(Melbourne, Australia), dissolved in 100 μl dimethyl sulfoxide and further diluted in 40% acetonitrile, 0.1 M HEPES (pH 7.4), where necessary. Characteristics of the seven synthetic peptides, designated as $Ep_{31}$, $Ep_{173}$, $Ep_{185}$, $Ep_{250}$, $Ep_{225}$, $Ep_{296}$ and $Ep_{304}$, have been listed in Table 1. A human immunodeficiency virus-1 (HIV-1) envelope peptide RYLRDQQLL (25) (residues 584–592, designated as $ENV_{584}$) and an EBV (Epstein-Barr virus) latent membrane protein 2 peptide TYGPVFMCL (26) (residues 419–427, EBV-LMP2419) were synthesized by Toray Research Center (Kamakura, Japan).

### Cell staining and flow cytometric analysis

Surface expression of HLA-A24 and Ep-CAM molecules was examined with the help of indirect immunofluorescence by using an anti-HLA-A24 monoclonal antibody (One Lambda, Inc., Canoga Park, CA), the anti-Ep-CAM monoclonal antibody and FITC-labelled (fluorescein isothiocyanate) anti-mouse IgG $F(ab')_2$ fragments (IMMUNOTECH, Marseille, France). MHC/peptide tetramers were produced as previously described (22, 27). The Ep-CAM-specific $CD8^+$ T cells were stained with PE-labelled HLA-A*2402 tetramers incorporating the Ep-CAM peptide, $Ep_{173}$ (designated as the HLA-A24/$Ep_{173}$ tetramer) or the HIV-1 peptide, $ENV_{584}$ (HLA-A24/$ENV_{584}$ tetramer). Flow cytometric analysis of the stained cells was performed by means of a FACSCalibur (Becton Dickinson, San Jose, CA) and the data were analysed with the help of CellQuest software (Becton Dickinson).

### MHC stabilization assay

The seven synthesized peptides were used in an MHC stabilization assay by using T2-A24 cells as described earlier (22). Briefly, T2-A24 cells ($2 \times 10^5$) were incubated with 200 μl of RPMI1640 containing 0.1% FCS and $5 \times 10^{-5}$ M β-mercaptoethanol and each of the peptides at a concentration of 10 μM at 26°C for 16 h, followed by incubation at 37°C for 3 h. Surface HLA-A24 molecules were then stained with the anti-A24 monoclonal antibody and FITC-labelled anti-mouse IgG. Expression was measured in the FACSCalibur, and mean fluorescence intensity (MFI) was recorded. The percentage of MFI increase was calculated as follows: percentage of MFI increase = 100 × (MFI with the given peptide − MFI without the peptide)/(MFI without the peptide).

**Characteristics of epithelial cell adhesion molecule (Ep-CAM) candidate peptides**

| Peptide designation | Amino acid sequence | Position | Sequence length | Score[a] | Percentage of MFI increase[b] |
|---|---|---|---|---|---|
| $Ep_{31}$ | NYKLAVNCF | 31–39 | 9 | 120 | 85 |
| $Ep_{173}$ | RYQLDPKFI | 173–181 | 9 | 150 | 102 |
| $Ep_{185}$ | LYENNVITI | 185–193 | 9 | 75 | 79 |
| $Ep_{225}$ | LFHSKKMDL | 225–233 | 9 | 20 | 29 |
| $Ep_{250}$ | YYVDEKAPEF | 250–259 | 10 | 198 | 57 |
| $Ep_{296}$ | KYEKAEIKEM | 296–305 | 10 | 83 | 24 |
| $Ep_{304}$ | EMGEMHREL | 304–312 | 9 | 5 | 16 |

[a]Estimated half-time of dissociation from HLA-A24 molecules (min), obtained with a computer program (World Wide Web site BioInformatics & Molecular Analysis Section (BIMAS) HLA peptide-binding predictions).
[b]Synthetic peptides were tested for binding to human HLA-A*2402 molecules in MHC stabilization assays as described in the section entitled 'Materials and methods.' MFI, mean fluorescence intensity.

*Table 1*

### Generation of Ep-CAM peptide-specific CTL lines and clones

Peripheral blood monocyte-derived dendritic cells (DCs) were generated as described previously (28). Briefly, plastic adherent cells were isolated from PBMCs and were cultured in RPMI1640 medium supplemented with 5% heat-inactivated human serum, 10 ng/ml of recombinant human interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) and 50 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (R&D Systems). On day 1 of incubation, 10 ng/ml of IL-1β PeproTech, Rocky Hill, NJ), 50 ng/ml of recombinant human tumour necrosis factor-α (TNF-α) (PeproTech) and 1 μM prostaglandin $E_2$ (Cayman Chemical Company, Ann Arbor, MI) was added for maturation. On days 2 or 3, the cells were harvested and were confirmed to express mature DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class-II molecules (data not shown). The DCs were pulsed with each of the synthetic peptides at a concentration of 10 μM in AIM-V medium (Gibco, Grand Island, NY) supplemented with $5 \times 10^{-5}$ M β-mercaptoethanol for 2–4 h at room temperature and were irradiated (33 Gy). Thereafter, the DCs ($1 \times 10^5$) were co-cultured with autologous $CD8^+$ T lymphocytes ($1 \times 10^6$) purified with the aid of CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in RPMI1640 medium supplemented with 10% pooled human serum, 25 ng/ml of recombinant human IL-7 (R&D Systems) and 5 ng/ml of recombinant human IL-12 (R&D Systems) in a culture tube. After culture for 7 days, the cells were stimulated again by adding $1 \times 10^5$ peptide-pulsed autologous DCs prepared as described above. After culture for 7 additional days, the cells were stimulated a third time in the same manner. One day after each restimulation, recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan) was added to a final concentration of 20 U/ml. If necessary, rapidly growing cells were split into two to three tubes and were fed with fresh culture medium containing 20 U/ml of IL-2.

In order to establish T-cell clones, limiting dilution of the polyclonal CTLs was performed (22). After 2-week culture in 96-well plates, the specificity of growing cells was examined with CTL–CTL killing assays as previously described (29). Clones that were

killed only when pulsed with the Ep-CAM peptide were transferred into flasks and were expanded as detailed above.

### Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (22). A total of $1 \times 10^3$ polyclonal $CD8^+$ T cells were co-cultured with peptide-pulsed T2-A24 cells ($5 \times 10^4$) in wells of the MultiScreen-HA plates (Millipore) coated with 10 μg/ml of anti-interferon-γ (IFN-γ) monoclonal antibody (R&D Systems). All assays were performed in duplicate. After probing with a polyclonal rabbit anti-IFN-γ antibody (Genzyme, Cambridge, MA), followed by exposure to peroxidase-conjugated goat anti-rabbit IgG (Genzyme), IFN-γ spots were visualized and were counted under a dissecting microscope.

### CTL assay

Target cells were labelled with $^{51}Cr$ as previously described (22). In some experiments, pre-determined amounts of blocking antibodies, W6/32 (anti-HLA class-I), MA2.1 (anti-HLA-A2) and A11.1 (anti-HLA-A24) were added to the wells 30 min before adding effector cells in order to determine the HLA restriction. The plates were incubated for 4 h at 37°C, and the supernatants were counted in a γ-counter. The percentage of specific $^{51}Cr$ release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

### Cold target inhibition assays

Cold target inhibition assays were performed as described previously (30). Briefly, T2-A24 cells were incubated with the peptide $Ep_{173}$ or EBV-$LMP2_{419}$ at a concentration of 10 μM for 1 h. After extensive washing, the indicated numbers of peptide-loaded cells were incubated with $2 \times 10^4$ cytotoxic effector cells for 1 h, and then $2 \times 10^3$ $^{51}Cr$-labelled PC9 cells were added to each well. Cytotoxicity was assessed as described above.

## Results

### Selection of potential HLA-A24-binding peptides within Ep-CAM proteins

The computer program was applied in order to identify potential HLA-A*2402-binding peptides within the amino acid sequence of Ep-CAM and to predict HLA-binding peptides, on the basis of estimation of the half-time dissociation of the HLA/peptide complex. The peptide sequences were reviewed according to peptide motifs for HLA-A24 molecules (31–33), and seven peptides were synthesized (Table 1). MHC stabilization assays were performed in order to test their HLA-A*2402-binding efficiency by using T2-A24 cells. Most peptides increased the HLA-A24 expression on the cells, indicating that they bound and stabilized the HLA complexes on the cell surface, but peptide $Ep_{304}$ gave a low value for the percentage of MFI (Table 1) and was excluded from further studies.

### Screening of peptides antigenic for anti-Ep-CAM polyclonal CTL lines by means of ELISPOT assay

In order to identify peptides recognized by Ep-CAM-specific CTLs in the context of HLA-A*2402 molecules, $CD8^+$ T cells of five HLA-$A24^+$ healthy donors were stimulated with autologous DCs pulsed with each of the six peptides. After three rounds of stimulation, T cell lines from four donors produced significant numbers of IFN-γ spots when incubated with T2-A24 cells pulsed with peptide $Ep_{173}$ (Fig. 1). Almost no spots were produced with control peptide $ENV_{584}$ (data not shown). After four-time stimulation, the CTL line established from donor 4 specifically stained with the HLA-A24/$Ep_{173}$ tetramer but not the HLA-A24/$ENV_{584}$ tetramer (37.2 *vs* 0.06% of the total $CD8^+$ T cells, Fig. 2A). The intensity of the tetramer-positive cells was homogeneous and stronger than that of tetramer-negative cells by two- to three-fold on a logarithmic scale.

We established a T-cell clone, designated as C27, from limiting dilution culture of the $Ep_{173}$-specific polyclonal CTL line of donor 4. The study with tetramers indicated that both polyclonal and monoclonal $Ep_{173}$-specific $CD8^+$ T cells had high-affinity antigen receptors directed to HLA-A*2402/$Ep_{173}$ complexes (Fig. 2B).

While an $Ep_{250}$-stimulated CTL line from donor 3 also specifically produced IFN-γ spots when incubated with $Ep_{250}$ (Fig. 1), the establishment of $Ep_{250}$-specific CTL clones was unsuccessful. We, thus, further evaluated the $Ep_{173}$-specific CTL clone.

### Characterization of the peptide $Ep_{173}$-specific $CD8^+$ CTL clone

Ep-CAM expression of cancer cell lines was examined by means of RT-PCR, Western blot analysis (Fig. 3) and indirect immunofluorescence (Table 2). The data showed concordance of results obtained with the three approaches. Twelve of 15 (80%) cancer cell lines appeared to express Ep-CAM. When HLA-A24 expression was examined with the help of indirect immunofluorescence by using an HLA-A24 monoclonal antibody, 10 were positive (Table 2).

As shown in Fig. 4(A), the $Ep_{173}$-specific CTL clone, C27, showed cytotoxicity to T2-A24 cells pulsed with $Ep_{173}$ at a peptide

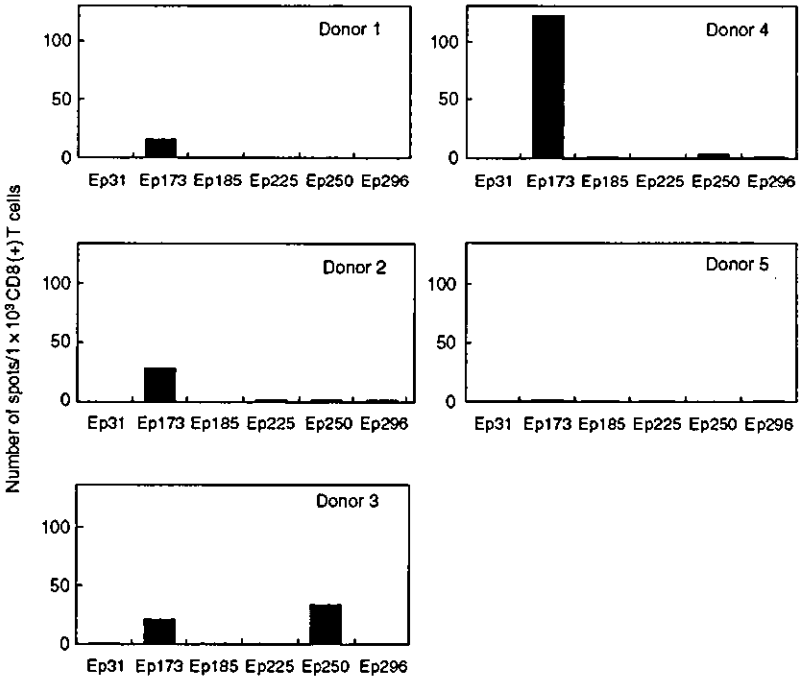


*Fig. 1.* **Evaluation of CD8⁺ T cell lines by means of enzyme-linked immunospot (ELISPOT) assay.** Aliquots of CD8⁺ T cells ($1 \times 10^3$) were incubated with T2-A24 cells pulsed with the indicated peptide (10 μM) in wells of the ELISPOT plate. All assays were performed in duplicate.

concentration as low as 100 pM, but not with control peptide EBV-$LMP2_{419}$. Data for cytotoxicity of C27 against various cancer cell lines have been shown in Fig. 5. C27 efficiently lysed lung cancer cell lines PC9, LU99, LC99A and LC-1/sq, an oral squamous cell carcinoma cell line HSC-2 and a gastric cancer cell line MKN45 that expressed both HLA-A24 and Ep-CAM. However, no killing was observed with HLA-A24⁺ Ep-CAM (11-18, COLO320 DM and A549-A24) or HLA-A24 (either Ep-CAM⁺ or Ep-CAM ) cell lines (QG56, A549 and MNK28). After the transfection of the HLA-A*2402 cDNA into HLA-A24 QG56 cells (QG56-A24), they were killed by C27. K562 cells were included in order to assess the degree of NK-like cytotoxicity of C27, which turned out to be negligible.

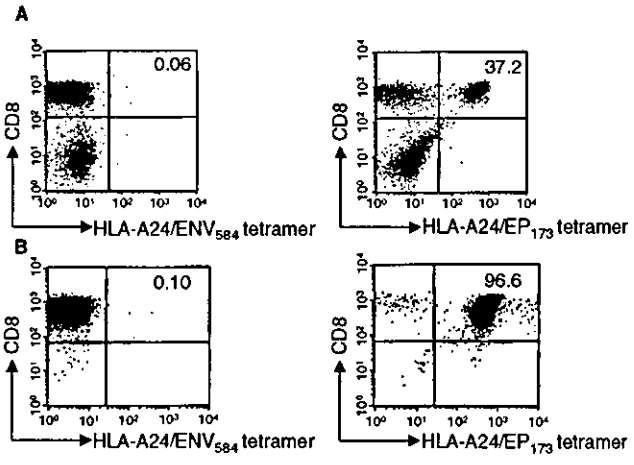


*Fig. 2.* **Tetramer staining of $Ep_{173}$ peptide-specific CD8⁺ T cells.** Polyclonal CD8⁺ T cells (A) and an $Ep_{173}$-specific cytotoxic T-lymphocyte (CTL) clone – C27 – (B) were stained with HLA-A24 tetramers incorporating $Ep_{173}$ or a control peptide, $ENV_{584}$. The percentages of tetramer-positive cells in CD8⁺ T cells have been shown. HLA, human leucocyte antigen.

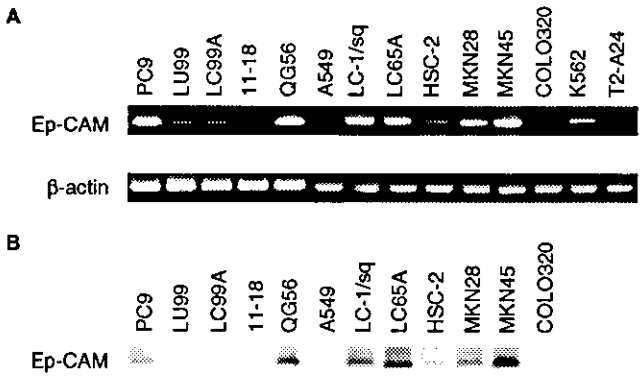


*Fig. 3.* **Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis of epithelial cell adhesion molecule (Ep-CAM) in cancer cell lines.** Semi-quantitative RT-PCR analysis was performed by using specific primers for Ep-CAM and β-actin (A). Western blot analysis of Ep-CAM was performed with a monoclonal antibody specific to Ep-CAM (B).

**Characteristics of the cell lines used**

| Cell line | Origin | Surface expression (MFI[a]) of Ep-CAM | HLA-A24 |
|---|---|---|---|
| Lung cancer | | | |
| LU99 | Giant cell carcinoma | + (22.55) | + (87.16) |
| PC9 | Adenocarcinoma | + (383.67) | + (43.53) |
| 11–18 | Adenocarcinoma | – (4.00) | + (73.67) |
| LC99A | Large cell carcinoma | + (15.32) | + (96.71) |
| LC65A | Small cell carcinoma | + (307.14) | – (3.67) |
| LC-1/sq | Squamous cell carcinoma | + (198.94) | + (70.56) |
| A549 | Adenocarcinoma | – (6.40) | – (3.21) |
| A549-A24 | Adenocarcinoma | Not done | + (109.73) |
| QG56 | Squamous cell carcinoma | + (229.13) | – (2.65) |
| QG56-A24 | Squamous cell carcinoma | Not done | + (84.12) |
| Gastric cancer | | | |
| MKN28 | Adenocarcinoma | + (722.59) | – (7.67) |
| MNK45 | Adenocarcinoma | + (823.25) | + (47.99) |
| Colon cancer | | | |
| COL0320DM | Adenocarcinoma | – (3.07) | + (35.35) |
| Others | | | |
| HSC-2 | Oral squamous cell carcinoma | + (57.15) | + (34.40) |
| K562 | Chronic myelogeneous leukaemia | + (41.45) | – (5.23) |
| T2-A24 | B × T hybrid cells | – (3.05) | + (197.75) |
| NHBE | Normal bronchial epithelial cells | + (152.56) | + (33.52) |

[a]Mean fluorescence intensity (MFI) was examined with the help of flow cytometric analysis after cell staining by using anti-Ep-CAM or anti-HLA-A24 monoclonal antibodies and FITC-labelled anti-mouse IgG $F(ab')_2$ fragments.

*Table 2*

These data demonstrated that $Ep_{173}$-specific CTLs kill tumour cells expressing both HLA-A24 and Ep-CAM.

We further examined whether C27 might recognize naturally processed peptides presented on the surfaces of tumour cells in the context of HLA-A24. Cytotoxicity of C27 against PC9 cells ($HLA\text{-}A24^+$ $Ep\text{-}CAM^+$ lung cancer cell line) was blocked by monoclonal antibodies specific to HLA-A24 or pan-class-I molecules, but not an anti-HLA-A2 monoclonal antibody (Fig. 4B), confirming the HLA-A24 restriction. Cold target inhibition assays demonstrated that C27-mediated cytotoxicity against PC9 cells was specifically inhibited in the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (Fig. 4C), indicating that C27 recognized peptides that were naturally processed and presented.

Because Ep-CAM is expressed in some sites of normal epithelial cells, there has been concern about potential autoimmune reactions after *in vivo* activation of T cells specific to the molecule. We, therefore, tested an HLA-A*2402-positive, normal human bronchial epithelial cell line, designated as NHBE, with clone C27. As demonstrated in Fig. 6(A), C27 exerted toxicity, especially at higher effector : target ratios (40 : 1 and 20 : 1), which was specifically blocked by T2-A24 cells pre-pulsed with the cognate peptide (Fig. 6B). At lower effector : target ratios (10 : 1 and 5 : 1), however, C27-mediated lysis of NHBE was apparently lower than that of cancer cell lines, such as PC9, LU99, LC99A and HSC-2 (Fig. 5).

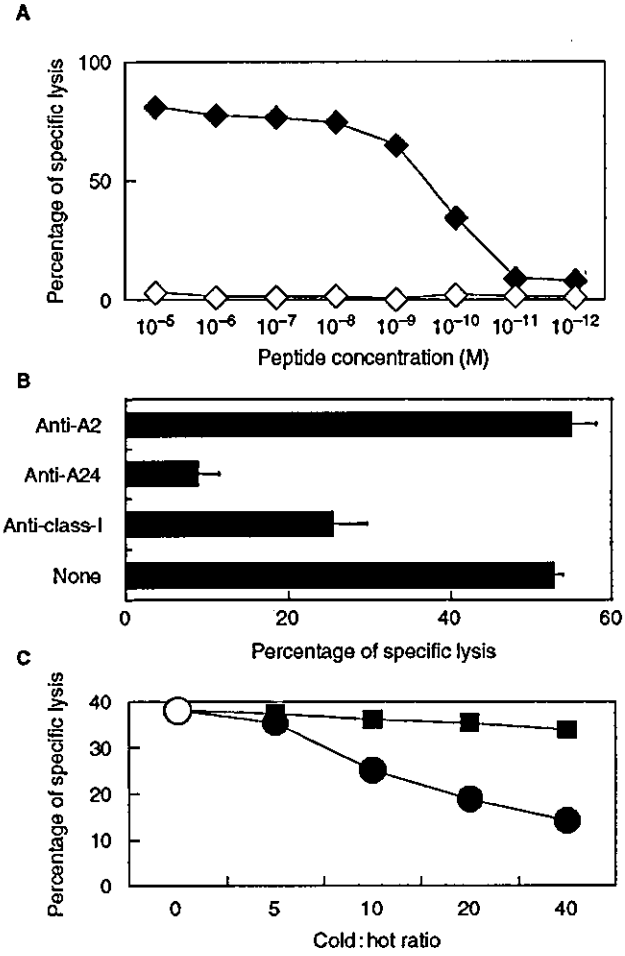


***Fig. 4.* Characterization of an $Ep_{173}$-specific cytotoxic T-lymphocyte (CTL) clone, C27.** An $Ep_{173}$-specific CTL clone, C27-mediated target cell (T2-A24) lysis in the presence of $Ep_{173}$ (◆) and control peptide $EBV\text{-}LMP2_{419}$ (◇) has been shown (A). C27-mediated target cell (PC9) lysis was blocked with monoclonal antibodies specific to HLA-A24 and pan-class-I molecules (B). C27-mediated target cell (PC9) lysis was blocked with T2-A24 cells that had been loaded with $Ep_{173}$ (●) but not with a control peptide $EBV\text{-}LMP2_{419}$ (■) (C). The cytotoxic assays were performed at effector to $^{51}Cr$-labelled target ratios of 1, 10 and 5, in experiments shown in A, B and C, respectively. HLA, human leucocyte antigen.

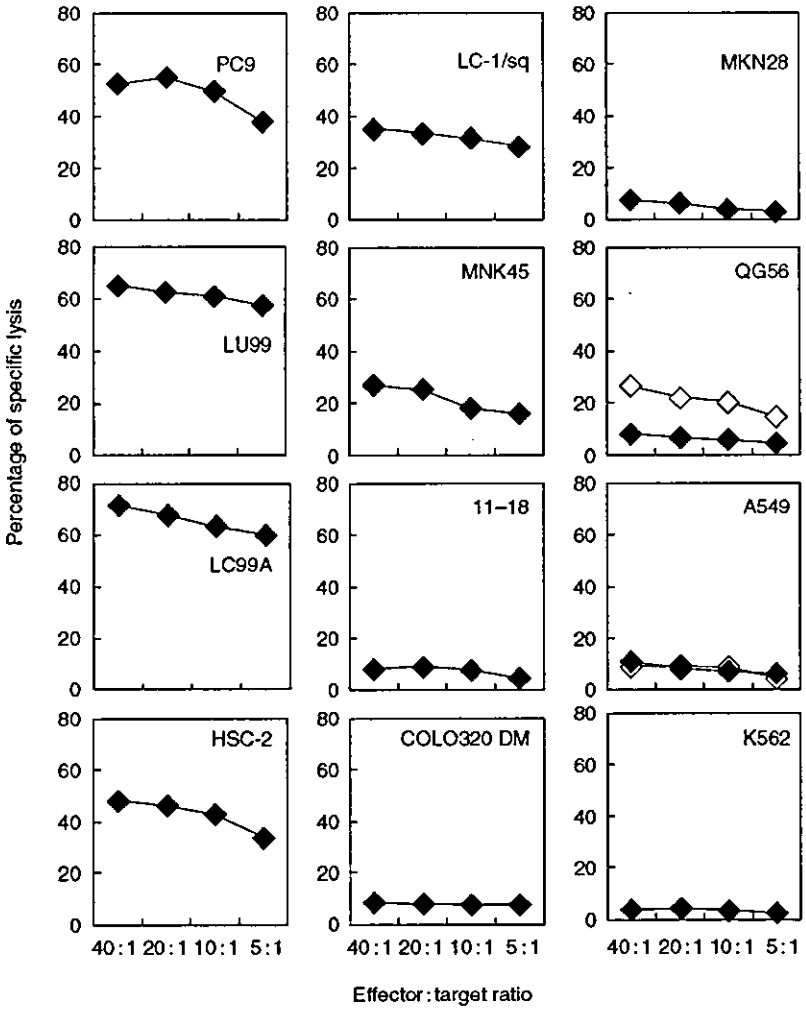


***Fig. 5.* Cytotoxicity of the clone C27 against cancer cell lines.** An $Ep_{173}$-specific cytotoxic T-lymphocyte (CTL) clone, C27-mediated lysis of various cancer cell lines. Cell lines in the left and the centre columns express HLA-A24 on their surface, whereas those in the right do not. All the target cell lines except for 11–18, COLO320DM and A549 express the Ep-CAM. HLA-A*2402 transfectants of QG56 and A549 cells were also used as target cells (◇). K562 was included in order to assess natural killer-like cytotoxicity. HLA, human leucocyte antigen.

## Discussion

According to recent findings, overexpression of Ep-CAM, a transmembrane protein mediating $Ca^{2+}$-independent cell–cell adhesion, is correlated with tumour grade (14, 34, 35), providing a useful marker for diagnosis of micrometastases (36) and a predictor of survival (14). The promoter region that regulates the Ep-CAM transcription has been cloned and it has shown to be negatively regulated by TNF-α (37).

Ep-CAM has become one of the major targets for immunotherapy with monoclonal antibody, because it is expressed in the vast majority of epithelial cell-derived cancers. The administration of Ep-CAM-specific murine monoclonal antibody (17-1A) into Duke's C colorectal cancer patients after surgery, for example, led to the prevention of distant metastasis and to prolonged survival after 7 years of follow-up evaluation (19). Recently, bispecific antibodies against Ep-CAM and CD3 (38, 39) or B7 (40) have been engineered in order to improve cytotoxicity with synergistic effects of antibodies and T lymphocytes. Considering the expression pattern, immunotherapy by using CTL directed against Ep-CAM appears attractive. Indeed, Ep-CAM peptides that elicited cellular immune responses restricted by HLA-A*0201 have recently been identified (4, 20). Of note, a natural T-cell response against Ep-CAM has been observed in colorectal cancer patients (41, 42). The available evidence, thus, suggests the possibility of therapeutic approaches using Ep-CAM as a target of CTLs.

We, in this study, explored immunogenic peptides derived from Ep-CAM that can elicit cellular immune responses against Ep-CAM$^+$ tumour cells in the context of HLA-A24, which is positive in nearly 20% of persons of European descent and more than 60% of Japanese. For that purpose, seven candidate peptides were first predicted from the Ep-CAM protein with a bioinformatic approach. Secondly, MHC

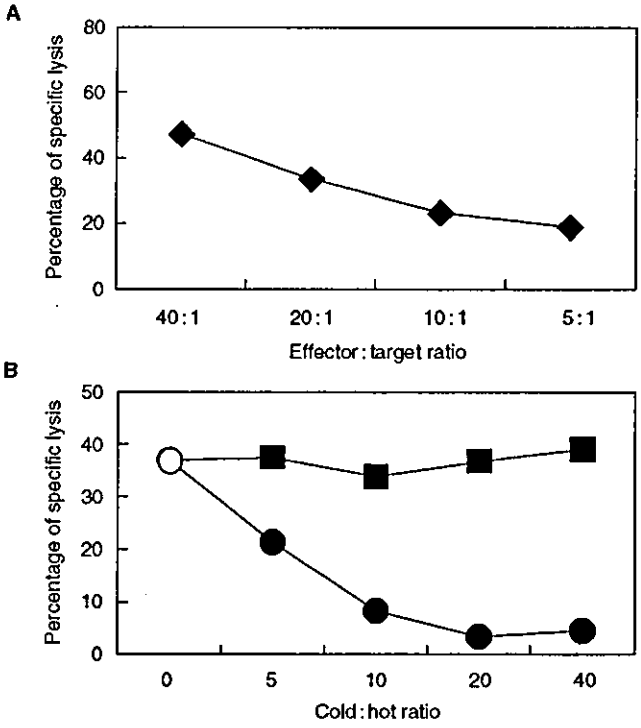


***Fig. 6.*** **Cytotoxicity of the clone C27 against normal bronchial epithelial cells.** C27-mediated lysis of normal bronchial epithelial cells (NHBE) expressing HLA-A24 and Ep-CAM (A) and C27-mediated NHBE cell lysis were blocked with T2-A24 cells pulsed with $Ep_{173}$ (●) but not with a control peptide EBV-$LMP2_{419}$ (■) (B). The effector to hot target ratio was 20. HLA, human leucocyte antigen.

stabilization assays were used in order to determine the binding affinity of the peptides with HLA-A*2402 molecules, revealing one peptide, $Ep_{173}$ (RYQLDPKFI), to have the highest affinity and $Ep_{304}$ (EMGEMHREL) the lowest. Thirdly, we successfully established $Ep_{173}$-specific polyclonal CTL lines from four of five healthy donors. A CTL clone, C27, demonstrated fine specificity for $Ep_{173}$, which was naturally presented on the surfaces of tumour cells, because C27-mediated PC9 cell lysis was blocked by both anti-HLA-A24 monoclonal antibody and $Ep_{173}$-pulsed cold target cells. An $Ep_{250}$-specific polyclonal CTL line was generated from donor 3, raising the possibility that $Ep_{250}$ is another CTL epitope presented by HLA-A*2402. We, so far, could not establish CTL clones specific to $Ep_{250}$, which are required for further evaluation of the peptide.

It is of note that there is no correlation between the level of expression of Ep-CAM and the degree of CTL-mediated lysis (Figs. 3 and 5; Table 2). The level of HLA expression does not seem to explain the issue. We speculate that the Ep-CAM may be more resistant to proteasome degradation in cells with high Ep-CAM expression and low or moderate sensitivity to CTL-mediated lysis (LC-1/sq, MKN45 and QG56). On the contrary, there may be a higher turnover rate of the Ep-CAM in cells with low expression and high sensitivity to CTL-mediated lysis (LU99, LC99A and HSC-2).

Although Ep-CAM is a candidate target for both humoral and cellular immunotherapy, a persistent difficulty arises because of its presentation on normal epithelial tissues. Indeed, the CTL clone, C27, lysed HLA-A24-positive normal bronchial epithelial cells in an epitope-specific fashion, especially at high effector : target ratios (Fig. 6). However, at lower effector : target ratios, such as 10 : 1 and 5 : 1, C27-mediated lysis of NHBE was apparently lower than that of C27-sensitive cancer cell lines (Fig. 5). In addition, C27-mediated lysis of NHBE was more efficiently inhibited by T2-A24 cells pulsed with the cognate peptide (Fig. 6B) than that of the PC9 lung cancer cell line (Fig. 4C), implicating a lower density of HLA-A*2402/$Ep_{173}$ complexes on the surface of NHBE cells.

It has been reported that an HLA-A*0201-restricted Ep-CAM-specific CTL line could not lyse normal bronchial epithelial cells under conditions, whereby epithelial tumour cells were efficiently killed (20). So far, immunization of colorectal carcinoma patients with a recombinant canarypox virus expressing Ep-CAM has been well tolerated and has been shown to induce anti-Ep-CAM CTL responses without causing autoimmune reactions (43). Interestingly, a monoclonal antibody specific to Ep-CAM did not localize to Ep-CAM-positive normal tissues of human Ep-CAM transgenic mice (6), indicating limited *in vivo* accessibility, which might explain any absence of autoimmunity. Another example of vaccination of tissue-specific self-antigen is provided by means of immunotherapy targeting CEA (carcino embryonic antigen). Greiner et al. (44) reported that vaccination of CEA-transgenic mice with a recombinant canarypox virus expressing CEA can generate substantial anti-tumour immunity with little or no autoimmunity. They proposed possible explanations including (i) differential susceptibility of tumour and normal tissues to the immune effector arms and (ii) blockage of the autoreactive T-cell activity by tolerizing antigen-presenting cells or the presence of regulatory T cells in order to terminate the response in the normal epithelia. Further studies are required in order to evaluate potential autoimmune reactions with $Ep_{173}$ immunization. Littermates of human Ep-CAM transgenic mice (6) bred with HLA-A*2402 transgenic mice would provide a suitable model to study this issue.

In conclusion, we present, in this study, a novel HLA-A*2402-restricted epitope, $Ep_{173}$ (RYQLDPKFI), which has the ability to induce $CD8^+$ T cells with high-affinity antigen receptors directed to HLA-A*2402/$Ep_{173}$ complexes. All the data suggest that the epitope-specific CTL responses may play some roles in both anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among populations possessing HLA-A*2402.

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# A Novel HLA-A*3303-Restricted Minor Histocompatibility Antigen Encoded by an Unconventional Open Reading Frame of Human TMSB4Y Gene[1]

Hiroki Torikai,[2*‡] Yoshiki Akatsuka,[2,3*] Mikinori Miyazaki,[*] Edus H. Warren III,[§] Taku Oba,[¶] Kunio Tsujimura,[*] Kazuo Motoyoshi,[‡] Yasuo Morishima,[†] Yoshihisa Kodera,[¶] Kiyotaka Kuzushima,[*] and Toshitada Takahashi[*]

**Female-to-male hemopoietic stem cell transplantation (HSCT) elicits T cell responses against male-specific minor histocompatibility (H-Y) Ags encoded by the Y chromosome. All previously identified H-Y Ags are encoded by conventional open reading frames, but we report in this study the identification of a novel H-Y Ag encoded in the 5′-untranslated region of the *TMSB4Y* gene. An HLA-A*3303-restricted CD8$^+$ CTL clone was isolated from a male patient after an HSCT from his HLA-identical sister. Using a panel of cell lines carrying Y chromosome terminal deletions, a narrow region controlling the susceptibility of these target cells to CTL recognition was localized. Minigene transfection and epitope reconstitution assays identified an 11-mer peptide, EVLLRPGLHFR, designated TMSB4Y/A33, whose first amino acid was located 405 bp upstream of the *TMSB4Y* initiation codon. Analysis of the precursor frequency of CTL specific for recipient minor histocompatibility Ags in post-HSCT peripheral blood T cells revealed that a significant fraction of the total donor CTL response in this patient was directed against the TMSB4Y epitope. Tetramer analysis continued to detect TMSB4Y/A33-specific CD8$^+$ T cells at least up to 700 days post-HSCT. This finding underscores the in vivo immunological relevance of minor histocompatibility Ags derived from unconventional open reading frame products.** *The Journal of Immunology,* **2004, 173: 7046–7054.**

Minor histocompatibility (minor H)[4] Ags are MHC-bound peptides derived from cellular proteins and are encoded by polymorphic genes, including Y chromosome-specific genes (1–3). Disparities in some minor H Ags in allogeneic hemopoietic stem cell transplantation (HSCT) have been shown to be associated with graft-vs-host disease (GVHD), graft rejection, or graft-vs-leukemia/lymphoma (GVL) effect (4–11). In the case of female to male HSCT, T cell clones specific for Y chromosome-encoded (H-Y) Ags were generated from the peripheral blood of recipients during GVHD or graft rejection, and their HLA class I or II epitopes have been identified, including *SMCY* (12, 13), *DFFRY* (14, 15), *UTY* (16, 17), *RPS4Y* (18), and *DBY* (19, 20). These five genes are among eight genes that have been reported to lie in the nonrecombining region of the human Y chromosome and have functional X homologues (21). Because all eight genes are sufficiently polymorphic with their X chromosome homologues to induce H-Y-specific T cell responses, it should be possible that more H-Y epitopes can be encoded either by the five genes that have proved to be immunogenic or by other Y chromosome genes (i.e., *ZFY*, *AMELY*, and *TMSB4Y*) for which H-Y epitopes have not yet been described.

In this study we report the identification of a novel human H-Y Ag, recognized by an HLA-A*3303-resricted CTL clone isolated from a male patient who developed chronic, but not acute, GVHD. The identified H-Y Ag is an 11-mer peptide, EVLLRPGLHFR, derived from *TMSB4Y*, a gene encoding thymosin $\beta$-4, Y isoform (22). Interestingly, the epitope identified in the *TMSB4Y* gene was encoded by the polymorphic region located 405 bp upstream of the initiation codon of the conventional open reading frame (ORF), whereas all minor H Ags identified to date are encoded by conventional ORF of the individual gene. There have been several reports describing CTL epitopes encoded by unconventional ORFs, such as untranslated regions (UTR) or alternative reading frames, most of which have been identified in tumor cells (reviewed in Ref. 23). To our knowledge, this is the first demonstration of a minor H Ag encoded in a region other than conventional coding region. Furthermore, we demonstrated, by CTL precursor (CTLp) frequency analysis, that a significant fraction of the total donor CTL responses in this patient was directed against the TMSB4Y epitope, and that the precursor remained detectable up to

*Division of Immunology, Aichi Cancer Center Research Institute, and †Department of Hematology and Chemotherapy, Aichi Cancer Center Hospital, Nagoya, Japan; ‡Third Department of Internal Medicine, National Defense Medical College, Tokorozawa, Japan; §Program in Immunology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; and ¶Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan

Received for publication August 4, 2004. Accepted for publication September 3, 2004.

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[1] This work was supported in part by a Grant-in-Aid for Scientific Research (C) (to Y.A.) and Scientific Research on Priority Areas (to T.T., K.T., and Y.A.), from the Ministry of Education, Culture, Science, Sports, and Technology, Japan; Research on Human Genome, Tissue Engineering Food Biotechnology (to Y.A.), and Second Team Comprehensive 10-year Strategy for Cancer Control (to T.T.), from the Ministry of Health, Labor, and Welfare, Japan; a grant from Aichi Cancer Research Foundation (to Y.A.); a Nagono Medical Research Grant (to K.K. and Y.A.); a grant of Entrusted Research with DNA Bank, RIKEN BioResource Center (to Y.A.); and a Lilly Clinical Investigator Award from the Damon Runyon Cancer Research Foundation (to E.H.W.).

[2] H.T. and Y.A. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Yoshiki Akatsuka, Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. E-mail address: yakatsuk@aichi-cc.jp

[4] Abbreviations used in this paper: minor H Ag, minor histocompatibility Ag; CI, confidence interval; CTLp, CTL precursor; DRiP, defective ribosomal product; GVHD, graft-vs-host disease; GVL, graft vs leukemia/lymphoma; HSCT, hemopoietic stem cell transplantation; H-Y Ag, Y chromosome-encoded Ag; LCL, B-lymphoblastoid cell line; ORF, open reading frame; UTR, untranslated region; STS, sequence-tagged site.



0022-1767/04/$02.00

700 days after HSCT. These findings underscore the in vivo immunological relevance of such a cryptic minor H Ag derived from unconventional ORF products.

## Materials and Methods

### *Cell cultures and Abs*

The HLA-A*3303-restricted $CD8^+$ CTL clone, 1B6, was isolated by limiting dilution from a cytotoxic T cell line generated from a PBMC sample obtained on day 50 post-HSCT from a 54-year-old man (HLA-A*2402/*3303, B*4403/*5401, Cw*0803/*1403) who had received his HLA-identical sister's marrow for treatment of chronic myelocytic leukemia. He did not develop acute GVHD, but did develop mild chronic GVHD of the skin and liver. The CTL clone was expanded as previously described (24) and frozen until use. B-lymphoblastoid cell lines (LCLs) were established from the donor and recipient and from normal volunteers. All blood or tissue samples were collected after obtaining written informed consent, and the study was approved by the institutional review board of Aichi Cancer Center.

The LCLs derived from individuals with Y chromosome deletions were provided by Dr. D. C. Page (Howard Hughes Medical Institute, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA), and a detailed analysis of these lines has been previously reported (25). LCLs selected according to their deletion pattern and other cell lines including Raji were retrovirally transduced with HLA-A*3303 cDNA as described previously (26). LCLs were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS (Immuno-Biological Laboratory, Gunma, Japan), 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin/streptomycin. Primary dermal fibroblast lines from skin and oral mucosa, bone marrow stromal cell lines, and 293T cells were grown in IMDM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FCS, 2 mM L-glutamine, and penicillin/streptomycin. mAbs, W6/32 (anti-pan HLA class I), HDR-1 (anti-HLA-DR), and A11.1M (anti-HLA-A24) were provided by Dr. K. Ito (Kurume University, Fukuoka, Japan).

### *Cytotoxicity assays*

Target cells were labeled with 0.1 mCi of $^{51}Cr$ for 2 h, and $1 \times 10^3$ target cells/well were mixed with CTL at various E:T cell ratios in a standard 4-h cytotoxicity assay using 96-well, round-bottom plates. All assays were performed at least in duplicate. Cells were treated with IFN-$\gamma$ (100 U/ml; Endogen, Woburn, MA) and TNF-$\alpha$ (10 ng/ml; Endogen) for 48 h where indicated. The percent specific lysis was calculated as follows: ((experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)) × 100. When necessary, allo-HLA-A24-specific CTL clones were used to confirm the susceptibility of the target cells.

### *Mapping of Y chromosome deletion mutant LCLs*

Oligonucleotide primer pairs specific for sequence-tagged sites (STSs) previously mapped to the Y chromosome (25) were used to PCR-amplify the corresponding Y chromosomal target sequences from genomic DNA of each LCL. Amplification of STSs was performed as reported previously (16). Aliquots of each PCR were separated in 2% agarose or 5% acrylamide gels, and cell lines were scored as positive or negative for the presence of each STS. DNA extracted from LCLs derived from normal male and female donors served as positive and negative controls, respectively.

### *Detection of expression of the candidate genes*

An RT-PCR assay was used to examine the expression of the candidate genes with cDNA synthesized from LCLs. PCR was performed in a total volume of 20 $\mu$l containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP, 0.5 $\mu$M of each gene-specific primer, and 1 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) on a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were separated in 2% agarose gels and visualized with ethidium bromide staining.

### *PCR cloning of TMSB4Y gene*

The conventional ORF sequence and full-length sequence of the *TMSB4Y* (GenBank accession no. NM_004202) were amplified from cDNA prepared from the recipient LCL and subcloned into a mammalian expression plasmid. The primer sequences used were as follows (*Hin*dIII and *Not*I sites are underlined, respectively): conventional ORF sense, 5′-TTAAGCTTCGCAGCCATGTCTGACAAACC-3′; conventional ORF antisense, 5′-ATGCGGCCGCCATGCCTGTTTAAGATTCGC-3′; full-length sense, 5′-TTAAGCTTTGGGAACAGACAGATCCTTTG-3′; and full-length antisense, 5′-ATGCGGCCGCTAGATTTCACTGCCCTCCCA-3′. PCR amplification was conducted in a total volume of 25 $\mu$l of 1× buffer containing 200 $\mu$M of each dNTP, 1.0 mM $MgSO_4$, 0.3 $\mu$M of each primer, and 1 U of KOD-Plus-DNA polymerase (TOYOBO, Osaka, Japan).

All products were digested with the restriction enzymes and ligated into *Hin*dIII-*Not*I-cut pEAK10 vector (Edge Biosystems, Gaithersburg, MD). The sequences of the cloned genes were verified by direct sequencing with BigDye Terminator kit (version 3.0, Applied Biosystems) on an ABI PRISM 3100 (Applied Biosystems).

### *Construction of truncated genes and minigenes for TMSB4Y*

Expression plasmids encoding truncated forms of the TMSB4Y cDNA were constructed by RT-PCR using antisense primers that produced 345, 552, 754, and 955 bp DNA fragments. All products were ligated into the pEAK10 vector as described above. Minigene expression plasmids encoding the minimal, N or C terminus-extended polypeptides of the epitope predicted by BIMAS software (http://bimas.dcrt.nih.gov/molbio/hla_bind/) (27) and SYFPEITHI software (http://syfpeithi.de) (28) were constructed as previously described (29). The constructs all encoded a Kozak sequence and initiator methionine (CCACC-ATG) and a stop codon (TAG). Pairs of sense and antisense oligonucleotides were designed to form cohesive ends for *Hin*dIII and *Not*I sites at the 5′ and 3′ ends after hybridization, respectively, and all products were ligated into the pEAK10 vector and verified by sequencing.

### *Electroporation of LCL*

The constructed vectors were introduced either into the donor LCL or into 293T cells. One million LCL were resuspended in 40 $\mu$l of OPTI-MEM I buffer (Invitrogen Life Technologies) and 4 $\mu$g of each plasmid in a 2-mm gap cuvette, and electroporated in an ECM 830 BTX Electro Square Porator (BTX, San Diego, CA) at 350 V and a pulse length of 1 ms. Then, cells were cultured in 4 ml of culture medium for 2 d, followed by selection with puromycin (0.7 $\mu$g/ml) for 3 d before use.

### *Transfection of 293T cells and cytokine release assays*

293T cells were retrovirally transduced with HLA-A*3303 cDNA and selected in the presence of 1 $\mu$g/ml puromycin (referred to as 293T-A33). Aliquots of the 293T-A33 cells were transiently cotransfected with pEAK10 vectors encoding full-length TMSB4Y, a C-terminal deletion mutant cDNA, or minigenes of TMSB4Y. 293T-A33 cells were plated the day before transfection at $4 \times 10^4$ cells/100 $\mu$l/well into 96-well, flat-bottom microtiter plates and transfected with 6 $\mu$l of RPMI 1640 containing 90 ng of plasmid DNA and 0.27 $\mu$l of FuGENE 6 (Roche, Indianapolis, IN). After 24 h at 37°C, 100 $\mu$l of a cell suspension containing $1 \times 10^4$ CTL clone 1B6 in IMDM containing 20 U/ml IL-2 was added. Supernatants from the cocultures were harvested after 24 h and assayed for the presence of IFN-$\gamma$ by ELISA.

### *Epitope reconstitution assay*

The candidate peptide epitope identified by the minigene experiments and the homologous TMSB4X-encoded peptide were synthesized by standard methods. $^{51}Cr$-labeled donor LCL were incubated for 30 min in medium containing 10-fold serial dilutions of the peptides and then used as target cells in standard cytotoxicity assays.

### *Real-time PCR assay for TMSB4Y expression*

cDNA from a panel of different human adult and fetal tissues were purchased from BD Clontech (MTC panels human I and II; Palo Alto, CA) or synthesized from total RNA of human lung (BD Clontech) or various cultured cells. PCR amplification and real-time quantification analysis were performed using the TaqMan assay according to the manufacturer's instructions. The following sequences were used as primers and TaqMan probe to detect the mRNA region encoding the epitope: 5′-GACTAGA AAGCGGGCGCAG-3′ (sense; nt 302–320), 5′-ACTTCCGCGTTCAA GTGGTT-3′ (antisense; nt 415–434), 5′-(FAM)-TCCCTTCTCGACACG GAGTCTATGTGTAGT-(MGB)-3′ (TMSB4Y probe; antisense; nt 366–382). For the internal control, a primer and probe set for human GAPDH (Applied Biosystems) was used. PCR was performed in a 1× TaqMan Universal PCR master mix containing 10 pmol of each sense and antisense primer and 2 pmol of probe in a total volume of 25 $\mu$l in the ABI PRISM 7700HT Sequence Detector System (Applied Biosystems). The temperature profile was 50°C for 2 min, 95°C for 10 min, and then 95°C for 15 s and 62°C for 1 min for 40 cycles. Samples were quantified using relative standard curves for each amplification. All results are normalized with

respect to the internal control and are expressed relative to the levels found in a pool of male PBMC.

*Limiting dilution-based CTLp frequency assay*

The proportion of CTLp specific for the TMSB4Y peptide among the total CTLp against the recipient minor H Ags was quantitated using a standard limiting dilution assay. Purified $CD8^+$ T cells from the PBMC obtained at days 50 and 146 post-HSCT were cultured at 2-fold serial dilutions with 33 Gy-irradiated $3 \times 10^4$ CD40-activated B (CD40-B) cells generated from pre-HSCT recipient PBMC in 96-well, round-bottom plates in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% pooled human serum. IL-2 (50 U/ml) was added on days 2 and 5 after each restimulation with CD40-B cells. For each dilution, there were at least 12 replicates. After three rounds of stimulation, a split-well analysis was performed for peptide-specific cytotoxicity against $^{51}Cr$-radiolabeled recipient PHA blasts or donor PHA blasts pulsed with TMSB4Y peptide or unpulsed. The supernatants were measured in a gamma counter after 4-h incubation. The wells were considered to be positive for lytic activity if the total cpm released by effector cells was $>2.5 \times$ SD above control wells (mean cpm released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated by L-Calc software (StemCell Technologies, Vancouver, Canada).

*Tetramer construction and flow cytometric analysis*

MHC-peptide tetramers were produced as described previously (30). In brief, HLA-A*3303 H chain and $\beta_2$-microglobulin (cloned in $pHN1^+$ vector; provided by the late Dr. D. C. Wiley, Howard Hughes Medical Institute, Harvard University, Cambridge, MA) were produced in XA90. The C terminus of the H chain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA/$\beta_2$-microglobulin/peptide complexes were folded in vitro in the presence of the peptide. The MHC complex was biotinylated and then converted into tetramers with PE-labeled streptavidin. For staining, PBMC or T cell lines were incubated with the tetramer at a concentration of 20 $\mu$g/ml at room temperature for 15 min, followed by FITC-conjugated anti-CD3 (BD Biosciences, San Diego, CA) and Tricolor anti-CD8 mAb (Caltag Laboratories, Burlingame, CA) on ice for 15 min. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

## Results

*A $CD8^+$ CTL clone shows cytotoxicity against an H-Y Ag presented on HLA-A*3303$^+$ LCL*

$CD8^+$ CTL clone 1B6 efficiently lysed recipient LCL and PHA blasts, but not donor LCL (Fig. 1*A*). Addition of anti-pan HLA class I mAb, but not anti-HLA-A24 or anti-HLA-DR mAbs, significantly inhibited lysis of recipient LCL by 1B6. Transduction of HLA-A*3303 cDNA into a male LCL conferred susceptibility to 1B6, indicating that the clone was restricted by HLA-A*3303 (Fig. 1*B*). 1B6 showed very weak cytotoxicity against dermal or oral fibroblasts or against keratinocytes generated from HLA-A*3303-positive male individuals, whereas these targets were lysed moderately (i.e., 25–35%) by CTL specific for HLA-A24 alloantigen, which is shared by these targets. Even when they were treated with cytokines (IFN-$\gamma$ and TNF-$\alpha$), 1B6 still demonstrated relatively weak cytotoxicity, although HLA-A24-allospecific CTL induced robust cytotoxicity (Fig. 1*C*). Finally, 1B6 showed lytic activity only against male, but not female, LCLs transfected with HLA-A*3303 cDNA, indicating that the clone was specific for a H-Y Ag (data not shown).

*The gene encoding the minor H Ag maps to deletion interval 5D on the Y chromosome*

Cytotoxicity assay-based mapping was conducted to determine the location on the Y chromosome of the minor H gene encoding the epitope for 1B6. First, various LCLs were typed for terminal deletions of the Y chromosomes using the technique of STS content mapping (16, 21). Of these, a panel of LCLs with distinct terminal deletions was selected for transfection with HLA-A*3303 cDNA and assayed for susceptibility to 1B6.

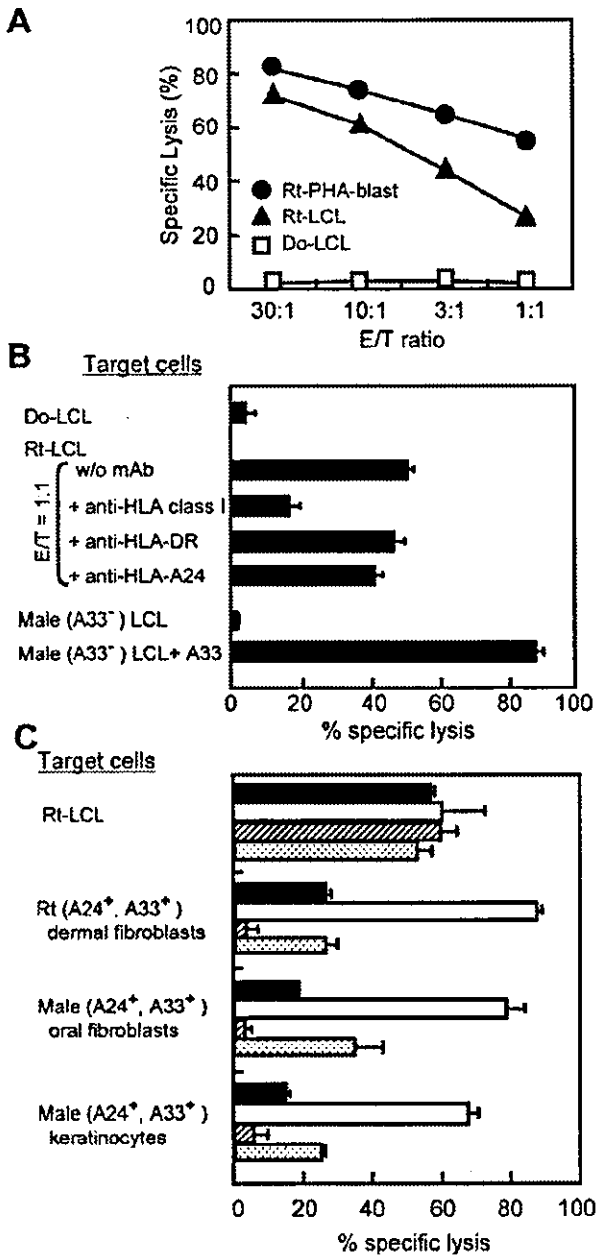


FIGURE 1. Specificity of the HLA-A*3303-restricted CTL clone, 1B6, analyzed in standard $^{51}Cr$ release assays. *A*, 1B6 recognition of target cells derived from recipient (Rt) LCL, PHA blasts, or donor (Do) LCL at the E:T cell ratios indicated. *B*, Ab blocking of the cytolysis was performed with anti-HLA mAbs (E:T cell ratio, 1:1). HLA-A*3303-negative male LCL with or without HLA-A*3303 transduction were tested at an E:T cell ratio of 10:1. *C*, Cytolytic activity of 1B6 or allo-HLA-A*2402-specific CTL against cytokine-treated or untreated HLA-A*3303 and A*2402-positive male B-LCL, dermal fibroblasts, oral fibroblasts, and keratinocytes was tested at an E:T cell ratio of 10:1. The cytokine treatment used was incubation of targets cells with 100 U/ml IFN-$\gamma$ and 10 ng/ml TNF-$\alpha$ for 48 h before $^{51}Cr$ labeling. The lysis of cytokine-treated cells by 1B6 (■) or allo-specific CTL (□) and of cytokine untreated cells by 1B6 (▤) or allo-specific CTL (▨) is shown.

Fig. 2*A* shows the 43-interval deletion map of the 7 LCLs and their susceptibility to 1B6. LCL WHY10 and WHY12 that were lysed by 1B6 share only deletion intervals 5C and 5D, indicating that the region controlling the expression of this minor H Ag is

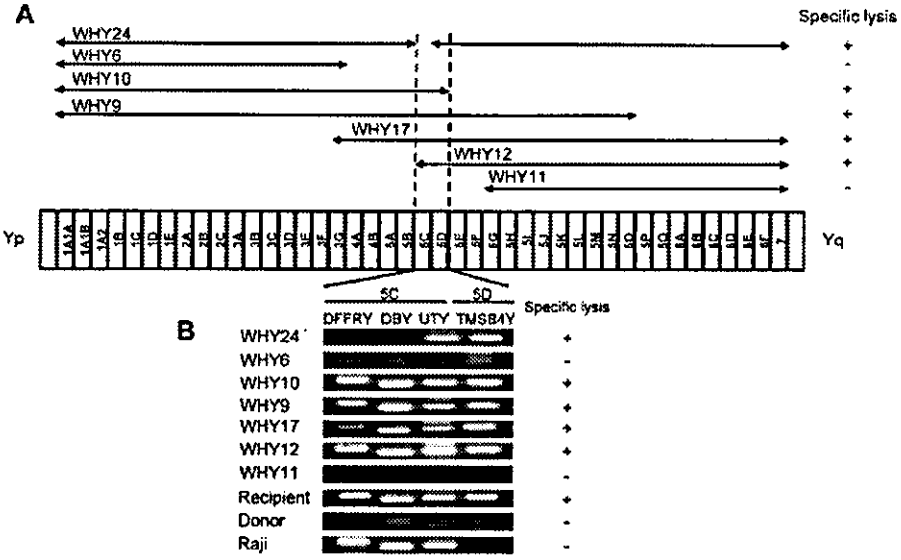


**FIGURE 2.** Mapping of the gene on the Y chromosome that encodes the minor H Ag recognized by 1B6. *A*, Genetic map of the LCLs carrying various terminal deletions of Y chromosome and their susceptibility to 1B6. Appropriate LCLs were selected based on their pattern of terminal deletions (16, 21, 25), transduced with HLA-A*3303 cDNA, and tested in standard $^{51}$Cr release assays. The presence of the region encoding the minor H Ag in each LCL line is determined by its susceptibility to 1B6 (indicated in the *right column*). Bidirectional arrows indicate the conserved region(s) from deletion and are related to the 43-interval deletion map of the Y chromosome (21, 25). Vertical dotted bars indicate the region predicted to encode the minor H Ag. *B*, mRNA expression of four genes encoded in the deletion intervals 5C and 5D in selected cell lines and their recognition by 1B6. Female donor LCL served as a negative control. HLA-A*3303 transfected Raji cells, a Burkitt lymphoma cell line derived from a male patient, were also analyzed.

located within these two deletion intervals. The region was further narrowed down using the results from LCL WHY24, which was found to lack deletion interval 5C, but was nevertheless lysed by 1B6. Collectively, these results indicate that the gene encoding the minor H Ag maps to deletion interval 5D. Four genes, *DFFRY*, *DBY*, *UTY*, and *TMSB4Y*, all of which have X homologues, are encoded within deletion intervals 5C and 5D (21, 25). We examined mRNA expression of these four genes among the seven LCLs by RT-PCR (Fig. 2*B*). As expected from the results of deletion mapping, WHY6 and WHY11 were negative for the expression of all four genes; WHY10, WHY9, WHY17, and WHY12 were all positive. Because WHY24 was positive for the expression of TMSB4Y and UTY and was lysed by 1B6, the minor H Ag was encoded by either *UTY* or *TMSB4Y*. In addition, during the course of specificity analysis, we found that HLA-A*3303-transduced Raji cells were not killed by 1B6, although Raji cells are of male origin. RT-PCR analysis showed that they were negative for expression of TMSB4Y as shown in Fig. 2*B*. Moreover, female LCLs from the patient's HSCT donor transfected with any of three isoforms of *UTY* cDNA were not lysed by 1B6 (data not shown). These results indicated that *TMSB4Y* most likely encoded the minor H Ag.

### *The 5′ untranslated region of the TMSB4Y gene encodes the minor H Ag*

To determine whether *TMSB4Y* indeed encodes the minor H epitope recognized by 1B6, we first tested CTL recognition of the female donor LCL transduced with the reported TMSB4Y ORF comprising 43 aa. However, 1B6 did not lyse the transfectant (Fig. 3*A*), suggesting either that the epitope is encoded not by *TMSB4Y* but by another gene located in deletion interval 5D, or that it is encoded elsewhere in the ~1.7-kb TMSB4Y cDNA. Recently, cryptic CTL epitopes encoded by alternative sources such as noncoding regions and nonconventional ORF have been described in both murine and human tumor cells (23). Thus, we cloned the full-length TMSB4Y cDNA (GenBank accession no. NM_004202) and then transduced donor LCL with it. As shown in Fig. 3*B*, female LCL expressing full-length TMSB4Y cDNA were lysed efficiently. Because the alternative ORF that is able to encode the antigenic peptide was unknown, a series of 3′ terminal deletion mutants of the TMSB4Y cDNA were prepared and tested for recognition by 1B6 by IFN-γ ELISA. Although cells transfected with TMSB4Y cDNA fragments extending from nt 1–552 were recognized when expressed in HLA-A*3303-transduced 293T cells, transfection of the fragment encoding nt 1–345 was not (Fig. 3*C*). These results indicated that the epitope was encoded in the 5′UTR between nt 346 and 552, which is at least 240 nt upstream of the reported ORF for the TMSB4Y protein (Fig. 4*A*).

Among three reading frames in this region, only one initiator methionine (nt 362–364) was found in the same reading frame encoding the TMSB4Y protein, followed by a polypeptide consisting of 19 aa, EVLLRPGLHFRNSCPILTT. This 19-mer contains a nonamer, LLRPGLHFR, which has the reported peptide-binding motif for HLA-A*3303 (i.e., Ala, Ile, Leu, Phe, Tyr, or Val at position 2, and Arg at C terminus) (31), with a predicted dissociation score of 9.0 by BIMAS software (27). However, a minigene construct encoding LLRPGLHFR failed to stimulate 1B6. Additional experiments using minigene constructs with N or C extensions finally identified the minimal epitope as EVLLRPGLHFR (Figs. 3*D* and 4*A*). Both Arg at the C terminus and Glu at the N terminus were essential for recognition by 1B6, indicating that Val and Arg are the likely N- and C-terminal anchors, respectively. The X homologue of TMSB4Y, TMSB4X cDNA (GenBank accession no. NM_021109), encoding thymosin β4, has much shorter 5′- and 3′UTR; thus, no corresponding region was found (Fig. 4*B*). However, a recently reported splice variant of *TMSB4X*, which includes 1076 bp of *TMSB4X* intron 1 (GenBank accession no. AK055976), has an initiator methionine and a following 32 aa in its 5′UTR upstream *TMSB4X* conventional ORF, and potentially encodes ETLFLPGLHFR, which differs from the1B6

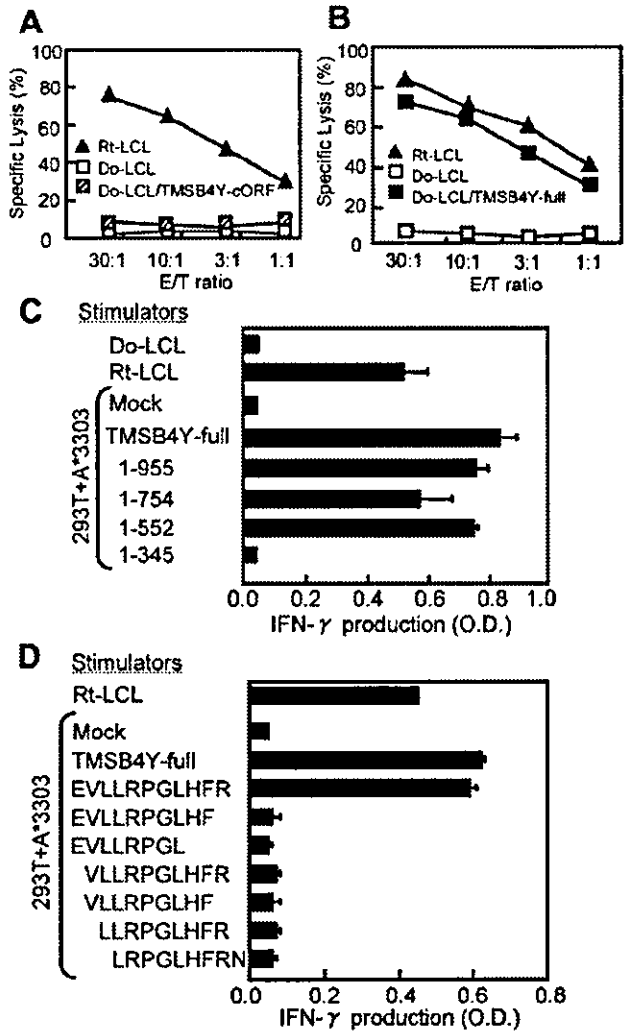


FIGURE 3. Localization of the minor H Ag epitope defined by 1B6 in TMSB4Y. *A*, Mammalian expression plasmid encoding the conventional ORF (cORF) of TMSB4Y, as identified in the deletion mapping (Fig. 2), was transfected into donor (Do) LCL, and recognition of transfected LCL by 1B6 was determined in a standard $^{51}Cr$ release assay. Recipient (Rt) LCL were used as a positive control. *B*, Plasmid encoding the full-length TMSB4Y cDNA containing the 5'- and 3'UTR (TMSB4Y-full; GenBank accession no. NM_004202) was transfected into donor LCL, and their susceptibility to 1B6 was tested as described above. *C*, Localization of the region encoding the minor H Ag by 3' deletion mutants of the TMSB4Y cDNA. HLA-A*3303-transduced 293 T cells were transfected with plasmids encoding various 3'-deleted TMSB4Y cDNAs terminating at 345, 552, 754, or 955 nt and cocultured with 1B6. Supernatants were harvested and assayed for the presence of IFN-γ by ELISA. The OD of each supernatant is shown. *D*, Identification of the 1B6 epitope. The susceptibilities of HLA-A*3303-transduced 293 T cells transfected with minigene constructs encoding nonamer peptide (LLRPGLHFR) predicted by BIMAS software (27) and selected N- or C-terminally extended peptides were tested by ELISA.

epitope, EVLLRPGLHFR, by three amino acids (underlined; Fig. 4, *B* and *C*).

We next synthesized these two 11-mer peptides, EVLLRPGLHFR and ETLFLPGLHFR, and tested the cytotoxicity of 1B6 against donor LCL pulsed with serial dilutions of each peptide. The titration of peptide EVLLRPGLHFR recognized by 1B6 gave half-maximal lysis at a concentration of 20 nM, whereas peptide ETLFLPGLHFR failed to sensitize the donor LCL at any concentration tested (Fig. 5). Thus, EVLLRPGLHFR defines the HLA-A*3303-restricted 1B6 epitope, and we designated it TMSB4Y/A33.

### *mRNA expression of the TMSB4Y gene is found in various tissues and cell types*

The X homologue of *TMSB4Y*, *TMSB4X*, has been shown to be expressed in a broad range of tissue types in rodents, with very high levels in spleen, thymus, and lung (32). To determine the distribution of *TMSB4Y* expression in different tissues, quantitative PCR analysis targeted to the 5'UTR of the mRNA was performed using a large panel of test samples derived from different tissues. PCR analysis demonstrated that the expression of *TMSB4Y* mRNA assessed by its 5'UTR was indeed observed in a wide range of normal tissues; from the highest expression in testis, prostate, pancreas, and hemopoietic cells to the lowest expression in dermal fibroblasts and skeletal muscles (~50-fold less than that in hemopoietic cells; data not shown). Expression of the mRNA in a panel of primary leukemic cells ranged from undetectable to levels similar to those seen in normal hemopoietic cells (data not shown).

### *TMSB4Y/A33-specific CD8$^+$ T cells are detectable in recipient post-transplant PBMC*

A split-well assay was used to estimate the relative frequencies in the post-HSCT PBMC of CTLp specific for the TMSB4Y/A33 minor H Ag and those specific for other minor H Ags expressed on the recipient's hemopoietic cells. As shown in Fig. 6*A*, the frequencies of CTLp reactive with recipient PHA blasts and TMSB4Y/A33 peptide-pulsed donor PHA blasts in peripheral blood obtained on day 50 post-HSCT from which the 1B6 was derived were 324 (95% confidence interval (CI), 213–493) and 96 (95% CI, 47–175) per $10^6$ peripheral blood CD8$^+$ cells, respectively, indicating that nearly a quarter of the CTL responses to recipient minor H Ags in this donor/recipient pair were indeed directed at the TMSB4Y/A33 minor H Ag. On day 146, the frequency of CTLp recognizing TMSB4Y peptide-pulsed donor PHA-blasts was 316 (95% CI, 216–464), and that for CTLp recognizing recipient PHA blasts was 3215 (95% CI, 2150–4808) per $10^6$ peripheral blood CD8$^+$ cells, demonstrating that even at the later time point the CTL responses against TMSB4Y/A33 continued to account for a significant fraction (10%) of the total donor CTL responses against recipient minor H Ags in this donor/recipient pair (Fig. 6*B*).

In additional experiments, an HLA/peptide tetramer was used to confirm the presence of TMSB4Y/A33-specific CTL in unstimulated post-HSCT PBMC (Fig. *C*, *left column*) as well as in T cell lines prepared by stimulating these PBMC with the same stimulators used in the CTLp assay (Fig. *C*, *right column*). The assays clearly detected TMSB4Y/A33-specific CD8$^+$ T cells in PBMC obtained on day 696 (0.35%), but for PBMCs obtained on day 50 and 146, the presence of TMSB4Y/A33-specific T cells was not clear because of the low number of PBMC available. After in vitro stimulation, tetramer-positive cells became detectable for the latter two samples, although direct comparison with the CTLp results was not possible due to the use of different culture conditions in the two assays.

## Discussion

In this study we have identified a gene, *TMSB4Y*, encoding a novel HLA-A*3303-restricted, H-Y Ag by testing HLA-A*3303-transfected cell lines carrying terminal deletions of the Y chromosome in cytotoxicity assays. This approach has previously been used to identify the HLA-B8-restricted H-Y Ag encoded by *UTY* (16).

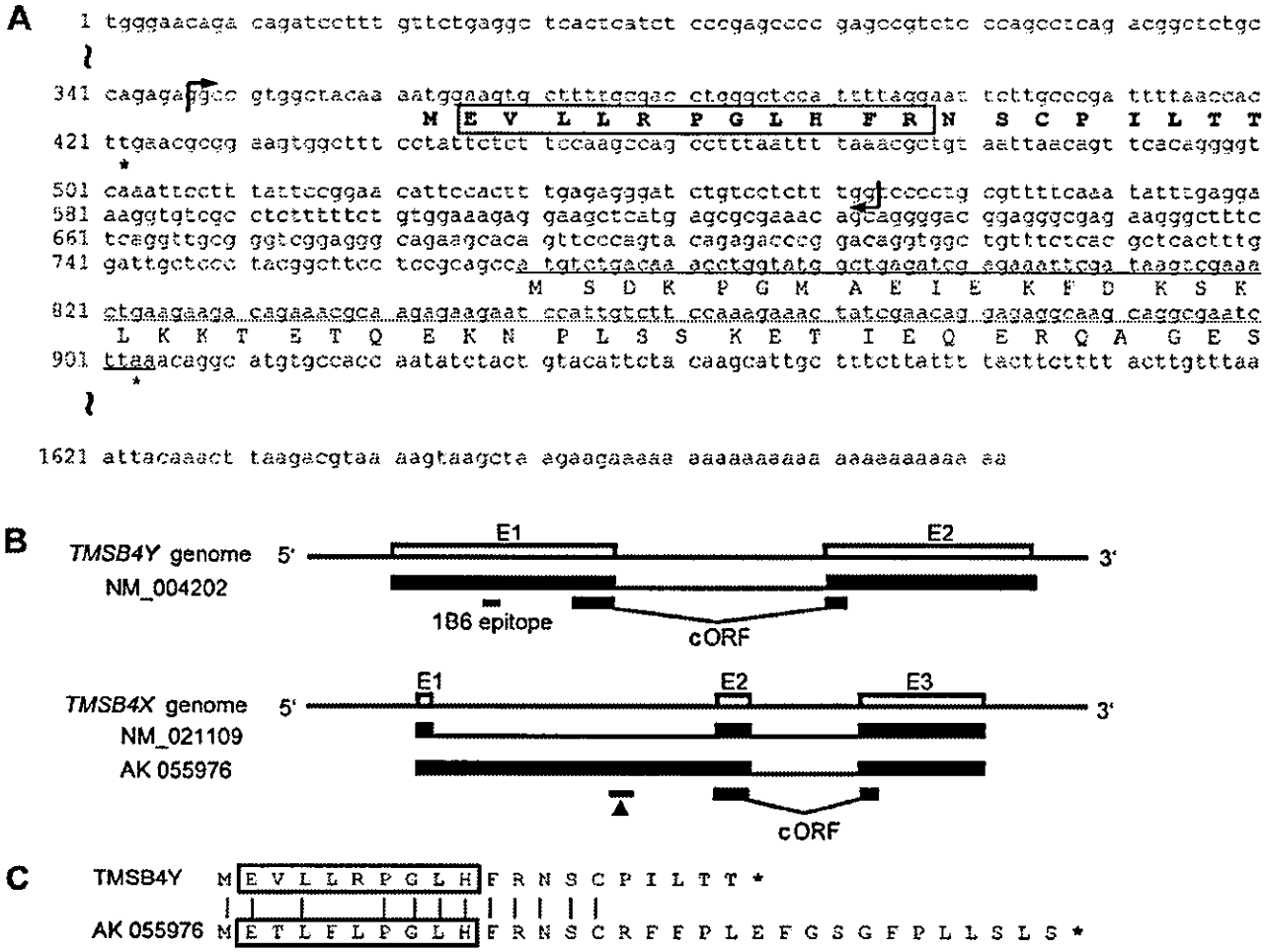


**FIGURE 4.** Location of the identified 1B6 epitope in the nucleotide and deduced amino acid sequences of the TMSB4Y cDNA (1702 bp; GenBank accession no. NM_004202). *A*, The deduced amino acid sequence is shown in one-letter designation below the nucleotide sequence for conventional ORF (the middle of the nucleotide sequence) and 5′UTR encoding the epitope recognized by 1B6. Asterisks indicate stop codons, and arrows indicate the region initially mapped by TMSB4Y cDNAs with nested 3′ deletions. The sequence corresponding to the identified peptide is boxed. *B*, Genomic organization of *TMSB4Y* and its X homologue, *TMSB4X*, and the relationship with their mRNAs. E1, E2, and E3 indicate exon 1, exon 2, and exon 3, respectively. The conventional ORF is indicated below the mRNA as cORF. The location of identified 1B6 epitope is shown below the 5′UTR of TMSB4Y cDNA. The putative X homologue peptide in a reported splice variant of *TMSB4X* containing its intron 1 (GenBank accession no. AK055976) is indicated by the arrowhead. *C*, Comparison of the deduced amino acid sequence in the 5′UTR encoding the 1B6 epitope in TMSB4Y and the putative X homologue (TMSB4X) peptide.

With the current discovery, all four identified genes that are encoded in deletion intervals 5C and 5D of the nonrecombining region of the human Y chromosome have been shown to encode at least one minor H Ag presented by class I or II HLA (14–17, 19, 20). Although the peptide sequence of the TMSB4Y/A33 minor H Ag identified in this study was 11 residues in length, and half-maximal lysis of peptide-pulsed female target cells was observed at a relatively high peptide concentration (~20 nM), it is likely that the 11-mer peptide is the minimal epitope, because it has a consensus Arg at the C terminus and a Val at the auxiliary anchor (position 2) (31). In addition, two computer algorithms predict that cleavage after the C-terminal Arg would be correctly performed by proteasomes (33, 34). Although all previously identified human H-Y Ags have homologue peptide on the ORF of their X homologous gene, it is not yet clear whether TMSB4Y/A33 minor H Ag has its homologue, because the longest cDNA clone (GenBank accession no. AK055976) assigned to be one of the splice variants of the *TMSB4X* gene containing the first intronic sequence was not detected by RT-PCR, whereas the full-length cDNA encoding thymosin β4 was readily detectable (data not shown). Thus, it is conceivable that the splice variant, AK055976, might be very rare or derived from a precursor mRNA.

Recently, evidence has been accumulating that cryptic polypeptides derived from noncoding regions, such as UTRs or introns, or encoded in alternative ORFs occasionally encode CTL epitopes for tumor or viral Ags in humans or mice (reviewed in Ref. 23). Of these, only one epitope is found in the 5′UTR of a cellular oncogene, c-*akt*, in the murine RLδ1 leukemia system (35). This unusual epitope is generated by insertion of the murine leukemia virus long terminal repeat into the exon of c-*akt*, resulting in transcription initiated at the cap site of the long terminal repeat. To the best of our knowledge, this is the first demonstration of a minor H Ag encoded outside a conventional ORF of a nonmutated gene. Although it is possible that the 19-residue ORF in the 5′UTR that encodes the epitope is an as yet unrecognized functional coding region, a search of the protein database, including the Protein-Protein Blast (http://www.ncbi.nlm.nih.gov/blast/), for amino acid sequence homology to this region did not identify any known functional domains.

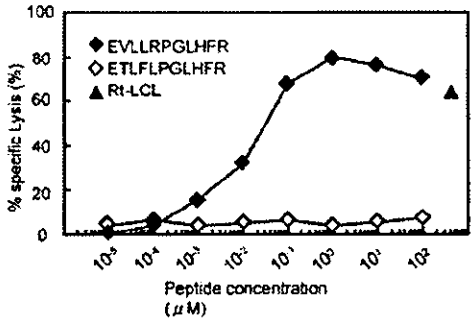


FIGURE 5. Evaluation of synthetic peptides for epitope reconstitution activity. Donor LCL (female) were labeled with $^{51}Cr$, then pulsed with serial dilutions of either EVLLRPGLHFR or putative female X homologue ETLFLPGLHFR (the mismatched amino acids are underlined) and used as targets for CTL clone 1B6 in a standard $^{51}Cr$ release assay.

Moreover, when the whole TMSB4Y genomic region was analyzed using GENESCAN software (36) (http://genes.mit.edu/GENSCAN.html), no ORF other than the reported ORF encoding the 34-mer polypeptide was predicted with a risk of <1.6% of false negative. These results strongly suggest that theTMSB4Y/A33 minor H Ag is not derived from a functional polypeptide, but, rather, that it is a subsidiary translation product of the TMSB4Y transcript.

Defective ribosomal products (DRiPs) consist of prematurely terminated polypeptides and misfolded polypeptides produced from translation of genuine mRNAs in the proper reading frame or are produced entropically due to the inevitable imperfections inherent to protein synthesis or folding (37). DRiPs, which account for 30% of newly synthesized proteins, have been suggested to be a major source of peptides presented on the cell surface by class I MHC (38). TMSB4Y was expressed in normal cells as well as transformed cells when assessed by quantitative PCR specific for the region encoding the TMSB4Y/A33 epitope, and the full-length mRNA was readily detected (data not shown). According to the definition of DRiPs, which is defective products from genuine mRNAs in the proper reading frame, TMSB4Y/A33 should be one of epitopes derived from cryptic polypeptides rather than DRiPs. In any case, the identification of a minor H Ag encoded outside the conventional ORF has important implications for the identification of other minor H Ag epitopes using genetic linkage analysis. Recently, we identified two minor H Ags using a similar approach (29), where we looked for peptides with potential HLA-binding sequence motifs that spanned nonsynonymous single nucleotide polymorphisms in the conventional ORF. However, the results of the current study suggest that not only conventional ORFs but also regions other than conventional ORFs should be taken into consideration when attempting to identify the epitope within the region mapped by linkage analysis.

Although the function of TMSB4Y is not yet known, its X chromosome homologue, *TMSB4X*, also known as *thymosin β4*, encodes a protein that plays an important role in the organization of the cytoskeleton, which binds to and sequesters actin monomers (G actin), leading to inhibition of actin polymerization (22). As expected from its function, thymosin β4 is highly expressed in metastatic melanoma cells together with fibronectin and RhoC, a member of the Rho GTPase family (39). Because Rho-like GTPases are suggested to be linked with HA-1 (40) and HA-3 (41) proteins in cytoskeleton rearrangement and have myosin 1G encoding HA-2 minor H Ag (42) as one of the downstream effector proteins (43), TMSB4Y/A33 derived from the Y homologue of thymosin β4 may also be classified as one of malignancy-associated minor H Ags according to the recent proposal by Spierings et al. (43).

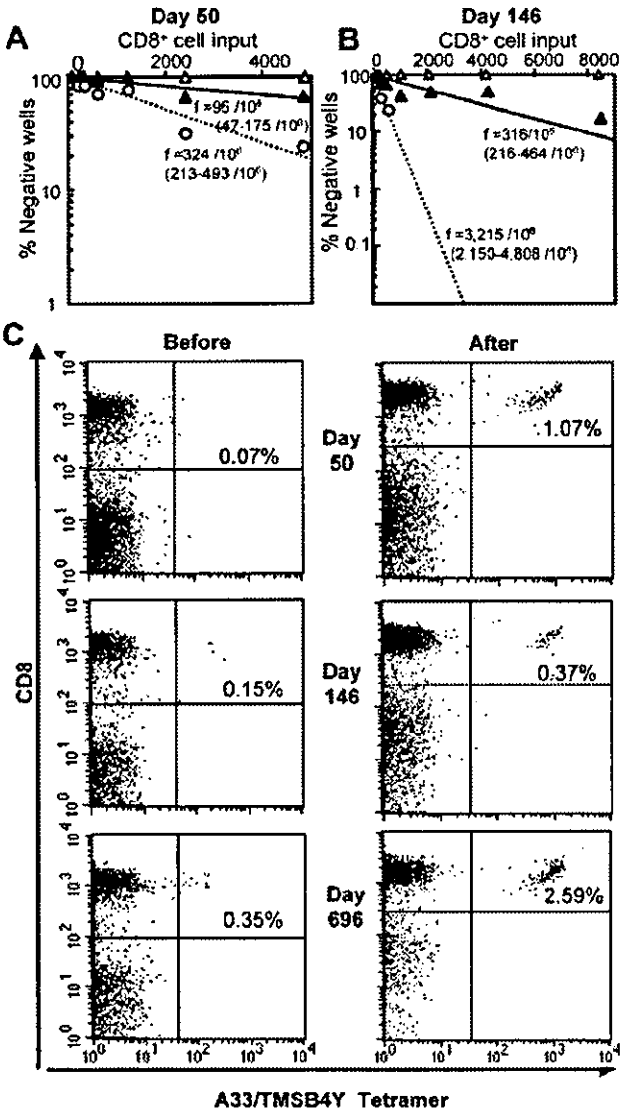


FIGURE 6. TMSB4Y/A33-specific CTLp frequency assay and tetramer analysis of post-HSCT PBMC. *A* and *B*, The proportion of CTL precursors specific for the identified TMSB4Y peptide among total CTLp against the recipient minor H Ags was quantitated using a standard limiting dilution assay. $CD8^+$ T cells from the PBMC on day 50 (*A*) or day 146 (*B*) post-HSCT were cultured at limiting dilution with irradiated CD40-B cells generated from pre-HSCT recipient PBMC. After three rounds of stimulation, a split-well analysis was performed for peptide-specific cytotoxicity against $^{51}Cr$-radiolabeled recipient PHA blasts (○) or donor PHA blasts pulsed with TMSB4Y peptide (▲) or unpulsed (△). The wells were considered to be positive for lytic activity if the total cpm released by effector cells was >2.5 × SD above that in control wells (mean cpm released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated with L-Calc software. *C*, Thawed post-HSCT (days 50, 146, and 696) PBMCs and T cell lines generated by stimulating the PBMCs three times with irradiated CD40-B cells generated from pre-HSCT recipient PBMC were stained with a PE-conjugated HLA-A33 tetramer incorporating the TMSB4Y/A33 peptide. The percentage of tetramer-positive cells of the total $CD3^+CD8^+$ cells is shown.

CTLp frequency assays revealed that the magnitude of the CTL response to the TMSB4Y/A33 epitope was early after HSCT and represented one-quarter of the measurable donor CTL responses to

recipient minor H Ags in this donor/recipient pair. This illustrates the extent to which such cryptic peptides may contribute to the diversity and immunogenicity of the total class I MHC-associated peptide pool in normal cells. In this regard, the relative immunogenicity of another minor H Ag, HB-1, which is derived from a polypeptide whose translation is initiated at a CUG instead of a conventional ATG codon exclusively in transformed B cells (44, 45), should also be of interest. Recently, Schwab et al. (46) have shown that the insertion into a 3′UTR of a sequence encoding an antigenic peptide elicits T cells specific for this peptide in vivo, which recognize at least DCs, B cells, and fibroblasts from mice carrying the transgene. In contrast, analysis of >200 endogenously derived HLA-B*1801-associated peptides from a human B cell line revealed that all the peptides were encoded by conventional ORFs from a wide variety of cellular genes (47), suggesting that the frequency of cryptic peptides being presented on class I MHC molecules is <1/200. Identification of more minor H Ags may answer the question of the significance of cryptic peptides over conventional peptides.

A recent study has suggested that CTL responses against minor H Ags encoded or regulated by genes on the Y chromosome contribute to a selective GVL effect against myeloid and lymphoid leukemias after female into male HSCT, even though recipients of this combination experience increased GVHD (6). An HLA-B8-restricted minor H Ag encoded by *UTY* has been shown to be a potential target for immunotherapy against hematological malignancies (16). It is noted in this regard that clone 1B6, used for defining TMSB4Y, was isolated from a patient who did not develop acute GVHD, and that its lytic activity against nonhemopoietic cells, including dermal/oral fibroblasts and bone marrow stromal fibroblasts, was significantly lower than that against LCL and PHA blasts, suggesting that TMSB4Y/A33 is a potential target of immunotherapy like UTY. However, the expression of the *TMSB4Y* transcript was found to not be restricted to normal hemopoietic cells and leukemia/lymphoma cells. In addition, the increase in A33/peptide tetramer-positive cells observed late after HSCT during late GVHD may suggest that this minor H Ag could be related to chronic GVHD rather than the GVL effect. Thus, additional studies of the polypeptide expression level derived from the *TMSB4Y* 5′UTR in various types of cells need to be conducted to elucidate whether this cryptic product can serve as a target for GVL.

## Acknowledgments

We thank Dr. Stanley R. Riddell for critically reading the manuscript. We also thank Yumi Nakao, Yasue Matsudaira, Keiko Nishida, and Hiromi Tamaki for their expert technical assistance.

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# Myeloma Cells Are Highly Sensitive to the Granule Exocytosis Pathway Mediated by WT1-Specific Cytotoxic T Lymphocytes

**Taichi Azuma,[1] Takemi Otsuki,[2] Kiyotaka Kuzushima,[3] Christopher J. Froelich,[4] Shigeru Fujita,[1] and Masaki Yasukawa[1]**

[1]First Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan; [2]Department of Hygiene, Kawasaki Medical School, Okayama, Japan; [3]Division of Immunology, Aichi Cancer Center Research Institute, Aichi, Japan; and [4]Evanston Northwestern Healthcare Research Institute, Evanston, Illinois

## ABSTRACT

***Purpose:*** **Because WT1 is a universal tumor antigen, we examined the sensitivity of myeloma cells to WT1-specific cytotoxic T lymphocyte (CTL)-mediated cytotoxicity.**

***Experimental Design:*** **WT1 expression in hematologic malignant cells was examined by quantitative reverse transcription-polymerase chain reaction. The cytotoxicity of a WT1-specific CTL clone against hematologic malignant cells, including myeloma cells, was examined by standard chromium-51 release assays. The extent of membrane damage induced by purified perforin was examined. Induction of WT1-specific CTLs from the patients with multiple myeloma (MM) was attempted, and we examined their function against myeloma cells.**

***Results:*** **The expression levels of WT1 mRNA in myeloma and lymphoma cells were significantly lower than that in acute leukemia cells. Although the WT1 expression levels in myeloma and lymphoma cells were almost same, only myeloma cells were lysed efficiently by WT1-specific CTLs in a HLA-restricted manner. The amounts of interferon-γ produced by WT1-specific CTLs in response to stimulation with myeloma cells and with lymphoma cells were almost the same, suggesting that WT1 protein is processed and expressed in the context of HLA class I molecules similarly on both myeloma and lymphoma cells. The extent of membrane damage induced by purified perforin appeared to be significantly higher in myeloma cells than in lymphoma cells. WT1-specific CTLs appeared to be present in patients with MM.**

***Conclusions:*** **The present study has shown that susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity and that WT1 is an ideal target antigen for cellular immunotherapy of MM.**

## INTRODUCTION

Multiple myeloma (MM) is a fatal hematologic malignancy characterized by monoclonal growth of plasma cells (1, 2). Although recent therapeutic approaches for MM, including high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation, have improved the overall survival rate, MM is still an incurable disease. Recently, treatment strategies targeting mechanisms whereby myeloma cells grow and survive in the bone marrow, including thalidomide and its potent immunomodulatory derivatives and the proteasome inhibitor bortezomib, have been developed and are expected to improve the outcome of patients with MM resistant to conventional treatment (3, 4); however, the therapeutic efficacy of these agents is limited. Because cancer immunotherapy is tumor specific and less toxic, it seems an ideal therapeutic strategy for MM. The identification of target antigens that are expressed preferentially in tumor cells but not in normal cells and are recognized by T lymphocytes is essential to the development of efficacious cellular immunotherapy; however, to date, only a limited number of MM-associated antigens that are recognized by T lymphocytes have been identified.

The *WT1* gene encodes a zinc finger transcription factor (5), and WT1 binds to the early growth response-1 DNA consensus sequence present in various growth factor gene promoters (6). Although WT1 was initially shown to act as a transcriptional repressor, the specific functions of WT1 in normal and neoplastic tissues remain to be fully elucidated. During normal ontogenesis, the *WT1* gene is expressed in a time- and tissue-dependent manner, mainly in the fetal kidney, testis, ovary, and supportive structures of mesodermal origin (7). In contrast, in adults, *WT1* gene expression is limited to very few tissues, including the splenic capsule and stroma, the Sertoli cells of the testis, the granulosa cells of the ovary, the podocytes of the kidney, and $CD34^+$ hematopoietic progenitor cells (8–10). With regard to malignant cells, it has been reported that most cases of acute leukemia and blast crisis of chronic myelogenous leukemia aberrantly overexpress WT1 (11–15). Previous studies have shown that the expression level of WT1 in B-lymphoma cells is significantly lower than that in acute leukemia (16); however, the details of WT1 expression in MM and other types

Received 5/4/04; revised 8/2/04; accepted 8/10/04.
Grant support: Grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant-in-Aid for Cancer Research 15-17 from the Ministry of Health, Labor and Welfare; Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists; the Welfide Medicinal Research Foundation; the Sagawa Cancer Research Foundation; the Cancer Research Foundation; the Yamanouchi Foundation for Research on Metabolic Disorders; the Uehara Memorial Foundation; the Takeda Science Foundation; and the Japan Medical Association. T. Azuma is a Research Fellow of the Japan Society for the Promotion of Science.
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Requests for reprints: Masaki Yasukawa, First Department of Internal Medicine, Ehime University School of Medicine, Shigenobu, Ehime 791-0295, Japan. Phone: 81-89-960-5296; Fax: 81-89-960-5299; E-mail: yasukawa@m.ehime-u.ac.jp.

of mature B-cell malignancies have not been reported. In the present study, we addressed the question of whether WT1 is expressed abundantly in myeloma cells and whether WT1-specific cytotoxic T lymphocytes (CTLs) can exert cytotoxicity against myeloma cells in an antigen-specific and HLA-restricted manner.

Another question raised in the present study is the nature of the mechanisms determining the sensitivity of target cells to CTL-mediated cytotoxicity. The mechanisms of cytotoxicity mediated by CTLs have been examined extensively, mainly in murine systems using various mutant and knockout mice, and various pathways have been identified. Among these, the granule exocytosis pathway mediated by perforin/granzymes and the Fas/Fas ligand pathway are thought to be the main mechanisms of CTL-mediated antigen-specific cytotoxicity (17). We recently used a combination of Fas-deficient target cells and perforin-deficient effector T lymphocytes to show that the granule exocytosis pathway is important in antigen-specific cytotoxicity mediated by human $CD4^+$ as well as $CD8^+$ CTLs (18–20). CTLs lyse target cells via recognition of the complex of target antigen-derived peptide and HLA molecule. Therefore, the susceptibility of target cells to antigen-specific cytotoxicity mediated by CTLs is thought to depend primarily on their expression levels of target antigen and HLA molecules. In the present study, we examined the mechanisms of cytotoxicity against myeloma cells mediated by WT1-specific CTLs, focusing on the sensitivity of target cells to perforin-mediated cytotoxicity. The data obtained from the present series of experiments revealed that WT1 expression levels in myeloma cells and lymphoma cells were both significantly lower than that in acute leukemia cells; however, myeloma cells, but not lymphoma cells, were lysed efficiently by WT1-specific CTLs. The extent of membrane damage induced by purified perforin in myeloma cells appeared to be significantly higher than that induced in lymphoma cells. In addition, WT1-specific CTL precursors were detected in peripheral blood of the patients with MM. On the basis of the present data, we discuss the feasibility of targeting WT1 in cellular immunotherapy for MM.

## MATERIALS AND METHODS

**Cell Separation and Cell Lines.** Bone marrow mononuclear cells were isolated from the patients with MM and healthy volunteers after obtaining informed consent and stored in liquid nitrogen until use. B-lymphoblastoid cell lines [B-(LCLs)] were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. LCLs were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The *HLA-A*2402* gene-transfected T2 cell line (T2-A24) was cultured in RPMI 1640 supplemented with 10% FCS and 800 μg/mL Geneticin (Life Technologies, Inc., Rockville, MD). All of the leukemia, myeloma, and lymphoma cell lines were cultured in RPMI 1640 supplemented with 10% FCS. All lymphoma cell lines used in the present study were established from patients with B-cell diffuse large non-Hodgkin's lymphoma or Burkitt's lymphoma. After obtaining consent from parents, cord blood was collected, and mononuclear cells were separated by Ficoll-Conray density gradient centrifugation. $CD34^+$ cells were isolated from cord blood mononuclear cells with immunomagnetic beads (MACS beads; Miltenyi Biotec, Auburn, CA) coated with anti-CD34 monoclonal antibody (MoAb). Immunomagnetic separations were performed according to the manufacturer's instructions.

**Generation of WT1 Peptide-Specific Cytotoxic T Lymphocytes.** WT1 peptide-specific CTLs were generated as described below. Peripheral blood mononuclear cells isolated from five HLA-A24–positive MM patients and three healthy volunteers after obtaining informed consent were plated in 96-well round-bottomed plates at $1 \times 10^5$ cells per well in the presence of the WT1-derived peptide WT1-T2 (CMTWNQMNL, residues 235–243) at a concentration of 10 μmol/L in RPMI 1640 supplemented with 10% human AB-type serum, 5 ng/mL human recombinant interleukin (IL)-7 (Genzyme, Boston, MA), and 100 pg/mL human recombinant IL-12 (Genzyme). After culturing for 7 days, the cells were restimulated by adding autologous mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan)-treated peripheral blood mononuclear cells and the WT1-T2 peptide at 10 μmol/L. After an additional 7 days of culture, the cells were restimulated in the same way. The next day, IL-2 (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 10 units/mL. Ten days after the final stimulation, the cells in 20 μL (about $2 \times 10^4$ T lymphocytes per well) in each culture well were tested by ELISPOT assays for their antigen specificity. For expansion of WT1 peptide-specific bulk CTLs, the cells that showed specific spots in ELISPOT assays were stimulated by adding MMC-treated T2-A24 cells and 10 μmol/L WT1-T2 peptide, and then the specificity and cytotoxicity of the growing cells were examined by detection of interferon (IFN)-γ production by enzyme-linked immunosorbent assay (ELISA; Endogen, Rockford, IL) and chromium-51 release assay, respectively.

**Establishment of a WT1 Peptide-Specific Cytotoxic T Lymphocyte Clone.** A CTL clone designated TAK-1, which specifically recognizes WT1-T2 peptide in the context of HLA-A*2402, was established as described previously (16, 21). Briefly, $CD8^+$ T lymphocytes were stimulated repeatedly with WT1-T2 peptide-loaded dendritic cells. The cytotoxicity of the growing cells was examined, and cells that exerted a cytotoxic effect on a WT1-T2 peptide-loaded autologous B-LCL were cloned by a limiting dilution method as described previously (22).

**Quantitative Analysis of WT1 Messenger RNA Expression.** Total RNA was extracted from samples with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed in a final volume of 50 μL with the One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reaction, carried out with 0.1 μg of total RNA from each sample, was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reverse transcription of the RNA was achieved at 48°C for 30 minutes, and polymerase chain reaction was performed with an enzyme activation step (10 minutes at 95°C) followed by 40 cycles of denaturation/annealing/extension (15 seconds at 95°C and 1 minute at 60°C). Sequences of primers and probes were as follows: *WT1* forward primer, 5′-CAACCACAGCACAGGGTACG-3′; *WT1* reverse primer, 5′-TCTG-

TATTGGGCTCCGCAG-3'; and probe, 5'-FAM-AGCGATA-ACCACACAACGCCCATCC-TAMRA-3'. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) quantitative analysis was performed with predeveloped TaqMan assay reagent target kits (Applied Biosystems). All analyses were performed in duplicate. To normalize differences in RNA degradation between the individual samples and in RNA loading for the RT-PCR procedure, the *WT1* expression level for a particular sample was defined as its *WT1* gene expression level divided by its *GAPDH* gene expression level (23). The *WT1* gene expression level of K562 leukemia cells, which strongly express *WT1*, was designated 1.0, and the levels for the experimental samples were calculated relative to this value (12).

**Flow Cytometric Analysis.** The expression levels of HLA class I molecules on myeloma and lymphoma cells were determined by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A, -B, -C MoAb (BD PharMingen, San Diego, CA). HLA-A24 expression on cells was examined by flow cytometry using an anti-HLA-A24 MoAb (One Lambda, Canoga Park, CA) with mouse IgG as the control. The stained cells were analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, CA). Measurement of mean fluorescence intensity and analysis of data were done with Cell Quest Software (Becton Dickinson).

**Purification of Perforin.** Human perforin was purified as described previously (24, 25). Briefly, perforin was extracted in 1 mol/L NaCl from granules of the human natural killer (NK) cell line YT and purified by ion metal affinity chromatography with an imidazole gradient in 10% betaine (IMAC; PerSeptive Biosystems, Cambridge, MA). Fractions with hemolytic activity as determined by sheep red blood cell assays were concentrated by Centricon ultrafiltration and stored in 2 mmol/L EDTA and 0.1% fatty acid-free bovine serum albumin until use. Purification of perforin was confirmed by SDS-PAGE and Western blotting (24).

**Cytotoxicity Assays.** $^{51}Cr$ release assays were performed as described previously (26). Briefly, $1 \times 10^4$ $^{51}Cr$ ($Na_2{}^{51}CrO_4$; New England Nuclear, Boston, MA)-labeled target cells and various numbers of effector cells in 200 μL of RPMI 1640 supplemented with 10% FCS were seeded into round-bottomed microtiter wells and incubated for 4 hours. In some experiments, the target cells were incubated with an anti-HLA class I framework MoAb (w6/32; American Type Culture Collection, Manassas, VA) or an anti-HLA-DR MoAb (L243; American Type Culture Collection) at an optimal concentration (10 μg/mL) for 30 minutes before adding effector cells to determine whether cytotoxicity was restricted by HLA class I. To determine whether WT1-specific CTLs lyse myeloma cells via recognition of the WT1 peptide, which is naturally processed in myeloma cells and expressed in the context of HLA-A24, cold target inhibition assay was performed. WT1 peptide-loaded and unloaded autologous LCL or HLA-A24–positive leukemia cell line MEG01, which was shown to be lysed by WT1-specific CTLs in a WT1-specific manner, was used as cold target cells. After incubation for 4 hours, 100 μL of supernatant were collected from each well. The percentage of specific lysis was calculated as follows: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm). Cytotoxicity mediated by purified perforin was measured by using 2-hour $^{51}Cr$ release assays and the trypan blue exclusion method. $^{51}Cr$-labeled target cells were incubated with various concentrations of purified perforin in the assay buffer [150 mmol/L NaCl, 20 mmol/L HEPES, and 2.5 mmol/L $CaCl_2$ (pH 7.4)] for 2 hours at 37°C. After incubation, supernatants were harvested after centrifugation of the microtiter plates, and radioactivity was determined. For trypan blue exclusion, the cells incubated with or without purified perforin were stained with trypan blue, and the percentages of stained cells were determined.

**Inhibition of Perforin-Mediated Cytotoxicity.** To examine the $Ca^{2+}$ dependency of the cytotoxicity, cytotoxicity assays were performed in the presence of EGTA (Sigma, St. Louis, MO) at various concentrations. To evaluate the role of perforin in CTL-mediated cytotoxicity, effector T cells were pretreated with concanamycin A (CMA; Wako Pure Chemical Industries, Osaka, Japan) at various concentrations for 2 hours and then incubated with the target cells in the presence of CMA. CMA is an inhibitor of vacuolar type $H^+$-ATPase that inhibits perforin-based cytotoxicity, mostly by accelerated degradation of perforin caused by an increase in the pH of lytic granules (27). Treatment with CMA at the concentration used in the present study showed no toxic effect against T lymphocytes and myeloma cells as determined from cell growth curves and $^{51}Cr$ release assays (data not shown).

**Detection of Interferon-γ Production.** The response of WT1-specific CTLs to various stimulator cells was examined by determining IFN-γ production. For the assays of IFN-γ production, $1 \times 10^5$ WT1-specific CTL clone cells or bulk CTLs and $5 \times 10^4$ MMC-treated tumor cells were suspended in 200 μL of RPMI 1640 supplemented with 10% FCS and cultured in flat-bottomed microtiter wells in the presence of 10 units/mL recombinant human IL-2. After 72 hours, the supernatants were collected from each well and assayed for IFN-γ production by ELISA.

**ELISPOT Assays.** ELISPOT assays were performed as described previously (28). Briefly, 96-well flat-bottomed MultiScreen-HA plates with a nitrocellulose base (Millipore; Millipore Corp., Bedford, MA) were coated with 10 μg/mL anti–IFN-γ MoAb (R&D Systems, Minneapolis, MN) and incubated overnight at 4°C. After washing with PBS, the plates were blocked with the culture medium for 1 hour at 37°C. T2-A24 cells ($5 \times 10^4$ cells) were pulsed with 10 μmol/L WT1-T2 peptide or PBS alone in RPMI 1640 with 10% FCS for 1 hour at room temperature, and then responder cells were seeded in each well. The plates were incubated in a 5% $CO_2$ incubator at 37°C for 20 hours and washed extensively with PBS containing 0.05% Tween 20. A polyclonal rabbit anti–IFN-γ antibody (Endgen, Woburn, MA) was added to individual wells and left for 90 minutes at room temperature, followed by exposure to peroxidase-conjugated goat antirabbit IgG (Zymed, San Francisco, CA) for an additional 90 minutes. For visualization of IFN-γ–specific spots, 100 μL of 0.1 mol/L sodium acetate buffer (pH 5.0) containing 3-amino-9-ethylcarbazole (Sigma) and 0.015% $H_2O_2$ were added to each well. After 40 minutes, the reaction was stopped by washing with water, and the plates were dried. Diffuse large spots were counted under a dissecting microscope.