Recognition of a B Cell Leukemia-Associated Minor Histocompatibility Antigen by CTL[1]

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CTL directed against minor histocompatibility Ags (mHag) play a major role in antileukemia reactivity after HLA-identical bone marrow transplantation. Some of these mHag are restricted to hemopoietic cells, others show a broad tissue expression. Therefore, antileukemia reactivity is often associated with graft-vs-host disease. Here, we report the identification of a B cell leukemia-associated mHag, HB-1, recognized by a CD8$^+$ CTL clone derived from peripheral blood of an acute lymphoblastic B cell leukemia patient who has been treated by HLA-matched bone marrow transplantation. Interestingly, the CTL clone that recognizes HB-1 exhibits specific cytotoxicity toward leukemic as well as EBV-transformed B cells, but not against untransformed B cells. Moreover, the CTL clone does not lyse PHA-stimulated T cell blasts, monocytes, and fibroblasts, indicating that HB-1 is mainly expressed by transformed B cells. Further analysis reveals that HB-1 is restricted by HLA-B44 (both *B*4402* and *B*4403*) and that 28% of HLA-B44-positive individuals express HB-1. These findings demonstrate that leukemia-associated mHag with a restricted tissue distribution, such as HB-1, elicit CTL reactivity in vivo. These Ags are of potential use in immunotherapy against leukemia because they generate antileukemia reactivity that is not associated with graft-vs-host disease. ***The Journal of Immunology,* 1997, 158: 560–565.**

T cell reactivity induced by disparities in minor histocompatibility Ags (mHag)[3] between donor and patient plays a major role in HLA-identical bone marrow transplantation (BMT) (1–3). CTL directed against mHag of the patient generally cause graft-vs-host disease (GVHD), which, although a complication in allogeneic BMT, is strongly associated with graft-vs-leukemia (GVL) reactivity (4, 5). Depletion of the allogeneic bone marrow graft from T cells to reduce occurrence of GVHD is correlated with an increased risk of recurrent leukemia (5). GVL reactivity is not observed in transplantation between identical twins in which anti-mHag responses are lacking (5). These clinical data indicate that CTL responses against mHag may be responsible for the GVL reactivity.

mHag are derived from intracellular proteins, and CTL that recognize these mHag in a MHC-restricted manner have been isolated from BMT recipients (6–9). Expression of some mHag is restricted to hemopoietic cells, including leukemic cells; others are expressed by cells of all tissues (8, 10–14). The identity of mHag, besides the recently identified HA-2 and H-Y antigenic peptides (15, 16), is unknown. HA-2 is most probably derived from an as yet unidentified non-filament-forming class I myosin protein and H-Y from the male-specific SMCY protein (15, 16).

Since mHag expressed by all host tissues induce GVHD and GVL, it is of great importance to identify leukemic restricted Ags because these exert antileukemia reactivity without GVHD. Clinical data and in vitro studies support the notion that GVL may indeed exist without the development of GVHD (4, 5, 13, 17, 18). Here, we investigated whether leukemia cell restricted mHag-specific CTL can be isolated from patients after HLA-identical BMT. We have identified a mHag-specific CTL clone directed against a new mHag, designated HB-1. HB-1 is mainly expressed by B-acute lymphoblastic leukemia (B-ALL) cells and by EBV-transformed B cells, and is recognized in association with HLA-B44.

Materials and Methods

mAb and immunofluorescence analysis

The following mAb were used for immunofluorescence analysis or for inhibition of cytotoxicity: TS2/18 (CD2), SPV-T3b (CD3), RIV-7 (CD4), WT82 (CD8), L15 (CD11a), F10.2 (CD54), TS2/9 (CD58), CRI304.3 (anti-TCRBV6S1), OT145 (anti-TCRBV6S7), E17.5F3 (anti-TCRBV17), W6/32 (anti-HLA-class I), and Q5/13 (anti-HLA-DR/DP). Immunofluorescence was performed by the indirect method. FITC-conjugated goat F(ab')$_2$ anti-mouse IgG and IgM (Tago Immunologics, Camarillo, CA) was used for staining followed by analysis by an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL).

CTL cultures

CD8$^+$ T cells were isolated from PBL of patient MP (a 42-yr-old woman with a B-ALL) 9 mo after an HLA-identical BMT using anti-CD8 immunomagnetic beads (Dynal, Olso, Norway). A CTL line was established by stimulating CD8$^+$ T cells (5 × 10^5/ml) with irradiated leukemic cells (10^6/ml) and autologous donor PBMC as feeder cells (2.5 × 10^5/ml) in IMDM (Life Technologies, Paisley, Scotland) plus 10% human serum. On day 7, cells were restimulated with irradiated leukemic cells from the patient (10^6/ml), and 100 U/ml IL-2 (Glaxo, Geneva, Switzerland) was added. From day 14 on, cultures were expanded and restimulated weekly with irradiated EBV transformed-lymphoblastoid cell lines (EBV-LCL) of the patient pre-BMT (10^6/ml), 100 U/ml IL-2, and 5 ng/ml IL-12 (Hoffmann-La Roche, Nutley, NJ).

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Received for publication July 1, 1996. Accepted for publication October 16, 1996.

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[1] This work was supported in part by grants from the Ank van Vlissingen Foundation and the Maurits and Anna de Kock Foundation.

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[3] Abbreviations used in this paper: mHag, minor histocompatibility antigen; BMT, bone marrow transplantation; GVHD, graft-vs-host disease; GVL, graft-vs-leukemia; B-ALL, B-acute lymphoblastic leukemia; EBV-LCL, Epstein-Barr virus lymphoblastoid cell line; ICAM-1, intercellular adhesion molecule 1; IMDM, Iscove's modified Dulbecco's medium; dNTP, 2'-deoxynucleoside 5'-triphosphate.

 0022-1767/97/$02.00

Target cells

Leukemic cells were collected from B-ALL patients at diagnosis. Fibroblast cell cultures were generated from bone marrow obtained from patient MP pre-BMT. Fibroblasts and EBV-LCL were cultured in IMDM plus 10% FCS. Monocytes were isolated after adherence to plastic. T cell blasts were generated by stimulating PBMC with 4 μg/ml PHA in IMDM plus 10% human serum for three days. T cell blasts were washed and further cultured with 100 U/ml IL-2 for three days. B cells were obtained by positive selection using anti-CD19 immunomagnetic beads (Dynal). B cell blasts were generated by stimulating 10^6 CD19$^+$ B cells with 5×10^4 CD32-transfected mouse fibroblastic L cells and 0.5 μg/ml CD40 mAb for 2 to 4 days. To increase susceptibility of B-ALL cells, fibroblasts, and CD40-stimulated B cells to specific CTL lysis, these cells were incubated with 10 ng/ml TNF-α (Boehringer Ingelheim, Alkmaar, The Netherlands) for 2 days.

Chromium release assay

Chromium release assays were performed as previously described (19). Fibroblast targets were labeled with 150 μCi ^{51}Cr for 18 h (20).

IFN-γ release assay

EBV-LCL and B cell blasts were tested for their ability to stimulate the production of IFN-γ by the CTL. Briefly, 10^4 CTL were cultured with 3×10^4 target cells in 200 μl IMDM plus 10% FCS and 25 U/ml IL-2. After 24 h, supernatant was collected and its IFN-γ content was determined by ELISA (CLB, Amsterdam, The Netherlands).

HLA-B44 subtyping

PCR cell lysates from 10^6 cells were prepared as described (21). HLA-B44 exon 3 DNA was amplified by PCR using 50 pmol B44EX3F primer (5'-TCCTCCGCGGGTATGACCAGG-3'), 50 pmol B44EX3R primer (5'-AGCGACTCCACGCACAGGCC-3'), 0.5 mM dNTPs and 2.5 U Taq polymerase (Life Technologies, Gaithersburg, MD) as previously described (22). PCR products were digested with *PvuII* to discriminate between *HLA-B*4402* and *-B*4403*.

Cloning and sequencing of TCRB gene rearrangement

Total RNA from 10^6 cells was extracted using the RNAzol method (Cinna/Biotecx Laboratories, Friendswood, TX) and reverse transcribed using an oligo(dT) primer and reverse transcriptase (Life Technologies, Gaithersburg, MD). TCRB cDNA was amplified by PCR using 250 pmol Cβ-N2 primer (5'-CACAGCGACCTCGGGTGGG-3'), 250 pmol Vβ-37 primer (5'-CGGATCCT(GT)T(AT)(CT)TGGTA(TC)C(GA)(TA)CA-3'), 0.5 mM dNTPs and 2.5 U Taq polymerase as previously described (23). The PCR product was cloned into pCRII vector by using the TA cloning kit (Invitrogen, San Diego, CA). Transformants were sequenced by the dideoxynucleotide chain termination method, and sequencing products were resolved on polyacrylamide gels.

Results

Isolation of antileukemic CD8$^+$ CTL

To identify mHag expressed by leukemia cells, we isolated and expanded CTL from patient MP by stimulating CD8$^+$ T cells, obtained after HLA-identical BMT, with irradiated B-ALL cells and used autologous donor PBMC as feeder cells. This CTL culture showed specific cytotoxicity against EBV-LCL of patient origin (51% specific lysis; E:T ratio 10:1), whereas EBV-LCL of the HLA-identical donor were not lysed. The CTL were of donor origin and expressed TCR$\alpha\beta$ and CD8. Interestingly, TCR repertoire analysis of this CTL culture showed that 21 of 23 cloned TCRB cDNA exhibited an unique BV6S1-DEAPEG-JB2S1 rearrangement (Table I). Eighty percent of the cells expressed TCRBV6S1 analyzed by flow cytometry (Table I).

Next, TCRBV6S1-expressing cells were sorted by flow cytometry. This CTL clone, MP1, efficiently lysed EBV-LCL of patient MP, whereas EBV-LCL of the HLA-identical donor BP were not killed (Fig. 1*A*). Lysis of K562 cells was not observed. Furthermore, we observed that B-ALL cells of patient MP preincubated with TNF-α were lysed, whereas untreated B-ALL cells were not killed (Fig. 1*B*). Lysis of B-ALL cells was efficiently inhibited by CD3, CD8, and HLA-class I mAb, whereas mAb directed against CD4 and HLA-class II were ineffective (Fig. 1*C*). These results demonstrate that CTL clone MP1 is directed against an HLA-class I-restricted mHag, designated HB-1.

Table I. *TCRB expression of CTL culture MP1*[a]

No. Clones	TCRBV Usage	Positive Cells (%)[b]
21/23	6S1	80
1/23	6S7	2
1/23	17	4

[a] TCRB repertoire was analyzed by cloning and sequencing of rearranged TCRB cDNAs after PCR amplification, as described in *Materials and Methods.*
[b] Frequency of each detected TCRBV was analyzed by flow cytometry.

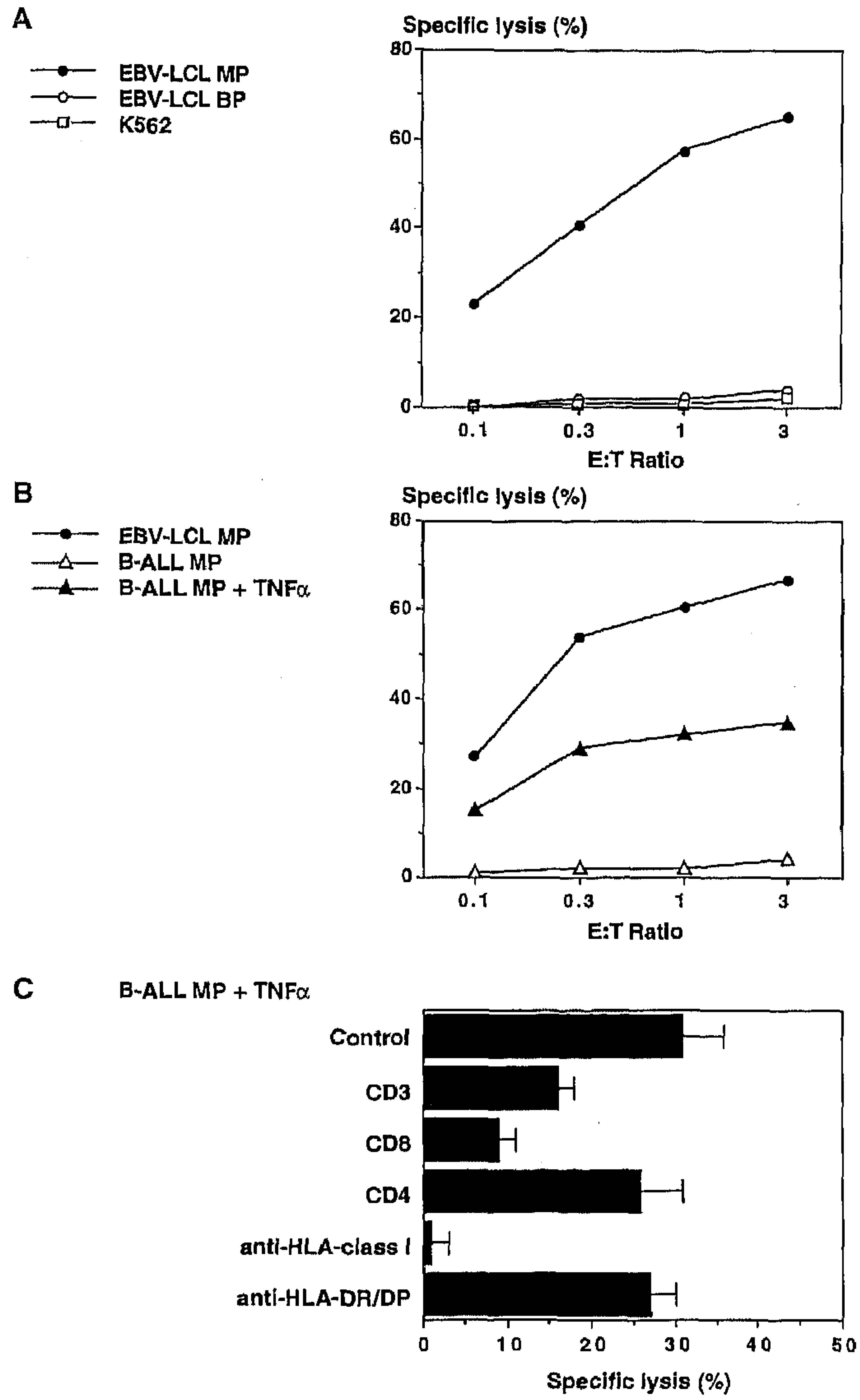

FIGURE 1. Specific cytotoxicity of CTL clone MP1. *A*, Cytotoxicity against K562 and EBV-LCL of patient MP and donor BP. *B*, Cytotoxicity against B-ALL cells of patient MP. B-ALL cells were untreated or treated with 100 U/ml TNF-α for 2 days. *C*, Inhibition of cytotoxicity against TNF-α-treated B-ALL cells. Blocking studies were performed using purified mAb (10 μg/ml), which was present during the assay. The E:T cell ratio was 1:1. One representative experiment of three is shown.

Table II. *Specific lysis by HB-1-specific CTL clone MP1 of a panel of EBV-LCL from relatives, unrelated sibling pairs, and unrelated individuals sharing one or more HLA class I Ags with patient MP*

EBV-LCL	Relation to Recipient	HLA Type				*B*44* Subtype	(%) Specific Lysis[a] 3:1	(%) Specific Lysis[a] 1:1
Relatives								
MP		A2	A33	B44	B60	03	63	52
BP	Donor	A2	A33	B44	B60	03	<	<
PP	Father	A3	A33	B7	B44	03	34	23
PG	Mother	A2	A26	B56	B60		<	<
RP	Sister	A26	A33	B44	B56	03	47	33
GP	Sister	A26	A33	B44	B56	03	33	30
EP	Sister	A2	A3	B7	B60		<	<
P	Brother	A2	A33	B44	B60	03	<	<
Unrelated sibling pairs								
RO1		A2	A33	B58	B62		<	<
RO2		A2	A33	B58	B62		<	<
LA1		A2	A33	B7	B58		<	<
LA2		A2	A33	B7	B58		<	<
HS1		A2	A24	B37	B44	02	<	<
HS2		A2	A24	B37	B44	02	<	<
VH1		A2	A23	B18	B44	03	36	34
VH2		A2	A23	B18	B44	03	<	<
VN1		A1	A2	B7	B44	02	<	<
VN2		A1	A2	B7	B44	02	<	<
GE1		A2	A11	B8	B44	02	<	<
GE2		A2	A11	B8	B44	02	<	<
Unrelated individuals								
KG		A2	A31	B44	B60	02	25	23
HM		A1	A3	B7	B44	03	<	<
OD		A11	A24	B35	B44	02	<	<
OO		A2	A3	B35	B44	02	<	<
BR		A2		B44	B60	02	20	15
ZM		A2		B44	B60	02	<	<
WG		A2	A29	B44	B49	03	31	22
GY		A2	A3	B18	B44	02	<	<
RB		A2	A11	B44	B51	02	<	<
BA		A2		B44		02	<	<

[a] < indicates <10% specific lysis.

Identification of the restriction element of HB-1

To determine the HLA molecule that presents HB-1 to CTL clone MP1, we tested EBV-LCL of relatives of patient MP. The results in Table II demonstrate that CTL clone MP1 recognizes HB-1 on EBV-LCL of three family members sharing expression of HLA-A33 and -B44 with the patient. Like EBV-LCL of the donor, EBV-LCL of one other HLA-A33, B44-positive family member does not express HB-1. These results demonstrate that HB-1 is recognized in association with HLA-A33 or -B44. To further define the HLA restriction molecule, we tested EBV-LCL of six sibling pairs unrelated to patient MP expressing HLA-A33 or -B44. EBV-LCL of one of these individuals, sharing only HLA-B44 with patient MP, were lysed by CTL clone MP1, thus demonstrating that HB-1 is presented by HLA-B44 (Table II). The observation that EBV-LCL of three out of ten randomly selected unrelated HLA-B44-positive individuals were also lysed confirms these data (Table II).

Expression of HB-1 by B-ALL cells

Since the HB-1-specific CTL clone MP1 was expanded by stimulation with B-ALL cells, we tested B-ALL cells of randomly selected HLA-B44-positive patients for recognition by CTL clone MP1. Leukemia cells of two out of eight B-ALL patients were HB-1 positive (Fig. 2). B-ALL cells of patient VR were only lysed after preincubation with TNF-α, like B-ALL cells of patient MP. Interestingly, B-ALL cells of patient SC were recognized by HB-1-specific CTL without TNF-α pretreatment (Fig. 2). To determine whether B-ALL cells in general show low susceptibility to CTL-mediated lysis and whether this can be enhanced by TNF-α, we tested these cells for lysis by an anti-HLA-A2 CTL. TNF-α preincubation of B-ALL cells of patient MP and VR increased significantly the susceptibility to lysis by the HLA-A2 allospecific CTL line 1E2 (Fig. 2). In an attempt to explain the enhanced susceptibility of B-ALL cells to HB-1 specific and anti-HLA-A2 CTL lysis upon TNF-α treatment, we analyzed expression of MHC class I and adhesion molecules LFA-1, LFA-3, and ICAM-1 of B-ALL cells incubated with and without TNF-α. TNF-α clearly enhanced expression of ICAM-1 and LFA-3 of B-ALL cells (Table III). Lysis of B-ALL cells incubated with TNF-α was completely inhibited by a combination of anti-LFA-3 and anti-ICAM-1 mAb (Table IV). These data demonstrate that HB-1 is expressed by these B-ALL cells, but that significant expression of adhesion molecules is a prerequisite for lysis of B-ALL cells by CTL clone MP1.

Tissue specificity of HB-1

To determine whether HB-1 is expressed by all host cells or shows a restricted tissue expression, we tested lysis of fibroblasts and normal hemopoietic cells of patient MP, and three other HLA-B44, HB-1-positive individuals. Interestingly, PHA-stimulated T cell blasts, monocytes, and TNF-α treated fibroblasts were not lysed by CTL clone MP1, indicating that HB-1 is restricted to the B cell lineage (Fig. 3*A*). All cell types were efficiently killed by the

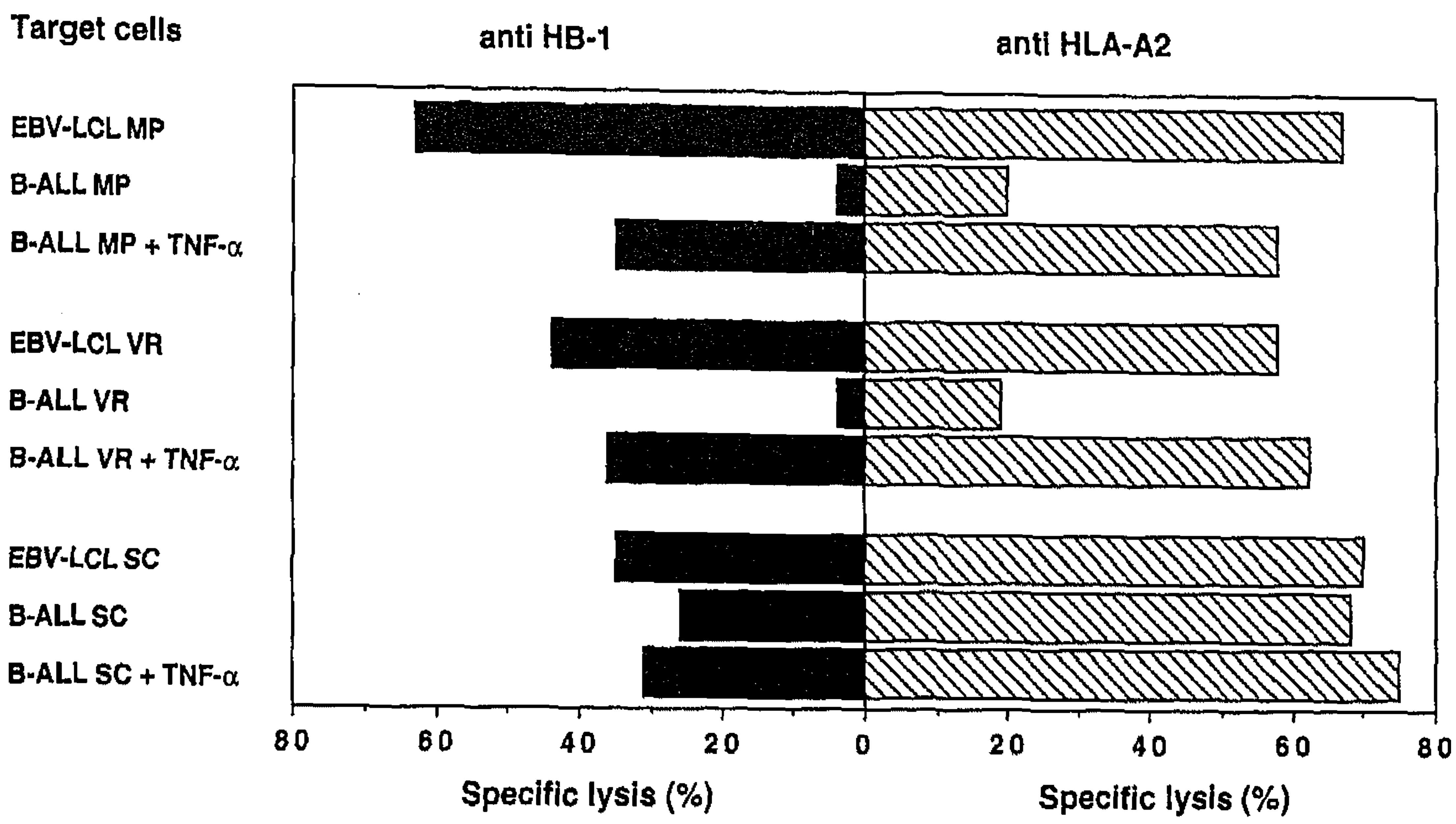

FIGURE 2. Specific cytotoxicity of CTL clone MP1 against B-ALL cells of three patients. HLA-B44 subtype of patient MP is B4403 and of patients VR and SC is B4402. B-ALL cells were untreated or treated with 100 U/ml TNF-α for 2 days. The E:T cell ratio was 1:1.

Table III. *Effect of TNF-α on expression of MHC class I and adhesion molecules on B-ALL cells*

	Relative Fluorescence Intensity					
	B-ALL MP		B-ALL VR		B-ALL SC	
	−TNF-α	+TNF-α	−TNF-α	+TNF-α	−TNF-α	+TNF-α
Control	2	2	2	2	3	3
MHC class I	569	568	373	436	513	616
LFA-1	11	11	36	50	16	17
LFA-3	29	36	45	102	100	123
ICAM-1	3	46	3	11	66	92

Table IV. *Inhibition of cytotoxicity of CTL clone MP1 against TNF-α-treated B-ALL cells*

	(%) Specific Lysis[a]		
mAb[b]	B-ALL MP	B-ALL VR	B-ALL SC
Medium	26	59	35
LFA-3 + ICAM-1	5	8	9

[a] E:T cell ratio = 3:1.
[b] Blocking studies were performed using purified mAb (10 μg/ml), which was present during the assay.

HLA-A2 allospecific CTL line 1E2, indicating that all target cells were susceptible to CTL-mediated lysis (Fig. 3*A*). To investigate B cell-specific expression of HB-1 in more detail, we tested this expression of in vitro TNF-α and CD40-stimulated B cell blasts of three HLA-B44, HB-1-positive individuals. TNF-α/CD40-stimulated B cell blasts were unable to induce IFN-γ release of CTL clone MP1, whereas EBV-LCL of these individuals induced a significant release of IFN-γ (Fig. 3*B*). These results show that HB-1 is expressed by leukemic and EBV-transformed B cells, but not by activated B cells.

Discussion

In the present report, we demonstrate that $CD8^+$ CTL specific for leukemia-associated mHag are present within the T cell repertoire of a leukemia patient treated by HLA-matched BMT. We identified a first example of a human B cell lineage-specific mHag, designated HB-1. Of the mHag identified so far in humans, some are expressed by all tissues, others are exclusively expressed by hemopoietic cells (8, 10, 11). We found that HB-1-specific CTL were not reactive against PHA-stimulated T cell blasts, monocytes, and fibroblasts, but showed cytolytic reactivity against leukemic and EBV-transformed B cells. These data show restricted expression of HB-1 to cells of the B cell lineage. Previously, a B cell-specific mHag has been identified in mice (24). This Ag is exclusively expressed by mature B cells and B cell tumors. In contrast, HB-1 is not expressed by mature untransformed CD40-stimulated B cells. Therefore, HB-1 is clearly a B cell leukemia-associated Ag. Our results suggest that expression of HB-1 is induced by activation of a silent B cell gene in EBV and leukemia-transformed B cells. Further analysis of HB-1 awaits cloning of the encoding cDNA.

HB-1 is recognized in association with HLA-B44, which is a common HLA-B allele expressed by 23% of the Caucasian population (25). Among randomly selected HLA-B44-positive individuals we found that HB-1 is expressed by 3 of 10 EBV-LCL and by 2 of 8 B-ALL, resulting in a phenotype frequency of 28% (5/18). Since amino acid substitutions among HLA subtypes can affect the presentation of peptides to specific T cells, it is possible that subtype differences bias the phenotype frequency of HB-1. Five subtypes of HLA-B44 have been found, but the most frequently expressed subtypes are *HLA-B*4402* and *-B*4403* (26, 27). *HLA-B*4402* differs from *HLA-B*4403* by a single amino acid substitution from Asp (**4402*) to Leu (**4403*) in position 156 of the α_2 domain (28). Both *HLA-B*4402* and *-B*4403* were able to present HB-1 to CTL clone MP1 (Table II and Fig. 2). This is consistent with the finding that the peptide-binding motif of both subtypes is identical (29).

B-ALL cells of two out of three HLA-B44, HB-1-positive patients were only lysed by CTL clone MP1 after preincubation with TNF-α. This finding is in accordance with reports that lymphatic leukemia cells are less susceptible to lysis by CTL in vitro than myeloid leukemia cells and normal hemopoietic cells (14). We investigated whether the absence of several adhesion molecules by B-ALL cells might be the cause of low susceptibility to CTL lysis. We found that resistance to lysis correlated with low expression of ICAM-1 and LFA-3 by B-ALL cells. Susceptibility to CTL-mediated lysis of B-ALL cells was increased after incubation with TNF-α, which was clearly associated by an increase of ICAM-1 and LFA-3 expression.

Leukemic relapse after allogeneic BMT is a serious problem. Infusion of mHag-reactive donor T cells will always result in broad reactivity, inducing severe GVHD. Since HB-1 is expressed by B cells after malignant or EBV transformation, it may be an excellent Ag to develop immunotherapeutic protocols to eradicate

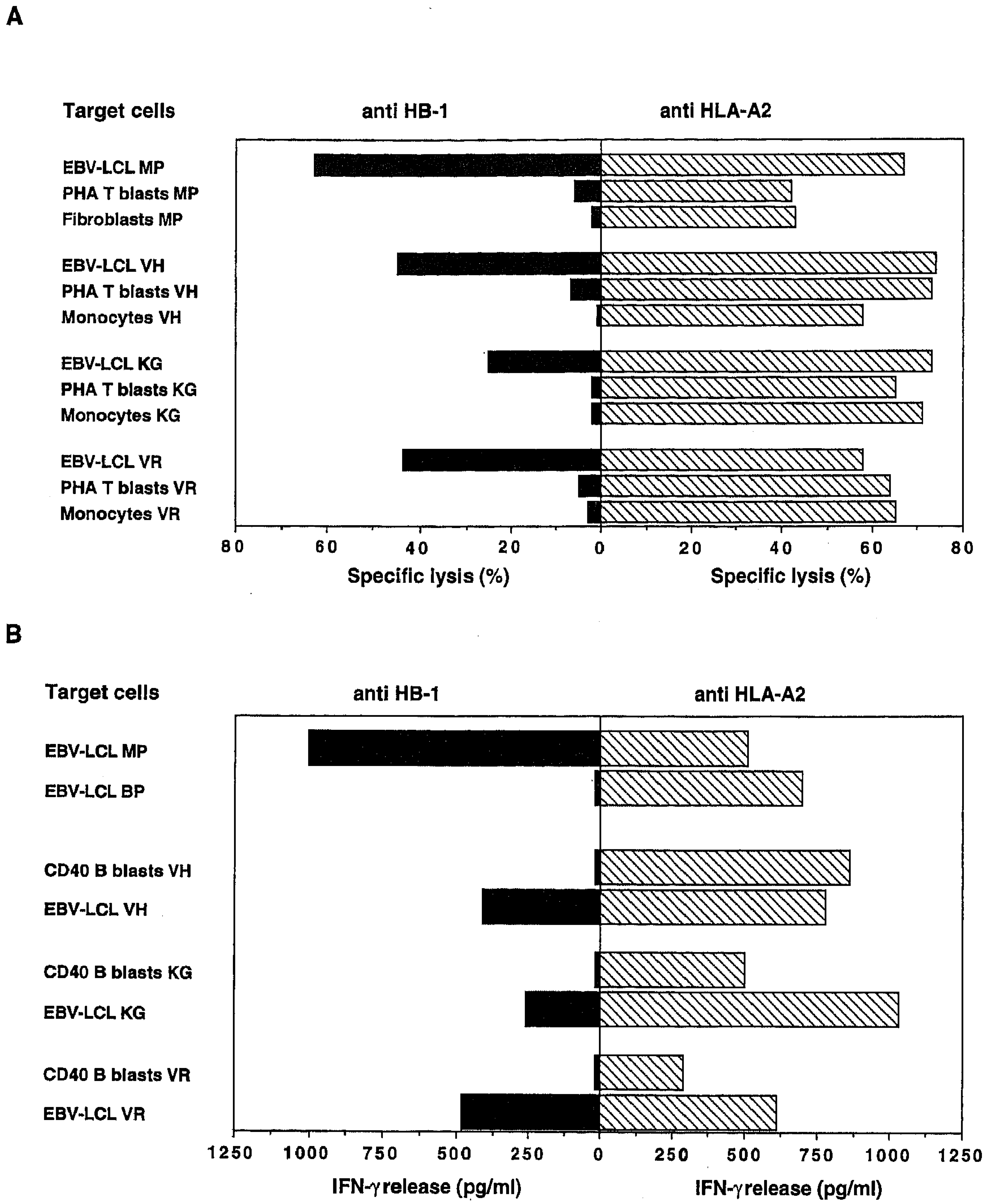

FIGURE 3. Tissue-specific expression of HB-1. *A*, Cytotoxicity against EBV-LCL, PHA-stimulated T cell blasts, monocytes, and fibroblasts of HLA-B44, HB-1-positive individuals. The E:T cell ratio was 1:1. *B*, Production of IFN-γ by CTL clone MP1 stimulated with CD40 activated B cell blasts and EBV-LCL of HLA-B44, HB-1-positive individuals. B cells were stimulated with CD40 and 100 U/ml TNF-α for 2 days. One representative experiment of two is shown.

residual B cell leukemia cells in BMT patients without the development of severe GVHD. The low ICAM-1 and LFA-3 expression by some B-ALL cells raises the issue of tumor escape to HB-1 specific CTL. However, serum levels of the inflammatory cytokines TNF-α and IFN-γ are increased in BMT recipients during GVH reactions and viral infections (30, 31). TNF-α and IFN-γ can induce or up-regulate expression of MHC and adhesion molecules on residual leukemia cells in BMT recipients. In our view, it is therefore likely that B-ALL cells in BMT recipients will be susceptible to mHag-specific CTL.

Acknowledgments

We thank Frans Maas for technical assistance and Aukje Zimmerman for the HLA-B44 subtyping.

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