell lymphoma, but splenectomy has not been performed
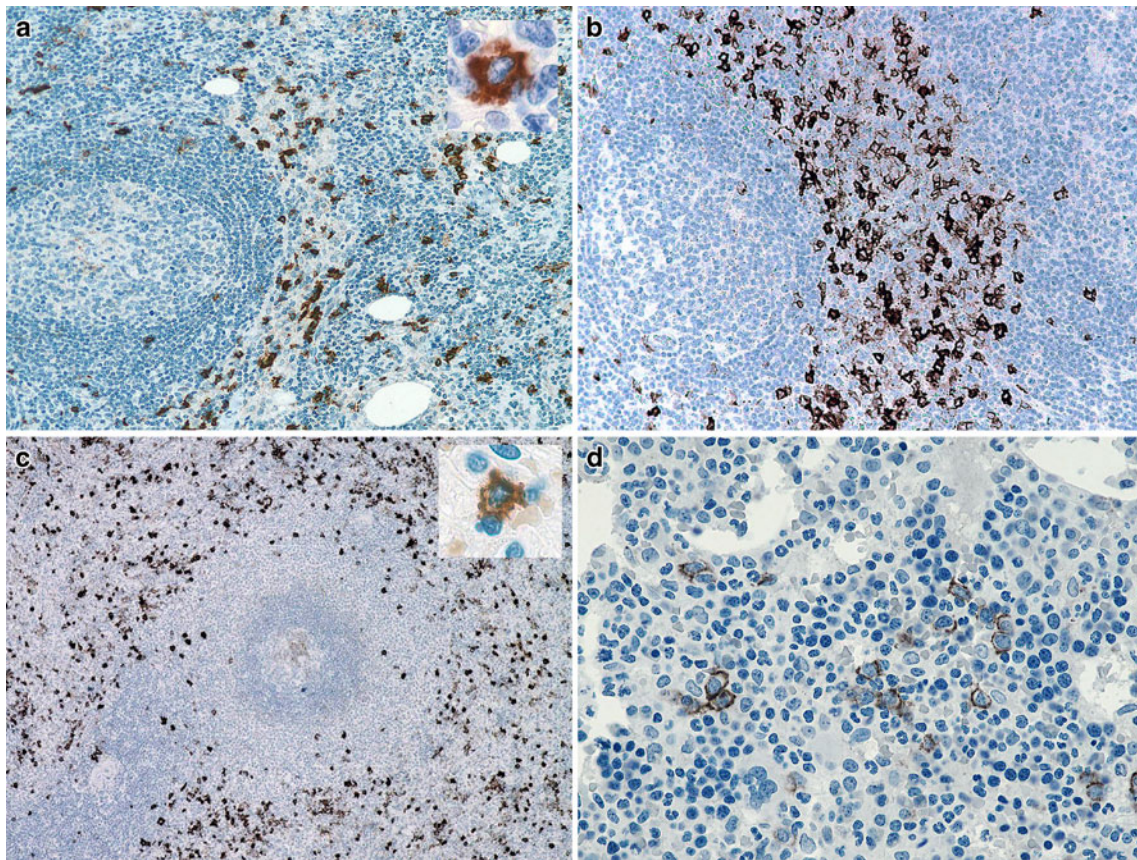
Fig. 1 a HBME-1-positive sinusoidal and perisinusoidal lymphocytes are demonstrated in a hyperplastic lymph node, showing a strikingly villous cell membrane (insert) (×200; *insert*, ×1,000). **b** The majority of the monocytoid B cells reveals HBME-1 staining in a toxoplasma lymphadenitis (×200). **c** Up to 10 % HBME-1-positive red pulp lymphocytes with villous cell surface (*insert*) and no follicular lymphoid cells are seen in a splenectomy specimen removed due to therapy-resistant ITP (×100; *insert*, ×1,000). **d** Scattered HBME-1 positive early erythroid precursor cells in a case of erythropoietic hyperplasia (×400)

until the selection into the study, since the bone marrow morphology alone, in most cases, is insufficient to separate SMZL and SDRPL [13, 15, 28, 29].

The bone marrow trephine biopsies were fixed for 12–24 h in neutral buffered formalin supplemented with methanol and glucose (Schaffer's fixative) [9]. Since our pilot study demonstrated significant reduction of HBME-1 staining after acidic decalcification, bone marrow trephines were decalcified in 12.5 % (*w/v*) EDTA (Sigma-Aldrich) solution (adjusted to pH 7.0 by cc NaOH) at 60 °C for 16–24 h. The other samples were fixed for 24–72 h in 10 % (*v/v*) neutral buffered formalin. Each specimen was routinely embedded into paraffin.

Tissue microarray construction

Tissue microarrays (TMAs) were made with the Tissue Micro-Array Builder instrument (Histopathology Ltd., Pécs, Hungary), according to the manufacturer's instructions. Briefly, the recipient paraffin block with 24 holes arranged in four columns and six rows was formed with the TMA Builder. Cores of 2.0 mm

from the donor paraffin blocks were punched out with the paraffin-punch extractor and were arrayed in the recipient paraffin block. Each sample of interest was represented at least in duplicates.

Table 2 HBME-1 staining pattern in nonneoplastic tissues

Tissue	HBME-1-positive cells
Bone marrow	Scattered interstitial lymphocytes with villous surface
	Some early erythroid precursors
Lymph node	Scattered sinusoidal B lymphocytes with villous surface, many monocytoid B cells
	Some follicular and extrafollicular dendritic cells
Spleen	Up to 10 % red pulp lymphocytes, few marginal zone cells
	Some chordal histiocytes with dendritic cell morphology
Tonsil	Scattered interfollicular lymphocytes with villous surface
	Some intra- and subepithelial lymphocytes
	Some follicular and extrafollicular dendritic cells
Chronic gastritis	Scattered lymphocytes with villous surface

Immunohistochemistry and evaluation of the reactions

The immunohistochemical reactions were executed on whole tissue sections or TMA slides. Briefly, 2–4-μm-thick sections were cut and dewaxed in xylene and graded ethanol. Following blocking of endogenous peroxidase activity in ethanol containing 1.5 % (v/v) H_2O_2, heat-induced antigen retrieval was performed using household electronic pressure cooker (Avair IDA) in Target Retrieval Solution pH 6.10 (DAKO; Glostrup, Denmark). After protein blocking in TRIS-buffered saline (pH 7.4) containing 5 % (w/v) low-fat milk powder, the sections were incubated with the primary antibodies HBME-1 (DAKO; 1:50), DBA.44 (Thermo Scientific, Rockford, IL, USA; 1:200), or CD20/L26 (DAKO; 1:200), at room temperature for 70 min. Detection was performed using Novolink polymer kit (Leica Biosystems/Novocastra), and nuclear staining was carried out with Mayer's hematoxylin.

To analyze the diagnostic value in HCL, HCL-v, SDRPL, SMZL, and SBCL cases, HBME-1 staining was evaluated in conjunction with CD20, and the results were compared against staining with DBA.44.

The samples were independently assessed by two of the authors (JTL and LK), and for discordant cases, consensus was reached by a second look evaluation made jointly. Since up to 10 % HBME-1-positive reactive lymphocytes can occur in hyperplastic lymphoid tissues, an arbitrary cut-off of 20 % staining of CD20-positive B cells for the HBME-1 or DBA.44 was defined as positive in neoplastic samples.

Multicolor immunofluorescence staining

To evaluate the phenotype of the HBME-1$^+$ cells, multicolor immunofluorescence staining was performed in tissue samples of reactive lymph node and spleen. Briefly, paraffin sections were cut and pretreated as shown above. Next, the sections were blocked with 10 % nonimmune goat serum and incubated with HBME-1 (1:50) in various combinations with CD20/L26 (1:200), CD35/Ber-MAC-DRC (1:50), CD68/KP1 (1:100), CD79α/JCB117 (1:40), IgD/rabbit polyclonal (1:200), IgM/ rabbit polyclonal (1:500), or CD3ε/rabbit polyclonal (1:500) antibodies (each primary antibody from DAKO) in performing double and triple immunofluorescence stains. The primary

Fig. 2 a Multicolor immunofluorescence staining for HBME-1 (green), CD20 (red), and IgD (blue) in a nonneoplastic splenic sample demonstrates that most HBME-1$^+$ lymphocytes are CD20$^+$ B cells and some of them coexpress immunoglobulin delta heavy chain (confocal microscopy; ×1,000)
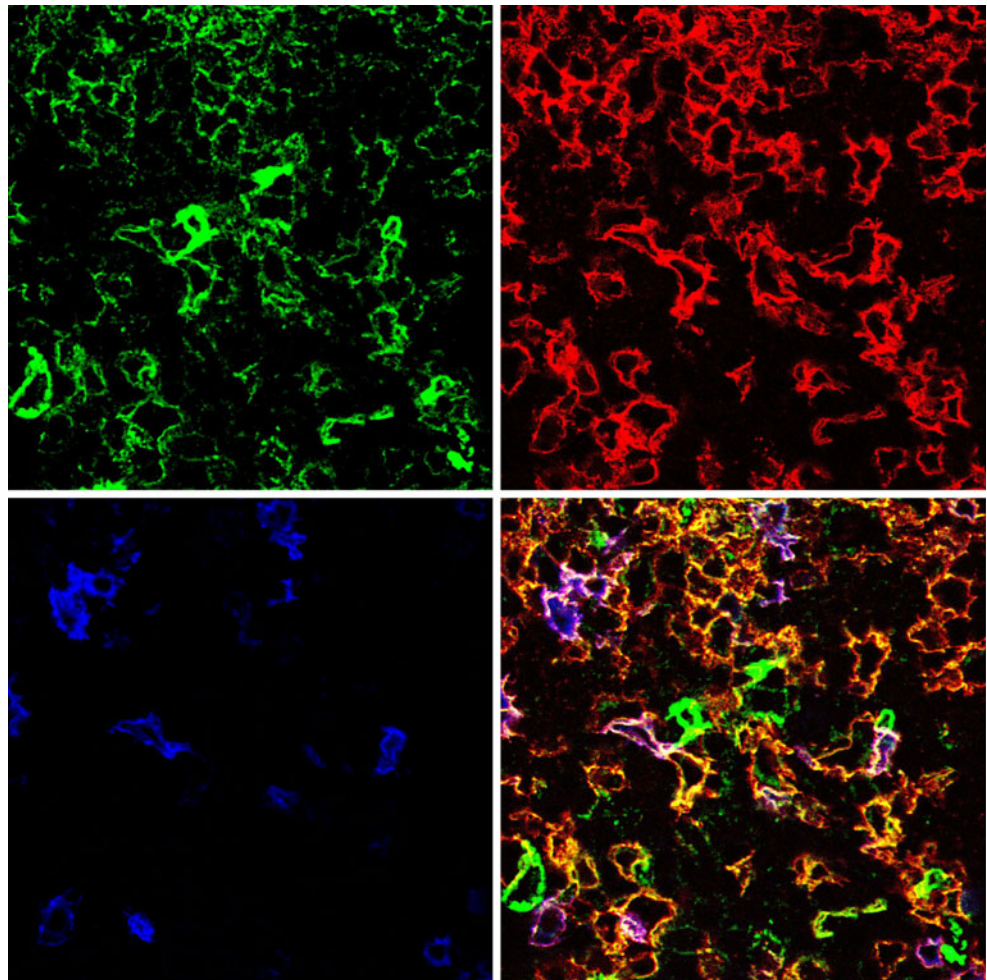
Table 3 Results of HBME-1 and DBA.44 immunophenotyping in B cell lymphomas

| Markers	HBME-1	DBA.44
HCL	69/72 (95.8 %)	70/72 (97.2 %)
HCL-v	5/13 (38.5 %)	11/13 (84.6 %)
SDRPL	4/8 (50 %)	5/8 (62.5 %)
SMZL	7/59 (11.9 %)	18/59 (30.5 %)
SBCL	7/37 (18.9 %)	12/37 (32.4 %)
MZL	6/51 (11.8 %)	2/11 (18.2 %)
MCL	2/33 (6.1 %)	2/21 (9.5 %)
B-CLL	0/49 (0 %)	0/11 (0 %)
WM	0/7 (0 %)	0/7 (0 %)
PC/MM	0/10 (0 %)	0/1 (0 %)
FL	3/21 (14.3 %)	0/2 (0 %)
DLBCL	1/41 (2.4 %)	1/5 (20 %)
BL	0/3 (0 %)	0/3 (0 %)

antibody combinations used were as follows: HBME-1/CD20, HBME-1/CD79a, HBME-1/IgM, HBME-1/CD3, HBME-1/CD68, HBME-1/CD20/IgD, HBME-1/CD35/IgD, and HBME-1/CD35/CD3. This step was followed by an incubation with fluorophore-conjugated secondary antibodies [FITC-goat F(ab′)2 antimouse IgM, 1:100; Cy3-goat antimouse IgM, 1:100; Cy3-goat antimouse IgG, 1:100; Cy5-goat antirabbit IgG, 1:100; FITC-goat antimouse IgG, 1:100; each from Life Technologies/Molecular Probes, Grand Island, NY, USA], mixed according to the species and immunoglobulin class of the primary antibodies applied. The sections were analyzed using a confocal laser scanning microscope (Leica TCS2, Leica, Bensheim, Germany).

Results

HBME-1 immunohistochemistry and multicolor immunofluorescence imaging in nonneoplastic samples

In the nonneoplastic samples, scattered HBME-1$^+$ sinusoidal and perisinusoidal lymphocytes were constantly encountered in the hyperplastic lymph nodes, showing a strikingly villous cell membrane, and similar cells could also be detected in the bone marrow and splenic red pulp. The positive cells were usually found in low number, but in the splenic specimens with marginal zone hyperplasia, removed due to traumatic rupture or in therapy-resistant idiopathic thrombocytopenic purpura (ITP), up
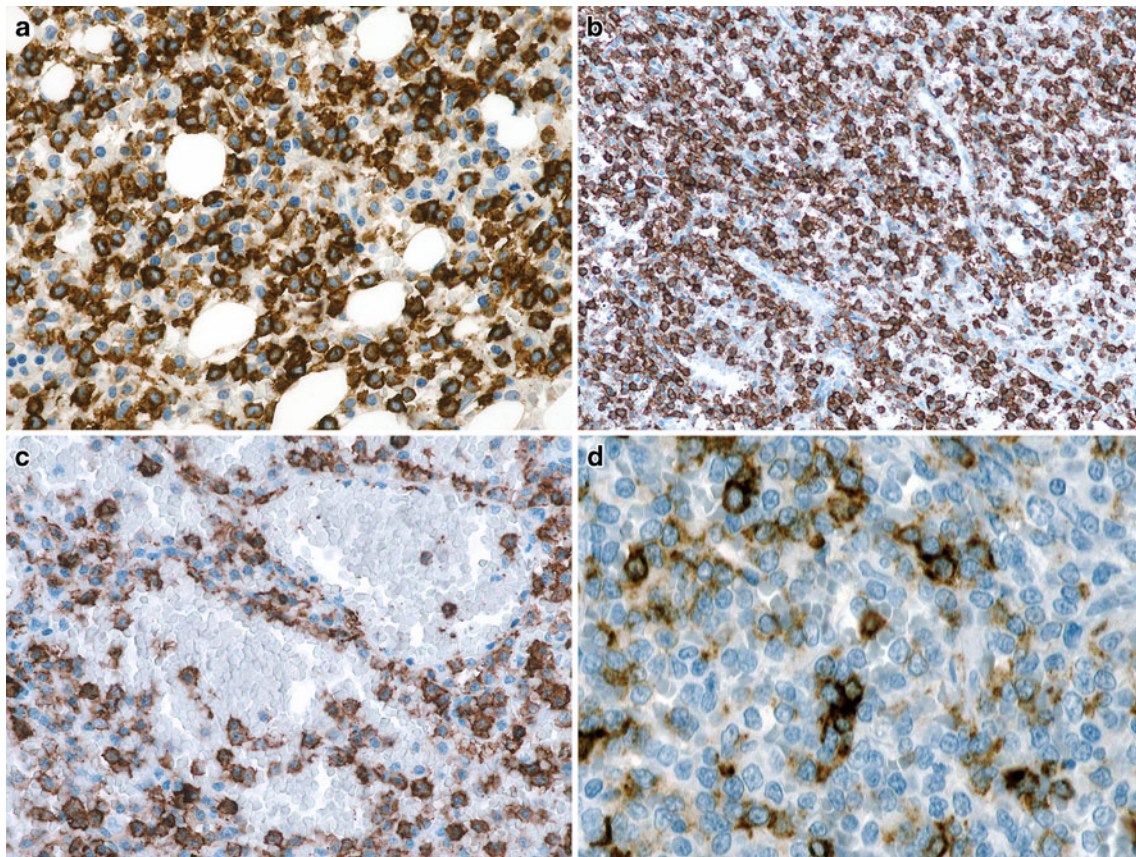
Fig. 3 **a** Bone marrow infiltration of a HCL case displays intense HBME-1 reactivity (×400). **b**, **c** The HBME-1 reactivity in splenic infiltration of HCL is shown (×100 and ×400). **d** Focal, mostly weak HBME-1 reactivity of the neoplastic red pulp B cells in a SDRPL case (×1,000)

to 10 % red pulp lymphocytes were detected, and a significant proportion of monocytoid B cells in toxoplasma lymphadenitis demonstrated HBME-1 staining (Fig. 1a–c). The staining pattern of the HBME-1 and DBA.44 revealed partly overlapping features with as main exception strong mantle zone staining found only with DBA.44. Apart from the villous lymphocytes, only a few nonlymphoid cells demonstrated HBME-1 staining. Some follicular dendritic cells and few extrafollicular or chordal reticular cells showed inconsistent weak positivity. In the normal bone marrow samples, an inconsistent low number of interstitial small lymphocytes with villous surface were found. Scattered early erythroid precursor cells, mostly in erythropoietic hyperplasia, also displayed HBME-1 staining (Fig. 1d). The staining pattern in nonneoplastic tissues is summarized in Table 2.

According to the multicolor immunofluorescence staining we used, the HBME-1$^+$ lymphocytes had a CD20$^+$/CD79a$^+$/IgM$^+$/CD35$^-$/CD3$^-$ B cell phenotype both in lymph node and spleen (Fig. 2). In addition, part of the splenic HBME-1$^+$ B lymphocytes also displayed IgD reactivity (Fig. 2). In nonlymphoid cells, HBME-1 stained few CD68$^+$ chordal histiocytes as well as weakly and inconsistently stained a few CD35$^+$ follicular dendritic cells.

Neoplastic samples

HBME-1 staining of tumor cells was exclusively detected in B cell neoplasms. The results are detailed in Table 3.

Nearly all HCL cases (69/72; 95.8 %) showed HBME-1 positivity (Fig. 3a–c), closely matching with the DBA.44 (70/72; 97.2 %). Comparing the proportion of the positive neoplastic cells, no considerable difference was noted for HBME-1 or DBA.44.

In other B cell lymphoproliferations of splenic origin, HBME-1 reactivity was found in 5/13 (39 %) of HCL-v, 4/8 (50 %) of SDRPL (Fig. 3d), 7/59 (12 %) of SMZL, and 7/37 (19 %) of SBCL cases. Weak and focal HBME-1 positivity was found in the majority of SMZL and SBCL cases. A higher DBA.44 reactivity rate was found in each of these splenic lymphomas—11/13 (85 %) of HCL-v, 5/8 (63 %) of SDRPL, 18/57 (31 %) of SMZL, and 12/37 (32 %) of SBCL.

Nonsplenic MZL cases revealed an HBME-1 reactivity rate (6/51; 12 %) similar to SMZL, which was also exceeded by DBA.44 (2/11; 18 %). Nodal and extranodal marginal zone lymphoma cases showed 13 % (4/30) and 10 % (2/21) HBME-1 reactivity, respectively. HBME-1 reactivity in nonsplenic MZL cases was associated with monocytoid B cell morphology. A small minority (6/164; 3.7 %) of the other B cell lymphomas demonstrated positivity with the HBME-1. Three cases (3/21; 14.3 %) of follicular lymphoma, two cases (2/33; 6 %) of mantle cell lymphoma (MCL), and one case (1/41; 2 %) of diffuse large B cell lymphoma of probably transformed MZL demonstrated tumor cell reactivity with HBME-1.

Apart from scattered reactive lymphocytes and some reticular cells, no HBME-1 reactivity was detected in the T cell lymphoma and Hodgkin's lymphoma cases tested.

Discussion

The monoclonal antibody to HBME-1 was raised against an epitope present in the membrane of mesothelial cells [2, 19]. The target epitope is located in microvilli [34]. We have accidentally found that HBME-1 reacts with scattered lymphocytes showing villous surface in hyperplastic lymphoid tissue. To evaluate its reaction pattern, we have performed a consecutive immunohistochemical study in a wide variety of nonneoplastic and neoplastic bone marrow and lymphoid tissue samples.

The HBME-1-positive lymphocytes were constantly demonstrated in nonneoplastic lymph node, tonsil, bone marrow, and spleen. The positive cells were detected in low number, but in some hyperplastic splenic tissues, up to 10 % of red pulp lymphocytes and a significant proportion of monocytoid B cells in toxoplasma lymphadenitis showed HBME-1 staining. Confocal microscopy with multicolor immunofluorescence staining confirmed that the HBME-1-positive lymphocytes represent B cells. The staining pattern of the HBME-1 and DBA.44 revealed overlapping features with as main exception strong mantle zone staining found only with the DBA.44. The majority of the HBME-1$^+$ lymphocytes displayed a villous cell membrane, which cells may represent a unique B cell population. The relationship between these villous lymphocytes and interfollicular large B cells with stellate morphology, described in peripheral lymphoid tissue [16], is yet to be elucidated.

Since HBME-1-positive reactive lymphocytes can be found in the splenic red pulp and most of these cells show a villous surface, one might expect the HBME-1 to be positive in B cell lymphoproliferations with villous membrane projections; therefore, HCL and its mimics were overrepresented in our series (overall 189/427 cases). HBME-1 staining of lymphoid tumor cells was exclusively detected in B cell neoplasms, supporting that the HBME-1 reacts with an epitope on B lymphoid cells.

As we anticipated, the HBME-1 staining was high in B cell lymphoproliferations with villous cells, including nearly all cases (96 %) of HCL. HCL is an uncommon B cell lymphoproliferation characterized by pancytopenia, leukemic cells with 'hairy' projections, interstitial bone marrow (BM) as well as splenic red pulp involvement, and an indolent clinical course [8]. Neoplastic cells, as detected by flow cytometry, show "pan B cell antigen" (e.g., CD19, CD20, and CD22) reactivity with coexpression of several non-B cell molecules, like CD11c, CD25, CD103, and CD123 [4, 8, 17, 31]. Since circulating neoplastic cells are typically presented in small number and HCL-associated reticulin fibrosis results in "dry tap" aspiration, the diagnosis is best made on BM trephine biopsy [8]. Several antibodies have been reported effective on fixed and paraffin-

embedded BM samples, including DBA.44 [11, 38], TRAP [12, 14, 38], cyclin D1 [22, 35], CD11c [37], CD25, and CD103 [23], but each of these markers has limitations. Furthermore, HCL cases negative for conventional markers have been described [3, 32, 35]. Recently, annexin A1 has been reported to have the highest specificity [7], but assessment of the reactivity with this marker is often very difficult, due to its strong expression also in the granulocytic series [7, 35]. Due to these aspects, a panel of immunohistochemical stains, complemented with CD20, might still have to be applied [7, 35]. Most recently, application of BRAF V600E mutation-specific antibody [1] and immunohistochemical assessment of MEK-ERK pathway activation status using phospho-ERK1/2 specific antibody [36] have been reported as particularly useful in the diagnosis of HCL [1]. In our hands, HBME-1 showed a very high reactivity rate (96 %) in HCL, which was concordant with that of DBA.44 in our current study (98 %), and also with Annexin A1 (96 %) found in a recent study [35]. Our study has established that HBME-1 is at least as sensitive as but appreciably more specific as DBA.44 in detecting HCL tumor cells, and can be particularly useful in the differential diagnosis of indolent B cell lymphomas with confluent interstitial bone marrow involvement. Since HBME-1 is widely applied in surgical pathology and can be part of the antibody inventory in most immunohistochemical laboratories, it constitutes a simple, inexpensive assay of HCL, easily fitted in the diagnostic antibody panel for routinely fixed and processed samples.

As it requires special therapy, HCL must be distinguished from HCL-v, SDRPL, SMZL, and from other indolent B cell lymphoproliferations with predominant bone marrow and splenic involvement [8]. HBME-1 is significantly less often positive in HCL-v and SDRPL (39 and 50 %, respectively) than in HCL, usually with weaker staining intensity. HCL-v is a rare clinico-pathological entity, showing morphologic and phenotypic overlaps with HCL, some features intermediate between B cell prolymphocytic leukemia and HCL, and resistance to conventional HCL therapy [18, 28, 33]. HCL-v cases also overlap with SDRPL, and both are defined as provisional entities in the 2008 update of the WHO classification [28]. Both show diffuse splenic red pulp involvement and DBA.44 reactivity [15, 18, 28], and the latter was also confirmed by our study. The SMZL and SBCL cases revealed similar low HBME-1 reactivity rate, since the great majority of our SBCL cases should comprise SMZL.

In nonsplenic B cell lymphomas, HBME-1 reactivity was found in nodal and extranodal MZL cases in a similar low rate to SMZL and was associated with monocytoid B cell morphology, which is parallel to HBME-1 staining of reactive monocytoid B cells in hyperplastic lymph nodes.

In conclusion, our study has established that HBME-1 reacts with a minor subpopulation of B cells exhibiting a villous surface, which has not been described previously. We propose that the HBME-1 can be a useful marker in the diagnosis of HCL and other indolent lymphoproliferations of villous B lymphocytes.

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