A Human Minor Histocompatibility Antigen Specific for B Cell Acute Lymphoblastic Leukemia

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Summary

Human minor histocompatibility antigens (mHags) play an important role in the induction of cytotoxic T lymphocyte (CTL) reactivity against leukemia after human histocompatibility leukocyte antigen (HLA)-identical allogeneic bone marrow transplantation (BMT). As most mHags are not leukemia specific but are also expressed by normal tissues, antileukemia reactivity is often associated with life-threatening graft-versus-host disease (GVHD). Here, we describe a novel mHag, HB-1, that elicits donor-derived CTL reactivity in a B cell acute lymphoblastic leukemia (B-ALL) patient treated by HLA-matched BMT. We identified the gene encoding the antigenic peptide recognized by HB-1-specific CTLs. Interestingly, expression of the HB-1 gene was only observed in B-ALL cells and Epstein-Barr virus-transformed B cells. The HB-1 gene-encoded peptide EEKRGSLHVW is recognized by the CTL in association with HLA-B44. Further analysis reveals that a polymorphism in the HB-1 gene generates a single amino acid exchange from His to Tyr at position 8 within this peptide. This amino acid substitution is critical for recognition by HB-1-specific CTLs. The restricted expression of the polymorphic HB-1 Ag by B-ALL cells and the ability to generate HB-1-specific CTLs in vitro using peptide-loaded dendritic cells offer novel opportunities to specifically target the immune system against B-ALL without the risk of evoking GVHD.

Key words: bone marrow transplantation • cytotoxic T lymphocytes • minor histocompatibility antigens • B cell acute lymphoblastic leukemia • tumor immunity

Transplantation with HLA-identical sibling bone marrow is successfully used to treat patients with leukemia. The therapeutic effect can be partly attributed to the elimination of residual host leukemic cells by donor-derived T cells, termed the graft-versus-leukemia reactivity (1). However, this donor-derived T cell reactivity generally causes GVHD, a life-threatening complication in allogeneic bone marrow transplantation (BMT) (1). The ability of donor-derived CTLs to recognize host minor histocompatibility Ags (mHags) as foreign peptides plays an important role in both GVHD and graft-versus-leukemia reactivity (2–4). mHags are HLA-associated peptides generated from polymorphic regions of proteins present in the target cells (5–7). Interestingly, as well as mHags with ubiquitous tissue distribution, mHags with expression restricted to hematopoietic cells or leukemic cells have been characterized (2, 8–11).

Although allogeneic BMT may cure many patients with leukemia, relapse of the tumor occurs in a significant number of patients, indicating that in these patients not all tumor cells are sufficiently eliminated or suppressed. Therefore, there is a continuous search for additional and more specific immunotherapeutic strategies in the treatment of leukemia in order to eliminate leukemic cells without in-
produc ing GVHD. We have focused our attention on CTLs that selectively recognize leukemic but not normal cells. Recently, we isolated from a BMT recipient an HLA-B44-restricted CTL clone recognizing a novel mHag named HB-1, which shows specificity for B cell acute lymphoblastic leukemia (B-ALL). Using this CTL, we have identified both the nucleotide sequence encoding the HB-1 Ag and the polymorphic CTL epitope. Furthermore, we show that the expression of the HB-1 gene is restricted to B-ALL and EBV-transformed B cells.

Materials and Methods

Cell Culture. All cell lines were cultured in IMDM (GIBCO BRL) supplemented with 10% FCS. CTL clone M P1 was isolated from PBLs of leukemia patient MP after HLA-identical BMT and grown in IMDM supplemented with 10% pooled human serum, irradiated EBV-transformed-lymphoblastoid cell line (EBV-LCL) of the patient pre-BMT (10^6 ml), irradiated allogeneic PBMCs (10^6/ml), 300 U/ml IL-2 (Glaxo), and 0.4 μg/ml PHA.

*DNA Library Construction.* The cDNA library was constructed using the Superscript Plasmid System (GIBCO BRL). Total RNA was isolated from EBV-LCL MP and poly(A)-mRNA was prepared by oligo-dt binding (Qiagen). mRNA was converted into cDNA using an oligo-dT primer that contains a NotI site at its 5′ end. The cDNA was ligated to SalI adapters digested with NotI, and ligated into SalI and NotI sites of the expression vector pSV-Sport1. Escherichia coli DH10B were electroporated with the recombinant plasmids and clones were selected with ampicillin. This library was divided into 1,500 pools of ~100 cDNA clones. Each pool was amplified for 4 h and plasmid DNA was extracted with Bio-Rad miniprep kits (Bio-Rad).

Isolation of the HLA-B*4403 Gene. Total RNA from EBV-LCL MP was extracted using a Trizol method (GIBCO BRL). R reverse transcription was performed on 2 μg of total RNA using an oligo-dt primer and Moloney's murine leukemia virus (Mo-MuLV) reverse transcriptase (GIBCO BRL). HLA cDNA was amplified by PCR using 50 pmol HLA-5UTR primer (5′-GACTCAGA(AT)CTCTCCCAAGG-3′), 50 pmol HLA-3UTR primer (5′-CTCAGTTTCAAGGCA-3′), 0.5 mM dNTPs, and 2.5 U Taq polymerase as previously described (12). After separation of the PCR product on a 1% agarose gel, the 1.2-kb DNA fragment was isolated and cloned into pCR3 vector after treatment with NotI and ligated into pCR3 vector using the unidirectional TA cloning method (Invitrogen). This cloned 1.2-kb DNA fragment was isolated and cloned into pCR3 vector and mutation constructs were produced by PCR using specific primers, and amplified products were subsequently cloned into vector pCR3 using the unidirectional TA cloning method (Invitrogen).

Production of truncated and mutated DNA constructs. Deletion and mutation constructs were produced by PCR using specific primers, and amplified products were subsequently cloned into vector pCR3 using the unidirectional TA cloning method (Invitrogen).

*H B-1 Polymorphism.* Total RNA from cells was extracted using the Trizol method (GIBCO BRL). R reverse transcription was performed on 2 μg of total RNA using an oligo-dt primer and Mo-MuLV reverse transcriptase (GIBCO BRL). HLA-B*4403 was amplified by PCR using 50 pmol HBL-F5 primer (5′-GAGGCTTCTGACCTCACATC-3′), 50 pmol HBL-GSP3 primer (5′-TTGGTCTCTGGCTCATCACACC-3′), 0.5 mM dNTPs and 2.5 U Taq polymerase (GIBCO BRL). The PCR was performed for 33 cycles (1 min at 94°C, 1 min 60°C, and 1 min 72°C). PCR products were digested with NlaIII to discriminate between HB-1 and HB-1′ alleles.

Peptides and C r-release Assays. Peptides were synthesized with a free COOH terminus by Fmoc peptide chemistry using a multiple synthesizer (ABIMED). Peptides (~90% pure as indicated by analytical HPLC) were dissolved in DMSO and stored at −20°C. Cr-release assays were performed as previously described (13). In peptide recognition assays, target cells were preincubated with various concentrations of peptide for 1 h at 37°C in a volume of 100 μl before the addition of effector cells. After 4 h of incubation at 37°C, 100 μl supernatant was collected and radioactivity was measured by a gamma counter.

Taqman™ PCR Assay for HB-1 Expression. Total RNA from cells was extracted using the Trizol method (GIBCO BRL). R reverse transcription was performed on 2 μg of total RNA using an oligo-dt primer and Mo-MuLV reverse transcriptase (GIBCO BRL). Of the total cDNA volume of 20 μl, 1 μl was used for each PCR reaction. PCR amplification and real time quantitation analysis were performed using the Taqman™ assay (14, 15). The following sequences were used as primers and Taqman™ probes: 5′-GGCAATGCGGCTGCAA-3′ (HB1-F5, sense), nucleotide (nt) 58-77), 5′-TTGTCCCTCGTCTACCATCACC-3′ (HB1-GSP3, antisense, nt 271-291), 5′-FAM)-TCCCTCTCGA-CAGGGAGTCTATGTGTA(T- (TAMRA)-3′ (HB1-probe, anti- nucleotide, nt 188-217), 5′-GGCAATCGGCTGCAA-3′ (Pbgd-F), 5′-GGTACACCCAGAATACG-3′ (Pbgd-R), and 5′-(OE) -CTCATCTTTGGCTGTITTTTTCCGCC- (TAMRA)-3′ (Pbgd-probe). The reaction mixture contains 1.25 U AmpliTaq Gold (PE-Applied Biosystems), 250 μM dNTPs 15 pmol sense, and 15 pmol antisense primer in a total reaction volume of 50 μl. The enzyme was activated by heating for 10 min at 95°C. A two-step PCR procedure of 60 s at 90°C and 15 s at 95°C was applied for 40 cycles. 6 m M MgCl2 and 100 mM probe for the HB-1 PCR, and 5 mM MgCl2 and 100 nM probe for the Pbgd PCR, was used. The PCR and Taqman™ analysis were performed in the ABI/PRISM 7700 Sequence Detector System (PE-Applied Biosystems). The system generates a real time amplification plot based upon the normalized fluorescence signal. Subsequently the threshold cycle (Ct), i.e., the fractional cycle number at which the number of amplified target reaches a fixed threshold, is determined. The Ct is proportional to the initial number of target copies in the sample (14, 15). We used the expression of the porphobilinogen deaminase (Pbgd) gene to normalize the HB-1 expression. Pbgd was used as an endogenous reference to correct for differences in the amount of total RNA added to the reaction. The Pbgd primers only allows amplification of cDNA derived from the Pbgd house-keeping gene (16, 17). H B-1 mRNA expression was quantified by determining calibration functions for HB-1 and Pbgd expression of a reference cell line. Therefore, 2 μg of RNA of the B-ALL cell...
line KM3 was reverse transcribed into cDNA and serially diluted into water. This cDNA serial dilution was prepared once, stored at −20°C, and used in all tests performed in this study. The linear calibration functions between the CT and the logarithm of the initial starting quantity (N) were $CT = -3.44\log(N) + 25.8$ and $CT = -3.44\log(N) + 21.9$ for HB-1 and Pbgd, respectively. HB-1 and Pbgd mRNA expression in all test samples were quantified using these calibration functions. At the same level of Pbgd expression the level of HB-1 expression of test samples was determined as a percentage of the HB-1 expression in cell line KM3.

Results

Identification of a cDNA Coding for the HB-1 Antigenic Peptide. We generated a cDNA library from an EBV-LCL that expresses the HB-1 Ag since it was efficiently lysed by CTL MP1. Using expression cloning, we isolated from this library a cDNA that upon transfection along with the HLA-B44 cDNA into COS-1 cells stimulated CTL MP1 to release IFN-γ (Fig. 1). The IFN-γ release was as high as that induced by EBV-LCL MP, from which the cDNA library was generated, and even higher than that induced by EBV-LCL as well as B-ALL cells of the HLA-B44, HB-1–positive patient VR. Untransformed CD40-stimulated B cells of patient VR could not stimulate CTL MP1 to release IFN-γ. Transfection of either of the aforementioned cDNAs alone failed to induce the production of IFN-γ by CTL MP1, indicating that the isolated cDNA clone encodes the HB-1 Ag.

The cDNA encoding the HB-1 Ag consists of 397 nucleotides with no significant homology to sequences presently recorded in data banks. To localize the region encoding the HB-1 epitope, we transfected COS-1 cells with truncated HB-1 cDNA constructs in combination with the HLA-B44 cDNA and tested their capacity of inducing IFN-γ release by the CTLs (Fig. 2). The smallest truncated construct that still encoded both the translation initiation codon and the peptide coding region contains the nucleotide sequence 100 to 165 (Fig. 2). The three possible translational reading frames within this sequence did not code for a peptide according to the described HLA-B44 binding motif, a Glu at position 2 and a Phe or Tyr at position 9 or 10 (18, 19). We next synthesized all 9-, 10-, and 11-mer peptides with a Glu residue at position 2 encoded by the translational reading frames within nucleotide sequence 100 to 165, and tested their ability to induce lysis by CTL MP1 upon loading on HB-1–negative target cells. The 10-mer EEKRGSLHVW was specifically recognized by CTL MP1, but not by HLA-B44–restricted, EBNA3C–specific CTLs (Fig. 3, A and B). CTL MP1 did not recognize the 9-mer peptide EEKRGSLHV (data not shown).

The open reading frame (ORF) encoding the EEKRGSLHVW CTL epitope contains a CTG translation initiation codon resulting in a putative 41-amino acid protein (Fig. 4). Substitution of this CTG into AAG resulted in a complete loss of the ability to stimulate IFN-γ release by CTL MP1 upon transfection into COS-1 cells (data not shown). Together, these data led us to conclude that within the HB-1 sequence the CTG at position 108–110 initiates the translation of a 41-amino acid protein from which the EEKRGSLHVW CTL epitope is generated.

The HB-1 Gene Is Only Expressed by Transformed B Cells. Interestingly, we observed that CTL MP1 exhibits specific cytotoxicity towards leukemia- and EBV-transformed B cells, but not against untransformed B cells, T cells, monocytes, and fibroblasts (8). Therefore, we studied the level of HB-1 gene expression in a large panel of tumor and nonmalignant cells using real time quantitative reverse tran-
scriptase PCR. All B-ALL samples expressed the HB-1 gene at a significant level exceeding 10% of that found in the reference B-ALL cell line KM3 (Fig. 5 A). In addition, 2 out of 14 B cell lymphomas and 2 out of 5 acute undifferentiated leukemias showed significant HB-1 expression. In contrast, all T-ALLs, multiple myelomas, acute myeloid leukemias, and nonhematological solid tumors lacked HB-1 expression. Since the number of HB-1 transcripts in B-ALL cells is even lower than that of the low copy gene Pbgd we concluded that HB-1 is a rare mRNA species. This notion was confirmed by the observation that we could not detect HB-1 expression by Northern blot analysis, whereas β-actin mRNA was readily detected (data not shown).

Analysis of the expression of the HB-1 gene in a panel of nonmalignant cells revealed significant levels in 90% of the EBV-transformed B cell lines, whereas no significant HB-1 transcription was observed in all other nonmalignant cell types (Fig. 5 B). Some B cell and PHA-stimulated T cell samples express very low levels of HB-1 (<10% of that found in the reference B-ALL cell line KM3; Fig. 5 B). PHA-stimulated T cell blasts with an HB-1 expression levels of 8 and 2.5% of that found in the KM3 cell line were

Figure 3. Identification of the HB-1 antigenic peptide. (A) Cytolytic activity by CTL clone M P1 against HLA-B44–positive target cells incubated with 5 μM of HB-1 peptide EEKRGLHVW. Controls included the HLA-B44–negative target cells incubated either without peptide or with the EBN3C 281–290 peptide EENNLD FVRF. (B) Cytolytic activity by the EBN3C-specific CTL against HLA-B44–positive target cells incubated with 5 μM of EBN3C peptide EENNLD FVRF. Controls included the HLA-B44–positive target cells incubated either without peptide or with the HB-1 peptide EEKRGLHVW.

Figure 4. Sequence of HB-1 cDNA and of the 41 amino acid–encoded protein starting from the CTG start codon (underlined) at nucleotide positions 108–110. The sequence corresponding to the HLA-B44–restricted HB-1 peptide is boxed. These sequence data are available from EMBL/GenBank/DDBJ under accession number AF103884.
not recognized by CTL MP1 (Fig. 6). Loading of these PHA-stimulated T cell blasts with the EEKRGSGLHVW peptide results in lysis that is as high as the killing of the HB-1-positive EBV-LCL. Analysis of HB-1 expression in normal tissues revealed only low (<10%) transcription levels in testis samples (Table I). Together, these results demonstrate that substantial HB-1 mRNA expression is observed only in B-ALL cells and in EBV-transformed B cells and that very low mRNA expression of HB-1 does not result in lysis by CTL MP1.

A Polymorphism in the HB-1 Gene Determines CTL Recognition. CTL MP1 lyse EBV-transformed B cells derived from the patient MP, whereas EBV-transformed B cells derived from the HLA-identical sibling donor BP are not recognized (8). Since the HB-1 gene was significantly expressed by both cell lines, these results suggested the presence of a polymorphism in this sequence. Analysis of the HB-1 sequence of the donor revealed that it differs from that of the patient at only one nucleotide (position 153: C to T), leading to an amino acid change from H to Y in the HB-1 peptide (Fig. 7 A). The corresponding alleles were named HB-1H and HB-1Y, respectively.

Studies of the HB-1 gene polymorphism in relatives of patient MP resulted in a clear correlation between recognition by CTL MP1 of EBV-LCL of HLA-B44-positive family members and the expression of the HB-1H allele (Fig. 7 B). To verify that only expression of the HB-1H allele leads to recognition by CTL MP1, we transfected HB-1H or HB-1Y cDNA along with HLA-B44 cDNA into COS-1 cells. Cells transfected with the HB-1H cDNA stimulated IFN-γ release by the CTL, whereas cells transfected with the HB-1Y cDNA did not (Fig. 8 A). In addition, EBV-transformed B cells incubated with the peptide encoded by the HB-1Y allele were not lysed by CTL MP1 (Fig. 8 B). This probably is not the result of defective binding of the HB-1Y peptide to HLA-B44 molecules, as both peptides appeared to bind with equal affinity to these HLA molecules (data not shown). These results demonstrate that at least two allelic forms of the HB-1 gene exist, and that only the HB-1H allele encodes the peptide that is recognized by CTL MP1.

Discussion

Over the past few years, a large number of CTL-defined tumor Ags and their encoding genes have been identified in melanoma (20). However, little is known about CTL-defined Ags encoded by genes with expression restricted to leukemia. Using biochemical methods to isolate and sequence antigenic peptides presented by MHC class I molecules, the first CTL epitopes on leukemia cells have recently been identified (6, 7, 21). Expression of the identified polymorphic Ags HA-1 and HA-2 is restricted to hematopoietic cells but they are not leukemia specific (2). Although these mHags have this limited tissue distribution, matching for HA-1 is associated with the occurrence of severe GVHD (22). To the best of our knowledge, the polymorphic HB-1 gene product described here is the first identified leukemia-associated mHag that is only significantly expressed by leukemia- and EBV-transformed B cells. In some normal cells, few HB-1 transcripts are present.
ent, but expression is too low for recognition by H-B-1-specific CTLs, as was also observed for a number of other tumor-associated Ags (20, 23–25). Low levels of transcription of the melanocyte-associated Ag gp100 have been found in nearly all normal tissues and tumor cell lines of nonmelanocytic origin, but no gp100 protein could be detected by either Western blot or cytotoxicity assays (23). Similarly, the Ag encoded by the N-acetylglucosaminyl-transferase V (GnTV) gene was not recognized by specific CTLs when transcription levels did not exceed 8% of that of the reference melanoma cell line (24). Finally, MAGE-1-specific CTLs are unable to recognize tumor cell lines expressing low levels (<10%) of the MAGE-1 gene when compared with melanoma cells that were efficiently lysed by CTL MP1. Open symbols indicate no lysis. Expression of H-B-1 alleles was determined by reverse transcriptase PCR amplification and digestion of PCR products with restriction enzyme NlaIII.

The HB-1 antigenic peptide is encoded by a sequence which starts with a CUG codon resulting in the translation of a short protein of 41 amino acids. Such an unusual initiation of translation at a CUG has been reported previously (26). Whether this product is the only protein encoded by the HB-1 gene or whether there are more proteins encoded by alternative ORFs is currently unknown. Although most eukaryotic mRNAs have a single ORF of which translation is usually initiated by an AUG codon, several human genes are bicistronic, encoding two proteins (27–29). For instance, the gp75 gene in melanoma has two overlapping ORFs resulting in two completely different proteins: (i) gp75 as recognized by IgG antibodies in the serum from melanoma patients, and (ii) a short protein of 24 amino acids from which an antigenic peptide recognized by CTLs is generated (27). Whether the generation of the short 41-amino acid protein from the HB-1 gene is the result of translation of an alternative ORF awaits further characterization of the gene.

At present we can not exclude the possibility that the HB-1 gene-encoded protein is a B cell differentiation Ag that is overexpressed in B-ALL cells and lost in mature B cells. Alternatively, the malignant transformation of progenitor B cells itself may induce H-B-1 expression. This latter possibility is supported by the finding that the HB-1 gene also shows significant expression in B cells that are transformed in vitro with EBV. These EBV-transformed B cell lines express all EBV gene-encoded proteins, whereas EBV-positive Burkitt’s lymphoma cell lines express only the EBNA1 protein (30). Since HB-1 is significantly expressed by EBV-transformed B cell lines and not by Burkitt’s lymphoma cell lines, it is tempting to speculate that expression of EBV gene-encoded proteins other than EBNA1 may induce H-B-1 mRNA transcription in mature B cells. Studies dealing with expression levels during B cell differentiation and the role of EBV transformation in inducing H-B-1 expression are currently under investigation.

The antigenic peptide EEKRGLSHVW encoded by the HB-1 gene is recognized in association with HLA-B44, a common HLA-B allele expressed by 23% of the Caucasian
population. Five subtypes of HLA-B44 have been identified, but the most frequently expressed subtypes are HLA-B*4402 and B*4403. These two subtypes differ only by a single amino acid substitution from Asp (*4402) to Leu (*4403) in position 156 of the α2 domain (18, 31, 32). Both HLA-B*4402 and -B*4403 are able to present the HB-1 peptide to CTL clone M1. The consensus peptide binding motif for HLA-B44 shows a predominance for Glu at position 2, and Tyr or Phe at position 9 or 10 (18, 19). The HB-1 peptide contains a Glu at position 2, but in contrast to the consensus motif it has a Trp at position 10, indicating that all amino acids with aromatic side chains facilitate binding to HLA-B44. A polymorphism in the HB-1 gene resulted in a single amino acid exchange from His to Tyr at position 8 of the HB-1 peptide. The anchor residues of the peptide are not involved in this substitution, and both the HB-1H and HB-1Y peptide bind with similar affinity to HLA-B44 molecules. Since the HB-1Y peptide is not recognized by CTL clone M1, the polymorphism appears to influence a TCR contact residue.

The molecular identification and characterization of the leukemia-associated mHAg HB-1 allows the opportunity to treat leukemia patients with immunotherapy specifically targeted to the tumor without the risk of inducing GVHD. For this, patients must be typed for the presence of the HB-1H allele and their tumor cells must significantly express the HB-1 gene. The mHAg HB-1 might turn out to be an excellent target against which specific CTLs can easily be generated from allogeneic donors and adoptively transferred into BMT recipients with relapsed B-ALL. We have already succeeded in generating HB-1–specific CTLs in vitro from peripheral blood of healthy HLA-B44–positive individuals by stimulating CD8-positive T cells with peptide-loaded autologous dendritic cells (our unpublished results). These induced CTLs displayed lysis of both peptide-loaded HB-1–negative target cells, and autologous HLA-B44–positive EBV-transformed B cells endogenously expressing the HB-1 Ag. The presence of HB-1–specific CTLs in the T cell repertoire of HB-1–positive individuals and the restricted expression of the HB-1 gene by B-ALL cells may also allow the use of HB-1–encoded Ags for vaccination protocols to induce specific immunity in B-ALL patients.

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