in 10 μl reaction containing 200 nmol/l G-probe, 100 nmol/l A-probe, 500 nmol/l each of the primers, 1X TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 μl DNA (corresponding to 50–100 ng DNA) in a MicroAmp optical 96-well plate. PCR cycling conditions were: predenature, 95°C for 10 min followed by 35 cycles of 92°C for 15 s and 60°C for 1 min in an GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were analysed on an ABI 7900HT with the aid of SDS 2.0 software (Applied Biosystems). ACC-1 was considered incompatible with GVHD/GVL if the donor was ACC-1^C homozygous and the patient was either ACC-1^Y homozygous or heterozygous (i.e. ACC-1^Y/ACC-1^Y or ACC-1^Y/ACC-1^C).

Tetramer construction and flow cytometric analysis

The MHC-peptide tetramers were produced as described previously (Altman *et al*, 1996; Kuzushima *et al*, 2001). In brief, HLA-A*2402 heavy chain and β2-microglobulin were produced in BL21(DE3) pLysS (Novagen, Madison, WI, USA). The C-terminus of the heavy chain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA/β2-microglobulin/peptide complexes were folded *in vitro* in the presence of the 9-mer ACC-1^Y peptide, DYLQYVLQI. The MHC was biotinylated by using recombinant BirA enzyme (Avidity, Denver, CO, USA) and then converted into tetramers with phycoerythrin-labelled streptavidin (Molecular Probes, Eugene, OR, USA). For staining, PBMC or T-cell lines were incubated with the tetramer at a concentration of 20 μg/ml at room temperature for 15 min followed by incubation with fluorescein isothiocyanate-conjugated anti-CD3 and Tricolor anti-CD8 monoclonal antibody (Caltag, Burlingame, CA, USA) on ice for 15 min. Cells were fixed with 0·1% formaldehyde before analysis with a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson, Mountain View, CA, USA).

Induction of ACC-1^Y-specific T-cell lines using peptide-pulsed CD40-activated B cells

CD40-activated B (CD40-B) cells were generated by incubating donor PBMC on a γ-irradiated (96 Gy) human CD40L-transfected NIH3T3 cell line (t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA) in the presence of human interleukin (IL)-4 (4 ng/ml; Ono Pharmaceutical, Osaka, Japan) and cyclosporin A (0·7 mg/ml; Sandoz, Basel, Switzerland) in 2 ml of Iscove's modified Dulbecco's medium (Invitrogen, San Diego, CA, USA) supplemented with 10% pooled human serum. The expanding cells were transferred onto freshly prepared t-CD40L cells and fed with cytokine-replenished medium every 3–4 d. Thawed PBMC collected at various time points following BMT (1×10^6) were co-cultured with γ-irradiated (33 Gy) peptide-pulsed (1 μmol/l) autologous CD40-B cells (1×10^6) in 2 ml Roswell Park Memorial Institute 1640 medium supplemented with 10% pooled human serum at 37°C in 5% CO_2. On days 7 and 15, cells were restimulated, and 1 d after each stimulation, recombinant human IL-2 (Chiron, Emeryville, CA, USA) was added to the cultures at the final concentration of 20 U/ml.

Cytotoxic assays

Target cells were labelled with 3·7 MBq of ^{51}Cr for 2 h, and 1×10^3 target cells/well were mixed with T-cell lines at various effector:target (E:T) ratios in a standard 4-h cytotoxicity assay using 96-well round-bottom plates. All assays were performed at least in duplicate. The percentage of specific lysis was calculated as follows:

$$\frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100.$$

Data collection and statistical analysis

Clinical data were collected using standardized forms provided by the JMDP. A final clinical survey of patients was carried out on 1 July 2001. Standard risk for leukaemia relapse was defined as acute leukaemia in first complete remission and CML in first chronic phase, whereas high risk was defined as a more advanced stage than standard risk. Homogeneity between ACC-1^Y incompatible and compatible patients was evaluated with the chi-squared test for qualitative variables and Mann–Whitney *U*-test was used for continuous variables. The probability of acute GVHD, chronic GVHD, leukaemia relapse and disease-free survival were calculated by the Kaplan–Meier method, and assessed by the log-rank test. The Cox proportional hazard model was applied for multivariate analysis. The following variables were evaluated: ACC-1 disparity, patient age (linear), donor age (linear), sex (donor/patient pair), risk of leukaemia relapse (standard *versus* high), preconditioning [total body irradiation (TBI) regimen *versus* non-TBI regimen] and GVHD prophylaxis (cyclosporine *versus* tacrolimus). Statistical significance was set at $P < 0·05$.

Results

Detection of ACC-1^Y-specific T cells in post-BMT PBMC and their in vitro expansion

Of 14 HLA-A24 positive patients enrolled in our study, only one patient had received an ACC-1 GVHD/GVL-direction incompatible transplant, and from whom the CTL clone used for defining ACC-1^Y epitope was established. The percentage of tetramer-binding cells in the peripheral $CD8^+$ cells collected on days 43 and 212 were 0·2% and 0·07% respectively (Fig 1A). After two cycles of *in vitro* stimulation with peptide-pulsed donor CD40-B cells, the percentage of tetramer-binding cells among $CD8^+$ cells in the T-cell lines increased dramatically (Fig 1B; 96% for day 43, 69% for day 75 and 7·2% for day 212). Figure 1C shows the growing

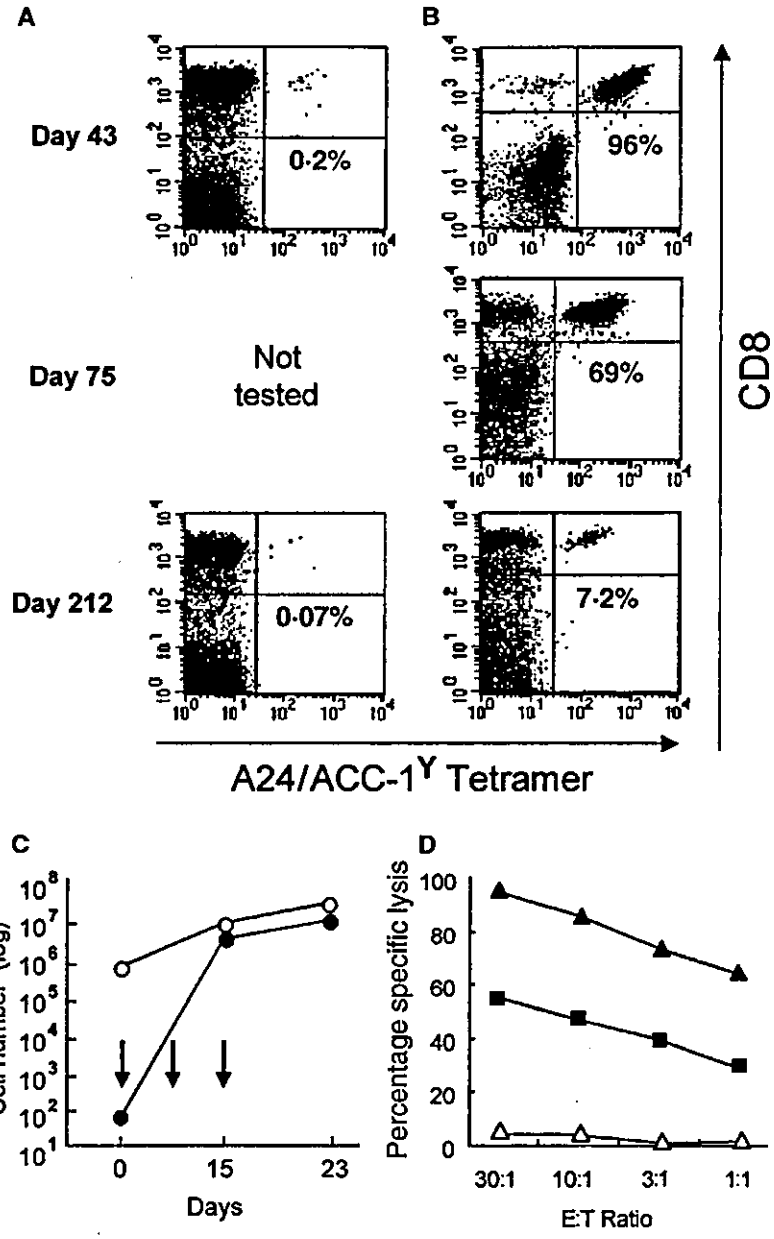

Fig 1. Detection of ACC-1^Y-specific T cells after bone marrow transplantation, and their expandability and function. (A) HLA-A24/ACC-1^Y tetramer was used to detect the presence of ACC-1^Y-specific $CD8^+$ cells in post-transplant PBMC collected on days 43 and 212 after transplant. The patient received marrow from his HLA identical, HLA-A24 positive and ACC-1 disparate brother (i.e. ACC-1^C homozygous). (B) The remaining PBMC used above and also PBMC collected on day 75 were stimulated twice with ACC-1^Y peptide-pulsed (1 μmol/l) activated donor B cells (see 'Materials and Methods') and assayed on day 15 for HLA-A24/ACC-1^Y tetramer reactivity. (C) The T-cell line induced from PBMC collected on day 43 as shown in (B) was further stimulated on day 15. The absolute number of growing cells (open circles) and HLA-A24/ACC-1^Y tetramer-positive cells (closed circles) were examined on days 7, 15, 23 after the first stimulation. The arrows indicate stimulation with peptide-pulsed activated donor B cells. (D) Cytotoxicity of the above T-cell lines tested on day 15 against donor LCL (open triangles), ACC-1^Y peptide pulsed (10 μmol/l) donor LCL (closed triangles) and patient LCL at the E:T ratio shown. Data shown are representative of two independent experiments performed in triplicate.

absolute number of tetramer-binding $CD8^+$ T cells following stimulations for PBMC obtained on day 43. The T-cell line lysed ACC-1^Y peptide pulsed donor LCL and patient LCL but not donor LCL pulsed with antigenicity negative ACC-1^C peptide, suggesting the *in vitro* expanded T-cell line retained ACC-1^Y-specific cytotoxic function. These results indicate that ACC-1^Y-specific T cells persisted in the patient peripheral blood for at least 7 months following BMT and they were expandable *in vitro* with ACC-1^Y peptide-pulsed antigen presenting cells.

Table II. Multivariate analysis for factors affecting clinical outcome following bone marrow transplantation.

Outcome and factor	Odds/hazard ratio (95% CI)	*P*-value
Acute GVHD (grades II–IV)		
ACC-1 disparity	0·91 (0·52–1·58)	0·73
Patient age	0·98 (0·97–1·00)	0·04
Chronic GVHD		
ACC-1 disparity	1·01 (0·59–1·73)	0·96
Donor age	1·04 (1·01–1·07)	0·007
Relapse		
ACC-1 disparity	1·04 (0·51–2·14)	0·91
Risk of leukaemia	3·17 (1·78–5·62)	<0·0001
Disease-free survival		
ACC-1 disparity	1·00 (0·65–1·55)	0·99
Risk of leukaemia	2·12 (1·51–3·00)	<0·0001
Patient age	1·02 (1·01–1·03)	0·006
Donor age	1·03 (1·01–1·05)	0·02

GVHD, graft *versus* host disease; CI, confidence interval.

Statistical analysis

Among the selected 320 donor/patient pairs, genotyping detected 55 ACC-1 incompatible GVHD/GVL cases (17·2%). The characteristics of ACC-1 compatible and incompatible groups are listed in Table I. There were no statistical differences between these two groups for previously identified risk factors for GVHD (Weisdorf *et al*, 1991; Nash *et al*, 1992) such as patient age, donor age, sex (donor/patient pair), disease status, preconditioning regimen and GVHD prophylaxis.

There was no statistical difference in the occurrence of grades II–IV or III–IV acute GVHD between ACC-1 incompatible and compatible patients. Risk factors for acute and chronic GVHD, leukaemia relapse and disease-free survival were analysed by multivariate analysis (Table II). Patient age only correlated with acute GVHD grades II–IV. Other factors, including ACC-1 disparity [hazard risk, 0·9; 95% confidence interval (CI), 0·5–1·6; $P = 0·73$], were not significant. The ACC-1 disparity was not identified as a significant risk factor in terms of chronic GVHD (hazard risk, 1·0; 95% CI 0·6–1·7; $P = 0·96$), relapse (hazard risk, 1·0; 95% CI 0·5–2·1; $P = 0·91$) or disease-free survival (hazard risk, 1·0; 95% CI 0·6–1·5; $P = 0·99$) (Table II).

Discussion

Graft *versus* host disease still remains the most life-threatening complication following allo-HCT or subsequent DLI, although new immunosuppressive drugs or regimens, such as non-myeloablative conditioning, have been introduced. Various strategies to separate GVHD from GVL effects have been explored (reviewed in Farag *et al*, 2003; Kolb *et al*, 2004). Among these, immunotherapy against leukaemia-specific, or recipient haematopoietic cell-specific, antigens (i.e. mHAs) is expected to eradicate leukaemic cells without causing GVHD. ACC-1 encoded by a polymorphic region in *BCL2A1* is one of the promising candidate epitopes for such immunotherapy (Akatsuka *et al*, 2003). In this study, we first showed that ACC-1^Y-specific $CD8^+$ T cells persisted in peripheral blood for at least 7 months following BMT and that they were expandable by stimulating with peptide-pulsed antigen presenting cells without losing its specific cytotoxic activity. Unfortunately, we were unable to find more than a single case eligible for this analysis because ACC-1^Y disparate transplants are estimated to occur at the frequency of <10% between HLA-identical sibling pairs; searches for more cases to verify the reproducibility are underway.

HA-1, one of the mHAs currently being tested in adoptive immunotherapy (Mutis *et al*, 1999), was originally identified as a target for severe acute GVHD (Goulmy *et al*, 1996). Controversial results have been reported in subsequent studies (Tseng *et al*, 1999; Gallardo *et al*, 2001; Lin *et al*, 2001; Socie *et al*, 2001) in terms of its contribution to acute GVHD. Our study did not detect any association between ACC-1 disparity and the occurrence of severe acute GVHD while we found that ACC-1^Y-specific T cells were expandable at least in one patient. Although the ACC-1^C peptide is considered not to be presented (Akatsuka *et al*, 2003), in order to exclude potential reciprocal immunogenicity of the ACC-1^C allele, we performed another statistical analysis by removing donor/patient pairs in which the donor was ACC-1^Y homozygous and the patient was either ACC-1^C homozygous or heterozygous. This attempt did not change the statistical results (T. Nishida and Y. Akatsuka, unpublished observations). We also searched for cases that received HLA-B44 positive and ACC-2-disparate HCT; however, we found only 83 pairs of which 22 received ACC-2-disparate HCT. A preliminary analysis could not detect any clinical relevance, either.

A potential shortcoming of the current statistical study is the use of the recipients of unrelated BMT but not those of sibling BMT. However, it is noted that there has been at least one report that analysed unrelated umbilical cord HCT cases, in which HLA mismatching often occurs, for determining the effects of both cytokine/cytokine receptor polymorphisms and mHAs (Kögler *et al*, 2002). In our study, the donor and recipient were genotypically matched for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 to minimize HLA effects. Nevertheless, gene polymorphisms between the donor and patient are still more diverse in unrelated HCT cases than in HLA-identical sibling HCT cases, which may limit the statistical power to detect a significant association. Thus, it may be necessary to include other factors, such as polymorphisms in genes encoding cytokines or cytokine receptors (Cavet *et al*, 1999; Socie *et al*, 2001; Nordlander *et al*, 2002), or killer inhibitory receptors (Cook *et al*, 2003), that can affect clinical outcome for determining the impact of mHA disparity in future analyses.

We did not observe any favourable effect of ACC-1 disparity in terms of leukaemia relapse or disease-free survival. In a recent large-scale study, HA-1 disparity was not associated

with these outcomes, suggesting that the naturally occurring immunogenicity following HCT may not be satisfactory for inducing GVL effect (Lin *et al*, 2001). We thus believe that passive immunization by the adoptive transfer of haematopoietic cell-restricted mHA-specific cytotoxic T cells may be necessary to eradicate residual leukaemic cells efficiently following HCT. An attempt to induce ACC-1^{Y}-specific CTL from ACC-1^{Y}-negative donors by using ACC-1^{Y} peptide-pulsed antigen presenting cells is under way for therapeutic use as reported for HA-1 (Mutis *et al*, 1999).

In conclusion, our current results indicate that ACC-1 disparity seems less likely to be associated with the development of severe GVHD and suggest that adoptive immunotherapy targeting ACC-1 may be carried out without the risk of GVHD. However, because haematopoietic cell-specific expression of mHAs may not necessarily exclude the possibility of acute GVHD (Goulmy *et al*, 1996; Gallardo *et al*, 2001; Socie *et al*, 2001), probably due to residual recipient-derived antigen presenting cells remaining in tissues susceptible for GVHD early after HCT (Billiau *et al*, 2002), the timing after HCT and donor-chimaeric status should be carefully considered when infusing mHA-specific T cells for immunotherapy.

Acknowledgments

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References

Akatsuka, Y., Kondo, E., Taji, H., Morishima, Y., Yazaki, M., Obata, Y., Kodera, Y., Riddell, S.R. & Takahashi, T. (2002) Targeted cloning of cytotoxic T cells specific for minor histocompatibility antigens restricted by HLA class I molecules of interest. *Transplantation*, **74**, 1773–1780.

Akatsuka, Y., Nishida, T., Kondo, E., Miyazaki, M., Taji, H., Iida, H., Tsujimura, K., Yazaki, M., Naoe, T., Morishima, Y., Kodera, Y., Kuzushima, K. & Takahashi, T. (2003) Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. *Journal of Experimental Medicine*, **197**, 1489–1500.

Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J. & Davis, M.M. (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science*, **274**, 94–96.

Billiau, A.D., Fevery, S., Rutgeerts, O., Landuyt, W. & Waer, M. (2002) Crucial role of timing of donor lymphocyte infusion in generating dissociated graft-versus-host and graft-versus-leukemia responses in mice receiving allogeneic bone marrow transplants. *Blood*, **100**, 1894–1902.

Cavet, J., Middleton, P.G., Segall, M., Noreen, H., Davies, S.M. & Dickinson, A.M. (1999) Recipient tumor necrosis factor-alpha and interleukin-10 gene polymorphisms associate with early mortality and acute graft-versus-host disease severity in HLA-matched sibling bone marrow transplants. *Blood*, **94**, 3941–3946.

Cook, M.A., Milligan, D.W., Fegan, C.D., Darbyshire, P.J., Mahendra, P., Craddock, C.F., Moss, P.A.H. & Briggs, D.C. (2003) The impact of donor KIR and patient HLA-C genotypes on outcome following HLA-identical sibling haematopoietic stem cell transplantation for myeloid leukaemia. *Blood*, doi: 10.1182/blood-2003-02-0438.

Dazzi, F., Szydlo, R.M., Craddock, C., Cross, N.C., Kaeda, J., Chase, A., Olavarria, E., van Rhee, F., Kanfer, E., Apperley, J.F. & Goldman, J.M. (2000) Comparison of single-dose and escalating-dose regimens of donor lymphocyte infusion for relapse after allografting for chronic myeloid leukemia. *Blood*, **95**, 67–71.

Dickinson, A.M., Wang, X.N., Sviland, L., Vyth-Dreese, F.A., Jackson, G.H., Schumacher, T.N., Haanen, J.B., Mutis, T. & Goulmy, E. (2002) In situ dissection of the graft-versus-host activities of cytotoxic T cells specific for minor histocompatibility antigens. *Nature Medicine*, **8**, 410–414.

Dolstra, H., Fredrix, H., Maas, F., Coulie, P.G., Brasseur, F., Mensink, E., Adema, G.J., de Witte, T.M., Figdor, C.G. & van de Wiel-van Kemenade, E. (1999) A human minor histocompatibility antigen specific for B cell acute lymphoblastic leukemia. *Journal of Experimental Medicine*, **189**, 301–308.

Farag, S.S., Fehniger, T.A., Ruggeri, L., Velardi, A. & Caligiuri, M.A. (2003) Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood*, **100**, 1935–1947.

Gallardo, D., Arostegui, J.I., Balas, A., Torres, A., Caballero, D., Carreras, E., Brunet, S., Jimenez, A., Mataix, R., Serrano, D., Vallejo, C., Sanz, G., Solano, C., Rodriguez-Luaces, M., Marin, J., Baro, J., Sanz, C., Roman, J., Gonzalez, M., Martorell, J., Sierra, J., Martin, C., de la Camara, R. & Granena, A., GvHD Subcommittee of the Grupo Espanol de Trasplante Hemapoyetico (GETH) (2001) Disparity for the minor histocompatibility antigen HA-1 is associated with an increased risk of acute graft-versus-host disease (GvHD) but it does not affect chronic GvHD incidence, disease-free survival or overall survival after allogeneic human leucocyte antigen-identical sibling donor transplantation. *British Journal of Haematology*, **114**, 931–936.

Glucksberg, H., Storb, R., Fefer, A., Buckner, C.D., Neiman, P.E., Clift, R.A., Lerner, K.G. & Thomas, E.D. (1974) Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation*, **18**, 295–304.

Goulmy, E. (1997) Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy. *Immunological Reviews*, **157**, 125–140.

Goulmy, E., Schipper, R., Pool, J., Blokland, E., Falkenburg, J.H., Vossen, J., Gratwohl, A., Vogelsang, G.B., van Houwelingen, H.C. & van Rood, J.J. (1996) Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *New England Journal of Medicine*, **334**, 281–285.

Horowitz, M.M., Gale, R.P., Sondel, P.M., Goldman, J.M., Kersey, J., Kolb, H.J., Rimm, A.A., Ringden, O., Rozman, C. & Speck, B. (1990)

Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*, **75**, 555–562.

Kögler, G., Middleton, P.G., Wilke, M., Rocha, V., Esendam, B., Enczmann, J., Wernet, P., Gluckman, E., Querol, S., Lecchi, L., Goulmy, E. & Dickinson, A.M. (2002) Recipient cytokine genotypes for TNF-alpha and IL-10 and the minor histocompatibility antigens HY and CD31 codon 125 are not associated with occurrence or severity of acute GVHD in unrelated cord blood transplantation: a retrospective analysis. *Transplantation*, **74**, 1167–1175.

Kolb, H.J., Mittermuller, J., Clemm, C.H., Holler, E., Ledderose, G., Brehm, G., Heim, M. & Wilmanns, W. (1990) Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients. *Blood*, **76**, 2462–2465.

Kolb, H.J., Schmid, C., Barrett, A.J. & Schendel, D.J. (2004) Graft-versus-leukemia reactions in allogeneic chimeras. *Blood*, **103**, 767–776.

Kuzushima, K., Hayashi, N., Kimura, H. & Tsurumi, T. (2001) Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8+ T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. *Blood*, **98**, 1872–1881.

Lin, M.-T., Gooley, T., Hansen, J.A., Tseng, L.H., Martin, E.G., Singleton, K., Smith, A.G., Mickelson, E., Petersdorf, E.W. & Martin, P.J. (2001) Absence of statistically significant correlation between disparity for the minor histocompatibility antigen-HA-1 and outcome after allogeneic hematopoietic cell transplantation. *Blood*, **98**, 3172–3173.

Mackinnon, S., Papadopoulos, E.B., Carabasi, M.H., Reich, L., Collins, N.H., Boulad, F., Castro-Malaspina, H., Childs, B.H., Gillio, A.P. & Kernan, N.A. (1995) Adoptive immunotherapy evaluating escalating doses of donor leukocytes for relapse of chronic myeloid leukemia after bone marrow transplantation: separation of graft-versus-leukemia responses from graft-versus-host disease. *Blood*, **86**, 1261–1268.

Marijt, W.A.E., Heemskerk, M.H.M., Kloosterboer, F.M., Goulmy, E., Kester, M.G.D., van der Hoorn, M.A.W.G., van Luxemburg-Heys, S.A.P., Hoogeboom, M., Mutis, T., Drijfhout, J.W., van Rood, J.J., Willemze, R. & Falkenburg, J.H.F. (2003) Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 2742–2747.

Morishima, Y., Sasazuki, T., Inoko, H., Juji, T., Akaza, T., Yamamoto, K., Ishikawa, Y., Kato, S., Sao, H., Sakamaki, H., Kawa, K., Hamajima, N., Asano, S. & Kodera, Y. (2002) The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood*, **99**, 4200–4206.

Mutis, T., Verdijk, R., Schrama, E., Esendam, B., Brand, A. & Goulmy, E. (1999) Feasibility of immunotherapy of relapsed leukemia with ex vivo-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood*, **93**, 2336–2341.

Nash, R.A., Pepe, M.S., Storb, R., Longton, G., Pettinger, M., Anasetti, C., Appelbaum, F.R., Bowden, R.A., Deeg, H.J. & Doney, K. (1992) Acute graft-versus-host disease: analysis of risk factors after allogeneic marrow transplantation and prophylaxis with cyclosporine and methotrexate. *Blood*, **80**, 1838–1845.

Nordlander, A., Uzunel, M., Mattsson, J. & Remberger, M. (2002) The TNFd4 allele is correlated to moderate-to-severe acute graft-versus-host disease after allogeneic stem cell transplantation. *British Journal of Haematology*, **119**, 1133–1136.

Shulman, H.M., Sullivan, K.M., Weiden, P.L., McDonald, G.B., Striker, G.E., Sale, G.E., Hackman, R., Tsoi, M.S., Storb, R. & Thomas, E.D. (1980) Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *American Journal of Medicine*, **69**, 204–217.

Socie, G., Loiseau, P., Tamouza, R., Janin, A., Busson, M., Gluckman, E. & Charron, D. (2001) Both genetic and clinical factors predict the development of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Transplantation*, **72**, 699–706.

Tseng, L.H., Lin, M.T., Hansen, J.A., Gooley, T., Pei, J., Smith, A.G., Martin, E.G., Petersdorf, E.W. & Martin, P.J. (1999) Correlation between disparity for the minor histocompatibility antigen HA-1 and the development of acute graft-versus-host disease after allogeneic marrow transplantation. *Blood*, **94**, 2911–2914.

Warren, E.H., Gavin, M., Greenberg, P.D. & Riddell, S.R. (1998) Minor histocompatibility antigens as targets for T-cell therapy after bone marrow transplantation. *Current Opinion in Hematology*, **5**, 429–433.

Weiden, P.L., Sullivan, K.M., Flournov, N., Storb, R. & Thomas, E.D. (1981) Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow graft. *New England Journal of Medicine*, **300**, 1068–1073.

Weisdorf, D., Hakke, R., Blazar, B., Miller, W., McGlave, P., Ramsay, N., Kersey, J. & Filipovich, A. (1991) Risk factors for acute graft-versus-host disease in histocompatible donor bone marrow transplantation. *Transplantation*, **51**, 1197–1203.

Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles

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Cytomegalovirus (CMV)–specific T-cell immunity plays an important role in protection from CMV disease in immunocompromised patients. Identification of cytotoxic T-lymphocyte (CTL) epitopes is essential for monitoring T-cell immunity and also for immunotherapy. In this and previous studies, CMV-pp65–specific CTL lines were successfully generated from all of 11 CMV-seropositive healthy donors, using pp65-transduced CD40-activated B (CD40-B) cells as antigen-presenting cells. By use of enzyme-linked immunospot (ELISPOT) assays, individual CTL epitopes could be mapped with truncated forms of the pp65 gene. For human leukocyte antigen (HLA) alleles with a known binding motif, CTL epitopes within the defined regions were predicted by computer algorithm. For HLA alleles without a known binding motif (HLA-Cw*0801, -Cw*1202, and -Cw*1502), the epitopes were alternatively identified by step-by-step truncations of the pp65 gene. Through this study, a total of 14 novel CTL epitopes of CMV-pp65 were identified. Interestingly, 3 peptides were found to be presented by 2 different HLA class I alleles or subtypes. Moreover, use of CD40-B cells pulsed with a mixture of synthetic peptides led to generation of pp65-specific CTL lines from some of seronegative donors. The study thus demonstrated an efficient strategy for identifying CTL epitopes presented by a variety of HLA alleles. (Blood. 2004;103:630-638)

Introduction

Late-onset cytomegalovirus (CMV) disease in hematopoietic stem cell transplant recipients (later than day 100 after transplantation) remains a major cause of morbidity and mortality, despite the introduction of new antiviral agents; indeed, recent reports have rather indicated an increase in disease.[1-5] Absence of reconstitution of CMV-specific T-cell response at 3 to 4 months after transplantation or the use of immunosuppressive drugs is strongly associated with reactivation of CMV and the subsequent CMV disease.[2-9] Immunologic treatments, such as adoptive transfer of CMV-specific cytotoxic T-lymphocyte (CTL) clones[6,10] or CMV-specific T-cell lines,[11] have successfully protected patients at risk from CMV disease, indicating that T-cell immunity plays an important role in controlling CMV infection. Thus, immunologic monitoring of T-cell immunity against CMV is crucial to evaluate the status of immunocompromised patients.

Identification of the CTL epitopes derived from CMV is very valuable not only for monitoring antiviral immunity but also for ex vivo generation of antiviral CTLs for possible application in adoptive immunotherapy. Immunodominance of pp65 protein among CMV antigens has been reported,[12-14] but previously identified CTL epitopes derived from the pp65 protein were limited to frequent human leukocyte antigen (HLA) class I alleles. Moreover, the pp65 CTL epitope presented by HLA-A*2402, which is the most frequent allele in Japanese individuals, may not be immunodominant because the percentage of $CD8^+$ T cells detectable with the HLA-A*2402/pp65 tetramer in healthy seropositive individuals is relatively low (A*2402, 0.1%[15]; versus A*0201, 0.75%; and B*0702, 1.85%[16]), whereas A*0201- or B*0702-restricted pp65 epitopes are considered immunodominant in white individuals. Therefore, additional pp65 epitopes of clinical significance need to be identified.

We previously reported an efficient strategy for in vitro CTL generation starting with as little as 10 mL of blood.[17] By use of retroviral transduced CD40-activated B (CD40-B) cells as antigen-presenting cells (APCs), pp65-specific CTL lines could be generated from all of 4 CMV-seropositive healthy donors and found to be restricted by multiple HLA class I alleles, suggesting utility for epitope identification. In the present study, using a total of 11 pp65-specific CTL lines, including 7 newly generated ones, we attempted to identify novel CTL epitopes by enzyme-linked immunospot (ELISPOT) assay using stimulator cells transfected with truncated forms of the pp65 gene or linear expression fragments encoding various regions of the gene, with or without the help of computer algorithm–based epitope prediction. This approach was sufficiently sensitive to identify even the subdominant epitopes recognized by the CTL lines. Through this study, a total of

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14 novel CTL epitopes were identified. Immunogenicity of a part of the newly identified epitopes was validated by successful generation of pp65-specific CTL lines from CMV-seropositive and also from some CMV-seronegative donors.

Materials and methods

Donors and cells

Peripheral blood samples were obtained from 11 CMV-seropositive and 8 CMV-seronegative healthy donors after we obtained informed consent. The study was approved by the institutional review board of the Aichi Cancer Center. Informed consent was provided according to the Declaration of Helsinki. CMV seropositivity was analyzed with regard to the presence of CMV-specific immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay, and HLA typing was carried out at The HLA Laboratory (Kyoto, Japan; Table 1). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by centrifugation on a Ficoll (Amersham Biosciences, Uppsala, Sweden) density gradient, and $CD8^+$ and $CD8^-$ fractions were separated using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cryopreserved until use.

Plasmids and synthetic peptides

Plasmids, pcDNA3-pp65, pcDNA3–enhanced green fluorescent protein (EGFP), and pcDNA3.1 (Invitrogen, Tokyo, Japan) encoding HLA class I cDNA were constructed as previously described.[17,18] To generate pEAK10-pp65, a portion of pp65 DNA was transferred from pcDNA3-pp65 into the pEAK10 vector (Edge Biosystems, Gaithersburg, MD). A plasmid containing a mutant *pp65* gene defective for the immunodominant HLA-A*0201–restricted epitope (NLVPMVATV to NLVPMVAAATV) was constructed by polymerase chain reaction (PCR)–based mutagenesis using the following primers: sense, ATGGTGGCTGCAGCTACGGTTCAGGGTCAG; antisense, AACCGTAGCTGCAGCCACCATGGGCACCAG (the inserted nucleotides are underlined). This plasmid was termed pcDNA3-pp65$^{\Delta NLV}$. All peptides were purchased from Toray Research Center (Tokyo, Japan).

Table 1. Characteristics of HLA class I and CMV serostatus of donors

Blood donor	HLA-A	HLA-B	HLA-C	CMV serostatus
P01*	1101/2603	1501/-	0401/1502	+
P02*	2402/-	4001/4002	0304/1502	+
P03*	0207/-	4001/4601	0102/0401	+
P04	2402/-	3901/5201	0702/1202	+
P05	2402/-	3501/5101	0303/1402	+
P06*	0201/2402	1501/4002	0303/0702	+
P07	0201/0207	4006/4601	0102/0801	+
P08	2402/3303	4006/4403	0801/1403	+
P09	2402/-	4002/5101	0801/1202	+
P10	1101/2402	1501/5101	0303/1202	+
P11	2402/-	4601/5901	0102/-	+
N01*	2402/3303	4403/5401	0102/1403	−
N02*	0201/0206	3501/4006	0303/0801	−
N03	2402/3101	4002/4006	0304/0801	−
N04	0206/2402	3501/5502	0102/0401	−
N05	0207/3303	4403/4601	0103/1403	−
N06	2402/-	4001/5401	0102/0304	−
N07	2402/2420	5201/5502	0102/1202	−
N08	1101/2402	3501/5201	0303/1202	−

+ indicates CMV seropositive; and −, CMV seronegative.

*Donors participated in the previous study. P01, P02, P03, N01, N02, and P06 correspond to donors 1, 2, 3, 4, 5, and 6, respectively.[17]

Generation of CD40-activated B cells and EBV-transformed lymphoblastic cell lines (LCLs)

CD40-B cells were generated as previously described.[17,19] In brief, a thawed $CD8^-$ fraction of PBMCs was cultured on a γ-irradiated (96 Gy) human CD40L-transfected NIH3T3 cell line[20] (t-CD40L; kindly provided by Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA) in the presence of interleukin 4 (IL-4; 4 ng/mL, Ono Pharmaceutical, Osaka, Japan) and cyclosporin A (CsA; 0.7 μg/mL, Sandoz, Basel, Switzerland) in 2 mL of Iscove modified Dulbecco medium (Invitrogen) supplemented with 10% pooled human serum. The expanding cells were transferred onto freshly prepared t-CD40L cells and fed with cytokine-replenished medium without CsA every 3 to 4 days. LCLs were established from the CD40-B cells with supernatant of an Epstein-Barr virus (EBV)–producing cell line (B95-8; American Type Culture Collection, Manassas, VA) in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (FCS; IBL, Takasaki, Japan), referred to as "complete medium."

Retroviral transduction of CD40-B cells and LCLs

Retroviral transduction was conducted as previously described.[17] In brief, the retroviral construct, LZRSpBMN/pp65 (the backbone plasmid, LZRSpBMN-Z was kindly provided by Dr G. Nolan, Stanford University, Stanford, CA), pLBPC/pp65, or pLBPC/EGFP, was packaged in the Phoenix gibbon ape leukemia virus (GALV) cell line[21] (a gift from H.-P. Kiem, Fred Hutchinson Cancer Research Center; and from G. Nolan, Stanford University, Stanford, CA) using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). CD40-B cells and LCLs were infected with the retroviral supernatant in the presence of 10 μg/mL polybrene (Sigma, Chicago, IL), spun at 1000*g* for 1 hour at 32°C, and incubated. Two days after, LCLs transduced with pp65 (LCL/pp65) or EGFP (LCL/EGFP) were selected in the presence of puromycin (0.7 μg/mL; Edge Biosystems). Transduction efficiency were assessed as previously described.[17]

Generation of pp65-specific CTL lines using retrovirally transduced CD40-B cells

Thawed $CD8^+$ cells (1×10^6) were cocultured with γ-irradiated (33 Gy) autologous pp65-transduced CD40-B (CD40-B/pp65) cells (1×10^6) in 2 mL RPMI 1640 supplemented with 10% pooled human serum and recombinant human IL-7 (50 U/mL; Genzyme, Cambridge, MA) at 37°C in 5% CO_2. On days 7 and 14, $CD8^+$ cells were restimulated and one day after each stimulation recombinant human IL-2 (Chiron, Emeryville, CA) was added to the cultures at the final concentration of 20 U/mL. If necessary, rapidly growing cells were split into 2 to 3 wells and fed with fresh media containing 20 U/mL IL-2. Peptide-pulsed CD40-B cells were also prepared by incubation with 10 μM peptides derived from pp65 and used as APCs. For generation of CTL lines from seronegative donors, IL-12 (5 ng/mL; R&D systems, Minneapolis, MN) was added on day 0.

Construction of deletion mutants

To construct deletion mutants of the *pp65* gene, the plasmid, pcDNA3-pp65, was opened with *Apa*I and *Bam*HI, and progressive 3′ deletions were produced by exonuclease III treatment using the Erase-a-Base System (Promega, Madison, WI). After ligation each clone was sequenced. A total of 22 clones were selected and termed "Δpp65(1-XXX)"; XXX indicates the amino acid position of the C-terminus of each clone.

Epitope selection and construction of linear expression fragments

The epitopes within the defined regions of the pp65 protein from human CMV strain AD169 were predicted by "HLA Peptide Binding Predictions" on the BioInformatics & Molecular Analysis Section (BIMAS) website[22,23] and also by "HLA Epitope binding prediction" (beta testing version) in the HLA Ligand/Motif Database.[24]

Linear expression fragments encoding various regions of the pp65 gene were constructed by 2-step overlapping PCR. Targeted region-specific 5′ and 3′ primers incorporating additional sequences (single- and double-underlined, see

below) were designed (eg, 5′primer, TCGGATCCACCATGCAGTACGATCCCGTGG [30 bp] and 3′ primer, GACTCGAGCGCTAGAAGAGCGCAGCCACGG [30 bp] for QYDPVAALF [amino acids (aa's) 341-349]) and used for PCR (KOD Plus; Toyobo, Osaka, Japan) with a template plasmid, pEAK10-pp65. CMV promoter (P_{CMV}) and bovine growth hormone (BGH) polyadenylation signal (pA) were independently amplified from pcDNA3.1 by PCR using the following primers: 5′ P_{CMV}, CTTAGGGTTAGGCGTTTTGC; 3′ P_{CMV}, NNCATGGTGGATCCGAGCTCGGTA; 5′ pA, NNTAGCGCTCGAGTCTAGAGGG; 3′ pA, GGTTCTTTCCGCCTCAGAAG; "N" means a mixture of A/C/T/G. The 3′ P_{CMV} and 5′ pA primers contained overlapping sequences (underlined) with 5′ primer and 3′ primer, respectively, of the targeted region. The 3 PCR products, P_{CMV}, the targeted region, and pA, were conjugated by second PCR using primers, 5′ P_{CMV} and 3′ pA, and termed "P_{CMV}XXX-XXX" (each "XXX" indicates the amino acid positions of the N- or C-terminus).

ELISPOT assays

ELISPOT assays were performed as described earlier.[17,25] In brief, a MultiScreen-HA plate (MAHA S4510; Millipore, Bedford, MA) was coated with antihuman interferon γ (IFN-γ) monoclonal antibody (M700A; Endogen, Woburn, MA) and used as an ELISPOT plate. The 293T cells were cotransfected with plasmids encoding each of the individual donor's HLA class I alleles and either pcDNA3-pp65, deletion mutants, or PCR products of the linear expression fragment by TransIT-293 (Mirus, Madison, WI) and were used as stimulator cells after 2 days. The transfected 293T, LCL/pp65, or LCL/EGFP cells (with or without peptide pulsing) were mixed with 10^3 or more effector cells from the CTL lines generated. After cells had been incubated in 200 μL complete medium in a 96-well plate (3790; Costar Corning, Cambridge, MA) for 4 hours, all the aliquots were transferred into an ELISPOT plate and incubated for an additional 16 hours. To visualize spots, a biotin-labeled antihuman IFN-γ antibody (M701B; 1 μg/mL, Endogen), streptavidin-alkaline phosphatase (Biosource International, Camarillo, CA), and substrate were used. Spots were counted after computerized visualization using a scanner (Canon, Tokyo, Japan). For peptide titration assays, autologous LCL/EGFP cells were pulsed with various concentrations of synthetic peptides and then used as stimulator cells to see the differences in avidity of the effector cells from CMV-seronegative and CMV-seropositive donors.

Chromium release assay

LCL/pp65 or LCL/EGFP cells were labeled in 100 μL complete media with 3.7 MBq ^{51}Cr for 1 hour at 37°C. Dermal fibroblasts were infected overnight with CMV (strain AD169) supernatant in the presence of 3.7 MBq ^{51}Cr following 500 U/mL IFN-γ pretreatment for 24 hours. For peptide reconstitution assays, 1 μM of synthetic peptide was added 1 hour before introducing effector cells. After 4 hours incubation with effector cells, supernatants were counted in a gamma counter. The percentage of specific lysis was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Results

Generation and characterization of pp65-specific CTL lines from CMV-seropositive donors using pp65-transduced CD40-B cells as APCs

We previously reported successful generation of pp65-specific CTL lines from all enrolled CMV-seropositive donors using retrovirally transduced CD40-B cells as APCs. In this study, we extended the findings by including additional 7 seropositive donors (Table 1). All CTL lines, with $CD8^+$ phenotype, lysed pp65-transduced autologous LCLs efficiently, but not untransduced autologous LCLs, and the activities were inhibited by anti–HLA class I antibody (data not shown).

To determine the HLA restriction of these CTL lines we conducted ELISPOT assays using 293T cells transfected with the

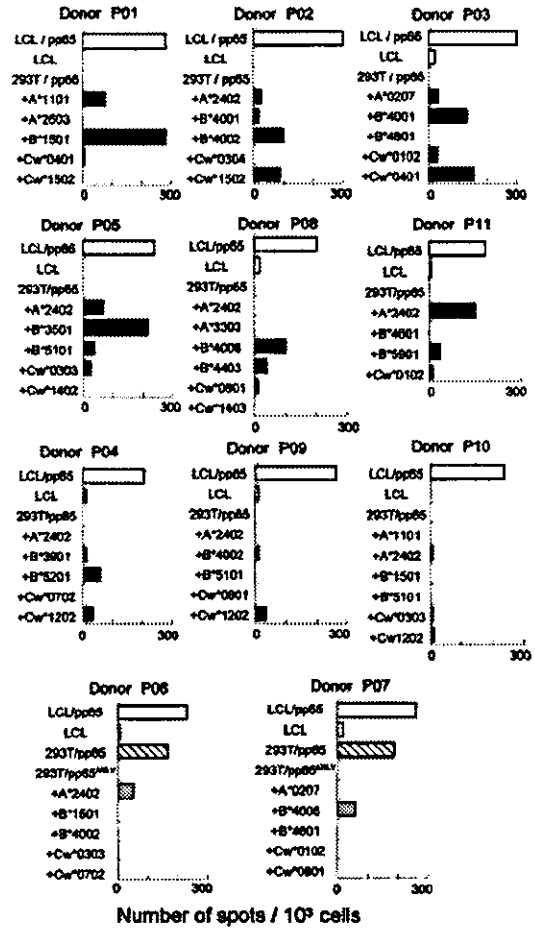

Figure 1. ELISPOT assay for the determination of HLA restriction of the CTL lines generated from CMV seropositive donors. CTL lines generated after the third stimulation with pp65-transduced CD40-B cells were tested for HLA restriction. ELISPOT assays were performed by incubating the CTL line with one of the following: autologous LCL, pp65-transduced autologous LCL (□), 293T cells transfected with both the pp65 gene and the individual HLA cDNA (■), or the *pp65* gene alone (▨). In the case of P06 and P07, CTL lines were found to recognize 293T cells transfected with the *pp65* gene alone, due to their endogenous expression of HLA-A*0201. Thus, 293T cells transfected with both mutant *pp65* gene (pcDNA3-pp65^{ANLV}), lacking a dominant A*0201-restricted epitope (NLVPMVATV), and individual HLA cDNA were used in these cases (□) (see "Materials and methods"). Each bar represents the number of spots per 10^3 cells.

pp65 gene plus one of the HLA class I alleles belonging to donors as stimulator cells (Figure 1). For instance, a major population of the CTL line generated from donor P01 was stimulated by 293T cells transfected with HLA-B*1501 and the *pp65* gene, while minor populations were restricted by HLA-A*1101 or -Cw*0401. Responses associated with the other HLA class I alleles were not detected when 10^3 cells of the CTL line were used. The results demonstrated that the P01 CTL lines recognized multiple pp65-derived epitopes presented by at least 3 different HLA class I alleles. Similarly in the cases of P02, P03, P05, P08, and P11, the CTL lines were restricted by multiple HLA class I alleles and the sum of the spots restricted to each allele was comparable to those produced with autologous LCL/pp65. In contrast, in the cases of P04, P09, and P10, the sum of the spots restricted to each HLA class I allele was much smaller than those with pp65-transduced LCLs (Figure 1).

The CTL lines from HLA-A*0201–positive donors (P06, P07) well recognized the transfectant with pp65 alone (Figure 1 ▨)

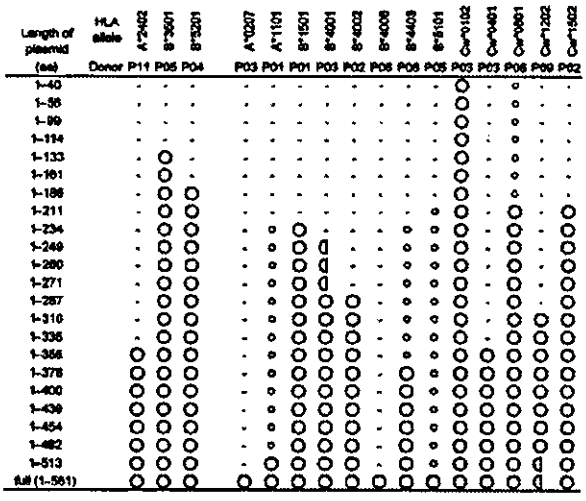

Figure 2. Localization of pp65-derived CTL epitopes estimated by ELISPOT assay using various pp65-deletion mutants. Amino acids (aa's) are numbered from the initial methionine. Each deletion mutant was transfected into 293T cells together with restricting HLA cDNA and was tested for the recognition by corresponding pp65-specific CTL line by ELISPOT assay. The number of spots over that of full-length pp65 or Δpp65(1-482) (only in Cw*1202) is indicated as follows: greater than 70% (large-sized circles), 20% to 70% (semicircles), less than 20% (small-sized circles). "—" indicates the absence of spots.

because 293T cells express HLA-A*0201 endogenously (data not shown). The mutant pp65 cDNA expression plasmid (pcDNA3-pp65$^{\Delta NLV}$), lacking the HLA-A*0201–restricted immunodominant epitope, enabled us to detect CTL responses associated with HLA alleles other than HLA-A*0201. The results revealed that HLA-A*2402 and -B*4006 were subdominant restricting HLA alleles for P06 and P07 CTL lines, respectively (Figure 1 ▨).

Identification of the regions encoding the pp65 epitopes restricted by individual HLA class I alleles

As an initial step toward defining the epitopes recognized by these pp65-specific CTL lines, C-terminus–truncated *pp65* genes were generated using the conventional exonuclease III deletion method to locate the regions encoding the epitopes (Figure 2). Thereafter, 293T cells were transfected with each of the deletion mutants plus restricting HLA class I cDNA and used for stimulation of each CTL line.

The P11 CTL line could recognize 293T cells transfected with HLA-A*2402 cDNA plus Δpp65(1-355) or longer deletion mutants, but not Δpp65(1-335) or shorter deletion mutants (Figure 2), indicating that the pp65 epitope presented by HLA-A*2402 should fully or partially be contained inside the region spanning between amino acid residues aa336 and aa355. Indeed, an HLA-A*2402–restricted epitope QYDPVAALF (aa's 341-349)[15] is found within the region. The results for HLA-B*3501 and -B*5201 were also consistent with the reported epitopes (IPSINVHHY [aa's 123-131][26] and QMWQARLTV [aa's 155-163],[27] respectively). These observations indicate that our strategy using deletion mutants worked effectively.

Next, we attempted to locate the regions containing pp65 CTL epitopes presented by other HLA class I alleles. As shown in Figure 2, such regions existed between the following amino acid residues: 514-561 (A*0207), 483-513 (A*1101), 212-234 (B*1501), 272-287(B*4002), 514-561 (B*4006), 356-378 (B*4403), 514-561 (B*5101), 1-40 (Cw*0102), 336-355 (Cw*0401), 187-211 (Cw*0801), 288-310 (Cw*1202), 187-211(Cw*1502). In the case of HLA-B*4001, the P03 CTL line strongly recognized not only Δpp65(1-287) or longer deletion mutants but also Δpp65(1-249), Δpp65(1-260), and Δpp65(1-271) to a lesser extent, suggesting the presence of 2 different HLA-B*4001–restricted epitopes. The existence of an additional subdominant epitope was also suggested in the cases of HLA-A*1101, -B*4403, -B*5101, and -Cw*0801 (Figure 2). As for HLA-Cw*1202, it is of note that the transfectants with the full-length plasmid produced a smaller number of spots than those with the shorter deletion mutants, such as Δpp65(1-482).

Identification of the pp65 epitopes presented by HLA alleles whose binding motif is predictable by computer algorithm

To predict the epitopes within the regions narrowed down by the deletion mutant experiments (Figure 2), amino acid sequences of the determined regions with a 10-aa extension to the N-terminus were analyzed by online computer algorithm software. The prediction results are listed in Table 2. For the HLA-A*0207–restricted epitope, A*0201 was alternatively selected because A*0207 was not available on the computer algorithm we used.[23,24] As for HLA-B*1501, the computer algorithm on the BIMAS website[22,23] depicted 3 candidate epitopes with similar scores (6, 4.4, and 4), one of which was also depicted by another algorithm[24] and subsequently adopted.

Table 2. Candidate epitopes predicted by computer algorithms

HLA restricted	Parameter submitted		Result predicted‡				Rank in whole pp65
	Range, aa†	HLA type	Position, aa	Sequence	Length	Score	
A*0207	504-561	A*0201	522-530	RIFAELEGV	9	39.8	12
A*1101	473-513	A*1101	501-509	ATVQGQNLK	9	1.5	3
B*1501	202-234	B*1501§	215-223	KMQVIGDQY§‖	9	117§	4
B*4001	225-249	B60	232-240	CEDVPSGKL	9	176	1
B*4001	262-287	B60	267-275	HERNGFTVL	9	160	2
B*4002	262-287	B61	267-275	HERNGFTVL	9	8	6
B*4006	504-561	B40	525-534	AELEGVWQPA	10	80	1
B*4403	346-380	B*4403	364-373	SEHPTFTSQY	10	720	1
B*5101	504-561	B*5101	547-555	LPGPCIAST	9	14.3	25
Cw*0102	1-40	Cw*0102§	7-15	RCPEMISVL§	9	200§	3
Cw*0401	336-378	Cw*0401	341-349	QYDPVAALF	9	216	4

†The different region between the shortest positive deletion mutant and the longest negative one (Figure 2) with a 10 aa extension to the N-terminus was submitted.

‡All results except for the marked ones§ were predicted by "HLA Peptide Binding Predictions" on BIMAS website.[23]

§Predicted by "HLA Epitope binding prediction" on the website of University of Oklahoma Health Sciences Center.[24]

‖By computer algorithm on BIMAS website, the peptide was assigned 3rd rank (score = 4) within the submitted range and 21st rank in the full-length pp65 gene, respectively.

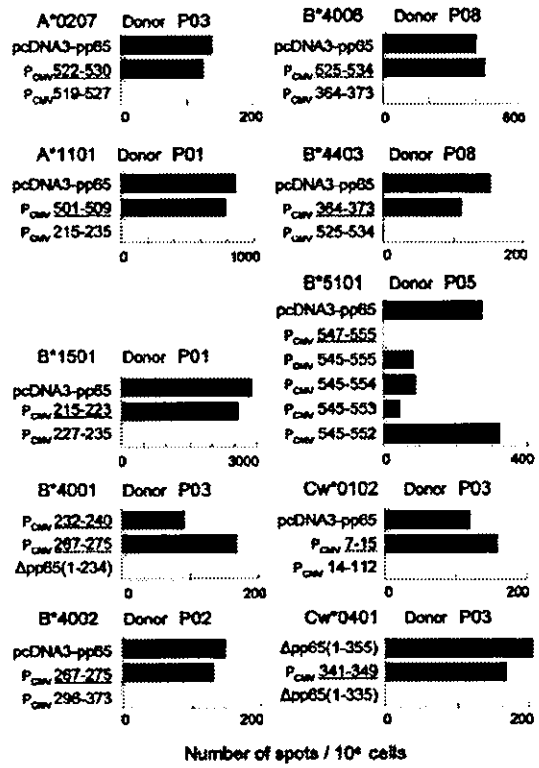

Figure 3. Recognition of the epitopes predicted by computer algorithm by the pp65-specific CTL lines. Linear expression fragments encoding various peptides including the predicted epitopes were generated and transfected into 293T cells together with restricting HLA cDNA. Recognition by the pp65-specific CTL lines was evaluated 48 hours later by ELISPOT assay. Numbers indicate the amino acid position of pp65 encoded by each construct. Linear expression fragments encoding the predicted epitopes are underlined. Each bar represents the number of spots per 10^4 cells.

To test the recognition of the predicted epitopes by each CTL line, linear expression fragments encoding various peptides including the predicted epitopes were generated by overlapping PCR (see "Materials and methods"), transfected into 293T cells together with restricting HLA class I cDNA, and evaluated by ELISPOT assay. As shown in Figure 3, all predicted epitopes except for the HLA-B*5101–restricted one were well recognized by the corresponding pp65-specific CTL, and the specificity was confirmed using irrelevant fragments. For instance, the P01 CTL line could be stimulated by 293T cells cotransfected with a fragment encoding aa's 215 to 223 and HLA-B*1501 comparably to the case with the full-length pp65 gene, but not at all with aa's 227 to 235, indicating that aa's 215 to 223 (KMQVIGDQY; nonamer) is at least one of the HLA-B*1501–restricted epitopes. With HLA-B*4001, both of the 2 fragments encoding the predicted epitopes (aa's 232-240 and aa's 267-275) were well recognized by P03 CTL line (Figure 3), confirming the results of ELISPOT assay using deletion mutants (Figure 2).

In the case of HLA-B*5101, the fragments encoding the predicted epitope (aa's 547-555) or the one with the second highest score (aa's 545-553) were not or only poorly recognized by the P05 CTL line (Figure 3). Thus various other fragments were tested and the one encoding octamer peptide (DALPGPCI; aa's 545-552) was found to be well recognized. This octamer has a binding motif consistent with that for HLA-B*5101.[28]

In summary, we successfully identified 11 new pp65-derived epitopes presented by 10 distinct HLA alleles (Table 3). Of interest is the fact that QYDPVAALF (aa's 341-349) restricted by HLA-Cw*0401 is identical to that restricted by HLA-A*2402.[15]

Identification of the pp65 epitopes presented by HLA alleles whose binding motif is not predictable by computer algorithm

Since algorithms that predict peptides binding to HLA-Cw*0801, -Cw*1202, and -Cw*1502 are currently not available, we performed step-by-step epitope mapping using the linear expression fragments. Based on the results with deletion mutants and HLA-Cw*1202 (Figure 2), fragments encoding the region from aa 267 to aa 292 or to aa 302 were tested. The P04 CTL line recognized the transfectant with the fragment encoding aa's 267 to 302, but not that with aa's 267 to 292 (Figure 4A left), suggesting that the epitope should be fully or partially contained between aa 293 and aa 302. Next, various fragments encoding the septamer to dodecamer peptides within this region were generated and tested. The fragments whose C-terminus was Phe302 were well recognized, but those with His301 or Gly303 at the C-terminus were not or only

Table 3. Summary of CTL epitopes derived from CMV-pp65

Newly identified epitopes				Previously reported epitopes				
HLA	Position, aa	Sequence	Length	HLA	Position, aa	Sequence	Length	Reference
A*0207	522-530	RIFAELEGV	9	A*0101	363-373	YSEHPTFTSQY	12	29,30
A*1101	501-509	ATVQGQNLK	9	A2	14-22	VLGPISGHV	10	31
B*1501	215-223	KMQVIGDQY	9	A2	120-138	MLNIPSINV	9	31
B*4001	232-240	CEDVPSGKL	9	A2	495-503	NLVPMVATV	9	14,32
B*4001	267-275	HERNGFTVL†	9	A*1101	16-24	GPISGHVLK	9	9,30
B*4002	267-275	HERNGFTVL†	9	A*2402	341-349	QYDPVAALF‡	9	15
B*4006	525-534	AELEGVWQPA	10	A*2402	369-379	FTSQYRIQGKL	11	9,30
B*4403	364-373	SEHPTFTSQY	10	A*2402	113-121	VYALPLKML	9	33
B*5101	545-552	DALPGPCI	8	A*6801/2	186-196	FVFPTKDVALR	11	30
Cw*0102	7-15	RCPEMISVL	9	B7	265-274	RPHERNGFTV	10	14,34
Cw*0401	341-349	QYDPVAALF‡	9	B7	417-426	TPRVTGGGAM	10	14,34
Cw*0801	198-206	VVCAHELVC§	9	B35(B*3501)	123-131	IPSINVHHY	9	26
Cw*1202	294-302	VAFTSHEHF	9	B35(B*3502)	188-195	FPTKDVAL	8	9,14,30
Cw*1502	198-206	VVCAHELVC§	9	B35(B*3503)	187-195	VFPTKDVAL	9	14,35
				B*3801/2	367-379	PTFTSQYRIQGKL	13	14,34
				B44(B*4402)	512-521	EFFWDANDIY	10	14,34,35
				B*5201	155-163	QMWQARLTV	9	27

†,‡,§ Same peptide found to be presented by different alleles or subtypes of HLA.

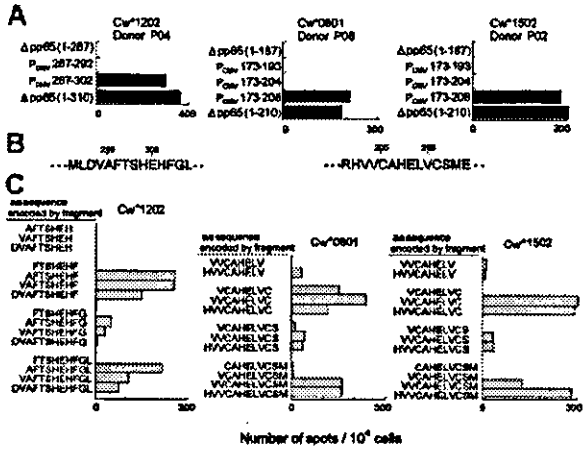

Figure 4. Identification of pp65-derived CTL epitopes for which a computer algorithm was not applicable. To narrow down the region containing the epitope, the linear expression fragments encoding further truncated forms of the deletion mutant, Δpp65(1-310) for HLA-Cw*1202 or Δpp65(1-210) for HLA-Cw*0801 and -Cw*1502, were generated and transfected into 293T cells together with restricting HLA cDNA. (A) Recognition by the pp65-specific CTL lines was evaluated 48 hours later by ELISPOT assay. (B) Amino acid sequence of pp65 around the defined region. (C) Based on the results shown in the upper panels, various linear expression fragments within the region were generated, transfected into 293T cells together with restricting HLA cDNA, and then tested for the recognition by corresponding pp65-specific CTL line using ELISPOT assay. Each bar represents the number of spots per 10^4 cells.

poorly recognized (Figure 4C left). The CTL lines were partially activated by the fragments ending at Leu304. Progressive N-terminal deletion revealed that Ala295 was crucial for recognition. The same results were obtained with the P09 CTL line (data not shown). Additional experiments with synthetic peptides revealed that the CTL clone could recognize VAFTSHEHF (aa's 294-302) at 2-log lower concentrations than AFTSHEHF (aa's 295-302) (data not shown), indicating that the nonamer VAFTSHEHF is the minimal epitope presented by HLA-Cw*1202.

In the case of HLA-Cw*0801, fragments encoding the region up to aa 193, aa 204, or aa 208 were also constructed for further narrowing the regions identified (Figures 2 and 4). Because the fragment encoding aa's 173 to 208, but not aa's 173 to 204, was recognized by the P08 CTL line, the C-terminus of the epitope should be located between aa's 205 and 208 (Figure 4A middle). ELISPOT assay using transfectants with various linear expression fragments revealed that those encoding the peptides ending at Cys206 or Met208 were well recognized (Figure 4C middle). The results also indicated that Val198 was required for recognition. Thus we determined that VVCAHELVC (aa's 198-206) is the minimal epitope presented by HLA-Cw*0801. Interestingly, the same nonamer (VVCAHELVC) was independently identified as a minimal epitope presented by HLA-Cw*1502 (Figure 4C right).

Generation of CTL lines from CMV-seropositive donors with CD40-B cells pulsed with newly identified pp65 epitopes

To confirm the immunogenicity of the newly identified epitopes, we tried to generate pp65-specific CTL using synthetic peptide-pulsed CD40-B cells as APCs. After the third stimulation cytolytic activity of the CTL lines was assessed. A CTL line from donor P03, induced by CEDVPSGKL presented by HLA-B*4001, could lyse HLA-B*4001–positive allogeneic LCLs (from donor N06) expressing pp65 antigen and also the peptide-pulsed LCL/EGFPs (Figure 5A). Similar results were also obtained with the other 5 epitopes.

Generation of pp65-specific CTL lines from seronegative donors with CD40-B cells pulsed with a mixture of newly or previously identified pp65 epitopes

In the current and previous studies we failed to generate pp65-specific CTL lines from all 8 seronegative donors using pp65-transduced CD40-B cells as APCs. To improve stimulation efficiency, we applied CD40-B cells pulsed with a mixture of newly or previously identified epitopes. One million $CD8^+$ T cells were stimulated 3 times with autologous CD40-B cells pulsed with a mixture of 2 to 4 synthetic peptides, which are presentable by HLA alleles belonging to each donor. The T-cell line from N02 produced IFN-γ spots after stimulation with 2 of the 4 peptides (Figure 6A). Peptide titration assay revealed that half-maximal number of spots for N02 line was obtained with approximately 3.0 nM of peptide IPSINVHHY, while that for P05 CTL line from a seropositive donor sharing HLA-B*3501 was 80 pM (Figure 6B top), indicating that a 38-fold higher peptide concentration was required in the N02 line. Nevertheless, the N02 could lyse autologous LCL/pp65s and also dermal fibroblasts infected with CMV although a lesser extent (9% at the effector-target [E/T] ratio of 25:1) (Figure 6C top). Two of the 4 peptides were recognized by the N03 T-cell line whose half-maximal spots for both peptides were obtained with 2.9 nM (peptide QYDPVAALF) and 14 nM (peptide HERNGFTVL), which were 35- and 77-fold higher than those for P02 CTL line from a seropositive donor sharing HLA-A*2402 and -B*4002, respectively. This N03 line lysed autologous LCL/pp65s and peptide-pulsed fibroblasts, but failed to lyse CMV-infected fibroblasts, suggesting the avidity of the line was not high enough for exerting cytotoxic activity against CMV-infected fibroblasts. On the other hand, in the case of N06 and N08, exogenously pulsed

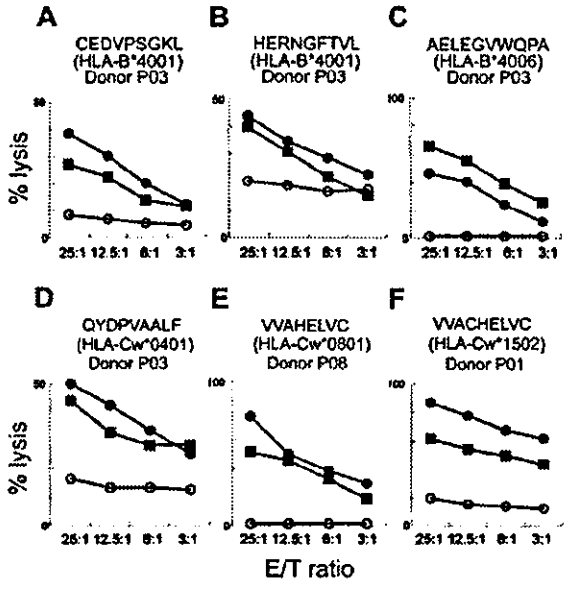

Figure 5. Cytolytic activity of the CTL lines generated by stimulation with CD40-B cells pulsed with newly identified epitopes. $CD8^+$ T cells from CMV-seropositive donors were stimulated with peptide-pulsed autologous CD40-B cells. CMVpp65-derived synthetic peptides used were CEDVPSGKL ($pp65^{232-240}$) to be presented by HLA-B*4001 (A), HERNGFTVL ($pp65^{287-275}$) by HLA-B*4001 (B), AELEGVWQPA ($pp65^{525-534}$) by HLA-B*4006 (C), QYDPVAALF ($pp65^{341-349}$) by HLA-Cw*0401 (D), VVCAHELVC ($pp65^{198-206}$) by HLA-Cw*0801 (E) or Cw*1502 (F). One week after the third stimulation, cytolysis of CTL lines was assessed by ^{51}Cr release assay against LCL/pp65 (■), LCL/EGFP (○), or 1 μM peptide-pulsed LCL/EGFP (●) over a range of E/T ratios as indicated. The origin of target cells was autologous (panel F) or allogeneic [panels A-B, N06 (B*4001+); panel D, P01(Cw*0401+); panels C and E, N03(B*4006, Cw*0801+)].

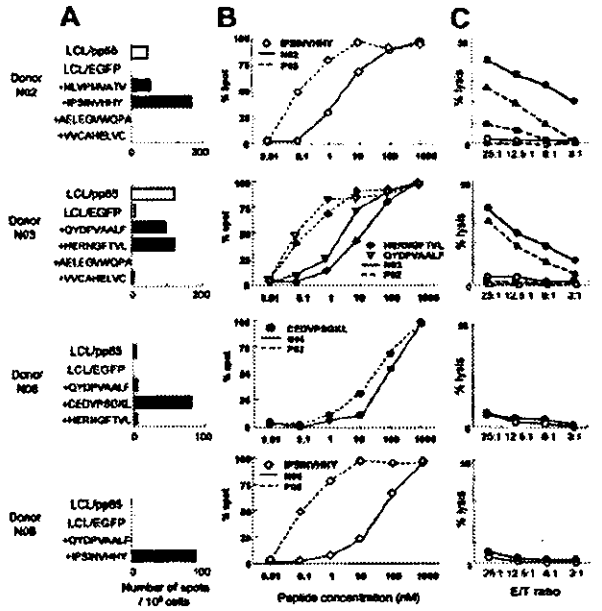

Figure 6. Effector cell activity of the T-cell lines generated from seronegative donors after stimulation with CD40-B cells pulsed with a mixture of antigenic peptides. CD8+ T cells from CMV-seronegative donors were stimulated 3 times with autologous CD40-B cells pulsed with a mixture of 2 to 4 peptides and tested for effector activity. CMVpp65-derived synthetic peptides used are listed in Table 3. The antigenicity of these peptides was proven in the experiments whose results are shown in Figure 5. (A) ELISPOT assay was conducted using LCL/pp65, LCL/EGFP (□), or LCL/EGFP pulsed with 1 μM of each peptide indicated (■). Each bar represents the number of spots per 10^3 cells. (B) Peptide titration was conducted using LCL/EGFP pulsed with various concentrations of the peptides indicated by ELISPOT assay. Percent spot was calculated for individual T-cell lines by dividing the number of spots at indicated peptide concentrations by the maximal number of spots × 100%. (C) Cytolysis of CTL lines was assessed against autologous LCL/pp65 (●), LCL/EGFP (○), HLA-matched dermal fibroblasts infected with CMV supernatant (■), with mock supernatant (□), or with 1 μM peptide mixture showing spots in the ELISPOT assay (▲) over a range of E/T ratios by ^{51}Cr release assay.

peptides (N06, CEDVPSGKL; N08, IPSINVHHY) on LCL/EGFPs were recognized (Figure 6A), whereas endogenously expressed pp65 in LCLs was not recognized (Figure 6A,C), which might partly be explained by the 10- to 20-fold higher peptide concentrations necessary for the half-maximal spots (65 nM for CEDVPSGKL in N06 and 36 nM for IPSNVHHY in N08; Figure 6B).

Discussion

The present study focused on the systematic identification of novel CTL epitopes derived from CMV-pp65. We combined biotechnology with 22 C-terminal truncations of the *pp65* gene at an average 22–amino acid interval (range, 11-48 aa's) and computer technology with algorithm-based prediction of peptide binding to certain HLA alleles. We were able to identify CTL epitopes using linear expression fragments constructed by overlapping PCR, even if a computer algorithm was not available for HLA alleles of interest. In general, to identify CTL epitopes, establishment of CTL clones and verification using ^{51}Cr release assay have frequently been used. In this study, for efficient detection of CTL responses, we adopted the ELISPOT assay[15] so that even responses to subdominant epitopes could be detected, such as the HLA-Cw*0102–restricted one in donor P03.

In the cases of P04, P09, and P10, the sum of the spots against 293T cells transfected with *pp65* gene plus each HLA class I allele was unexpectedly smaller than that against pp65-transduced LCLs (Figure 1). This could be due to differential processing and/or presentation of pp65 proteins in LCLs and 293T cells. In this sense, HLA-A*2402 and -Cw*1202 are the focus as presenting molecules because both are present in the cases. Since the CTL lines were poorly stained with HLA-A*2402 tetramers incorporating peptide, QYDPVAALF (data not shown), it is unlikely that HLA-A*2402–restricted epitopes are concerned in this issue. Indeed, defective processing and/or presentation of the HLA-Cw*1202–restricted epitope from full-length pp65 in 293T cells was demonstrated (Figure 2). Probably, in LCLs, the pp65 is more efficiently processed and presented to yield the HLA-Cw*1202–restricted epitope. The better processing might attribute to so-called immunoproteasomes equipped by LCLs. So far, simultaneous transfection with immunoproteasome components, such as large multifunctional protease 2 (LMP2), LMP7, and LMP10, or IFN-γ treatment did not improve the recognition of 293T transfectants (data not shown), suggesting that additional factors are involved. The reason why truncated pp65 were processed more efficiently is unclear but this could be due to relatively unstable pp65 protein prone to degradation and entry into the processing pathway. Additional studies are now in progress to address the question.

One of the interesting findings of this study is that linear expression fragments encoding the right epitopes with only a single amino acid extension at the C-terminus were not recognized by each CTL line efficiently, whereas N-terminal extension rarely affected the recognition (Figure 4). Aminopeptidases, such as endoplasmic reticulum aminopeptidase 1 (ERAP1)[36-38] or leucine aminopeptidase,[39] may be able to trim N-terminal extensions and create peptides with optimal length for binding to major histocompatibility complex (MHC). However, mammalian cells lack carboxypeptidases,[39-41] thus proteasome is solely responsible for creating the correct C-terminus of the epitopes. Although a very limited number of amino acids adjacent to both sides of a proteasomal cleavage site contribute to cleavage site selection,[42] only a single amino acid extension at the C-terminus may be too short to be removed efficiently by proteasomes. In addition, constitutive proteasomes might be dominant in 293T cells[43] rather than immunoproteasomes, which have greater efficacy and are expressed in LCLs or mature dendritic cells. This may partially explain why C-terminal–extended epitopes were poorly recognized in our experiments.

We identified 2 new epitopes presented by HLA-A molecules. One is RIFAELEGV, dominantly presented by HLA-A*0207 but not by HLA-A*0201. This result underscores differential peptide repertories that bind to HLA-A*0207 and -A*0201, probably influenced by a single amino acid substitution at the floor of the binding groove.[44] The other is presented by HLA-A*1101 (ATVQGQNLK; aa's 501-509): this seems to be the dominant epitope presented by the allele, although a subdominant one may be located between aa 211 and aa 234. Both the dominant and undefined subdominant epitopes are, however, different from those reported previously, GPISGHVLK; aa 16-24.[9,30] For HLA-B alleles we identified 7 new epitopes, 4 of which are restricted by the HLA-B40 group. Among them, HERNGFTVI (aa's 267-275) is presented by both HLA-B*4001 and -B*4002. HLA-B*4001 presents an additional epitope, CEDVPSGKL (aa's 232-240). An HLA-B*4403–restricted epitope found in this study (SEHPTFTSQY; aa364-373) differs from the HLA-B44–restricted one reported earlier (EFFWDANDIY; aa's 512-521).[14,34] HLA-B*4402 and -B*4403 are 2 major HLA-B44 subtypes in white individuals,[45,46] and HLA-B*4403 is most frequent HLA-B44 subtype in

Japanese individuals.[47-49] Because the reported epitope, EFFWDANDIY, was listed as the HLA-B*4402–restricted one,[35] these 2 epitopes might be restricted by a different subtype of HLA-B44. All the data imply that subtle differences in amino acid residues facing the groove of HLAs have an impact on the peptide binding and subsequent CMV-pp65–specific T-cell responses.

This paper describes, for the first time to our knowledge, pp65-specific epitopes presented by HLA-C alleles. A unique epitope, VVCAHELVC (aa's 198-206), was presented by both HLA-Cw*0801 and -Cw*1502. Since there seems to be no information on the peptide binding motif for these HLA alleles, the epitope was determined by gene engineering of pp65, followed by probing with CTL restricted to each HLA-C allele. The results should shed light on the structural basis of understanding the HLA-C molecules. Interestingly, an HLA-Cw*0401–restricted epitope, QYDPVAALF (aa's 341-349),[15] is also dominantly presented by HLA-A*2402 allele. The binding motifs of those 2 alleles are similar to each other (ie, Tyr, Pro, or Phe at the second position and Leu or Phe at the C-terminus in HLA-Cw*0401; and Tyr at the second position, and Ile, Leu, or Phe at the C-terminus in HLA-A*2402). From the point of immunotherapy, this single peptide has great advantages among populations where HLA-A*2402 and -Cw*0401 are common.

There are only a few reports of successful generation of pp65-specific CTL from CMV-seronegative donors. Kleihauer et al[50] showed that cytotoxic T-cell lines were generated from 2 of 11 seronegative donors starting with 3×10^6 PBMCs on stimulation with pp65 peptide–pulsed monocyte-derived dendritic cells. In our previous study, we failed to generate pp65-specific CTL lines from seronegative donors using pp65-transduced CD40-B cells as APCs, but in this study CTL lines that could lyse LCLs expressing endogenously processed peptides from the transduced pp65 gene were generated in 2 of 4 cases (N02 and N03) by using CD40-B cells pulsed with a mixture of the peptides as APCs. However CMV-infected fibroblasts were recognized weakly only by the CTL line from N02. Again, the better antigen processing by LCLs than fibroblasts might contribute to the better recognition by the CTL lines. Such CTLs may be able to only lyse CMV-infected hematopoietic cells in vivo. It is noted in this regard that CMV is reported to infect not only stroma and epithelial cells but also hematopoietic cells including $CD34^+$ stem cells, monocytes, and dendritic cells.[51-55] In the other 2 cases, the CTL lines could recognize only peptide-pulsed LCLs. This observation seems to reflect the results of peptide titration experiments by ELISPOT assay; the peptide concentration to yield half-maximal spots was found to be 10- to 20-fold lower in N02 and N03 lines compared with N06 and N08 lines, suggesting the lower avidity of the lines generated from N06 and N08. To overcome this problem, initial stimulation with peptides at a lower concentration may induce higher avidity CTLs.[56] Alternatively, more T-cell input at the time of initial stimulation may be needed to induce CTL lines from rare precursor T cells with higher affinity T-cell receptor since estimated precursor frequency of naive T cells against a single epitope is very low (for example, one in 5×10^6 of $CD8^+$ cells for lymphocytic choriomeningitis virus in mice[57]). Further efforts are now underway to establish better CTL induction conditions from seronegative donors.

In summary, we here identified 14 novel CTL epitopes derived from CMV-pp65 antigen restricted by HLA-A, -B, and -C alleles. These should be useful for immunologic monitoring of individuals expressing these HLA class I alleles and also for generation of pp65-specific CTLs from not only seropositive but also seronegative donors. In addition, our present approach of epitope identification applying deletion mutants and linear expression fragments, together with the efficient generation of CTL lines, may be applicable for other tumor-specific or viral antigens.

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References

1. Einsele H, Hebart H, Kauffmann-Schneider C, et al. Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. Bone Marrow Transplant. 2000; 25:757-763.
2. Boeckh M, Leisenring W, Riddell SR, et al. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. Blood. 2003;101:407-414.
3. Reusser P, Riddell SR, Meyers JD, Greenberg PD. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. Blood. 1991;78:1373-1380.
4. Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. Blood. 1994;83:1971-1979.
5. Krause H, Hebart H, Jahn G, Muller CA, Einsele H. Screening for CMV-specific T cell proliferation to identify patients at risk of developing late onset CMV disease. Bone Marrow Transplant. 1997;19: 1111-1116.
6. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. N Engl J Med. 1995;333:1038-1044.
7. Reusser P, Attenhofer R, Hebart H, Helg C, Chapuis B, Einsele H. Cytomegalovirus-specific T-cell immunity in recipients of autologous peripheral blood stem cell or bone marrow transplants. Blood. 1997;89:3873-3879.
8. Cwynarski K, Ainsworth J, Cobbold M, et al. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. Blood. 2001;97:1232-1240.
9. Hebart H, Daginik S, Stevanovic S, et al. Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon-gamma-enzyme-linked immunospot assay and flow cytometry in healthy individuals and in patients after allogeneic stem cell transplantation. Blood. 2002;99:3830-3837.
10. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science. 1992;257: 238-241.
11. Einsele H, Roosnek E, Rufer N, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. Blood. 2002;99:3916-3922.
12. Gilbert MJ, Riddell SR, Li CR, Greenberg PD. Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. J Virol. 1993;67:3461-3469.
13. McLaughlin-Taylor E, Pande H, Forman SJ, et al. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. J Med Virol. 1994;43:103-110.
14. Wills MR, Carmichael AJ, Mynard K, et al. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. J Virol. 1996;70: 7569-7579.
15. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood. 2001;98:1872-1881.
16. Gillespie GM, Wills MR, Appay V, et al. Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. J Virol. 2000;74:8140-8150.

17. Kondo E, Topp MS, Kiem HP, et al. Efficient generation of antigen-specific cytotoxic T cells using retrovirally transduced CD40-activated B cells. J Immunol. 2002;169:2164-2171.
18. Akatsuka Y, Goldberg TA, Kondo E, et al. Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines. Tissue Antigens. 2002;59:502-511.
19. Schultze JL, Michalak S, Seamon MJ, et al. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. J Clin Invest. 1997;100: 2757-2765.
20. Schultze JL, Cardoso AA, Freeman GJ, et al. Follicular lymphomas can be induced to present alloantigen efficiently: a conceptual model to improve their tumor immunogenicity. Proc Natl Acad Sci U S A. 1995;92:8200-8204.
21. Horn PA, Topp MS, Morris JC, Riddell SR, Kiem HP. Highly efficient gene transfer into baboon marrow repopulating cells using GALV-pseudotype oncoretroviral vectors produced by human packaging cells. Blood. 2002;100:3960-3967.
22. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol. 1994;152:163-175.
23. Taylor R. BioInformatics and Molecular Analysis Section (BIMAS) HLA peptide binding predictions. Available at: http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html. Accessed October 10, 2002.
24. Hildebrand WH, Muthuraman S. HLA Epitope binding prediction in HLA Ligand/Motif Database. Available at: http://hlaligand.ouhsc.edu/prediction.htm. Accessed December 15, 2002.
25. Geginat G, Schenk S, Skoberne M, Goebel W, Hof H. A novel approach of direct ex vivo epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from Listeria monocytogenes. J Immunol. 2001;166:1877-1884.
26. Gavin MA, Gilbert MJ, Riddell SR, Greenberg PD, Bevan MJ. Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. J Immunol. 1993; 151:3971-3980.
27. Kern F, Bunde T, Faulhaber N, et al. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. J Infect Dis. 2002;185: 1709-1716.
28. Kikuchi A, Sakaguchi T, Miwa K, et al. Binding of nonamer peptides to three HLA-B51 molecules which differ by a single amino acid substitution in the A-pocket. Immunogenetics. 1996;43:268-276.
29. Retiere C, Prod'homme V, Imbert-Marcille BM, Bonneville M, Vie H, Hallet MM. Generation of cytomegalovirus-specific human T-lymphocyte clones by using autologous B-lymphoblastoid cells with stable expression of pp65 or IE1 proteins: a tool to study the fine specificity of the antiviral response. J Virol. 2000;74:3948-3952.
30. Longmate J, York J, La Rosa C, et al. Population coverage by HLA class-I restricted cytotoxic T-lymphocyte epitopes. Immunogenetics. 2001;52: 165-173.
31. Solache A, Morgan CL, Dodi AI, et al. Identification of three HLA-A*0201-restricted cytotoxic T cell epitopes in the cytomegalovirus protein pp65 that are conserved between eight strains of the virus. J Immunol. 1999;163:5512-5518.
32. Diamond DJ, York J, Sun JY, Wright CL, Forman SJ. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. Blood. 1997;90:1751-1767.
33. Masuoka M, Yoshimuta T, Hamada M, et al. Identification of the HLA-A24 peptide epitope within cytomegalovirus protein pp65 recognized by CMV-specific cytotoxic T lymphocytes. Viral Immunol. 2001;14:369-377.
34. Weekes MP, Wills MR, Mynard K, Carmichael AJ, Sissons JG. The memory cytotoxic T-lymphocyte (CTL) response to human cytomegalovirus infection contains individual peptide-specific CTL clones that have undergone extensive expansion in vivo. J Virol. 1999;73:2099-2108.
35. Wills MR, Okecha G, Weekes MP, Gandhi MK, Sissons PJ, Carmichael AJ. Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. J Immunol. 2002;168:5455-5464.
36. York IA, Chang SC, Saric T, et al. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. Nat Immunol. 2002;3:1177-1184.
37. Saric T, Chang SC, Hattori A, et al. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. Nat Immunol. 2002;3:1169-1176.
38. Serwold T, Gonzalez F, Kim J, Jacob R, Shastri N. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. Nature. 2002;419:480-483.
39. Beninga J, Rock KL, Goldberg AL. Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. J Biol Chem. 1998;273:18734-18742.
40. Craiu A, Akopian T, Goldberg A, Rock KL. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. Proc Natl Acad Sci U S A. 1997; 94:10850-10855.
41. Saric T, Beninga J, Graef CI, Akopian TN, Rock KL, Goldberg AL. Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase. J Biol Chem. 2001;276:36474-36481.
42. Nussbaum AK, Dick TP, Keilholz W, et al. Cleavage motifs of the yeast 20S proteasome beta subunits deduced from digests of enolase 1. Proc Natl Acad Sci U S A. 1998;95:12504-12509.
43. Schultz ES, Chapiro J, Lurquin C, et al. The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome. J Exp Med. 2002;195:391-399.
44. Sudo T, Kamikawaji N, Kimura A, et al. Differences in MHC class I self peptide repertoires among HLA-A2 subtypes. J Immunol. 1995;155: 4749-4756.
45. Yao Z, Keller E, Scholz S, McNicholas A, Volgger A, Albert ED. Identification of two major HLA-B44 subtypes and a novel B44 sequence (B*4404): oligotyping and solid phase sequencing of polymerase chain reaction products. Hum Immunol. 1995;42:54-60.
46. Petersdorf EW, Setoda T, Smith AG, Hansen JA. Analysis of HLA-B*44 alleles encoded on extended HLA haplotypes by direct automated sequencing. Tissue Antigens. 1994;44:211-216.
47. Tanaka H, Akaza T, Juji T. Report of the Japanese Central Bone Marrow Data Center. Clin Transpl. 1996:139-144.
48. Tokunaga K, Ishikawa Y, Ogawa A, et al. Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. Immunogenetics. 1997; 46:199-205.
49. Saito S, Ota S, Yamada E, Inoko H, Ota M. Allele frequencies and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population. Tissue Antigens. 2000;56:522-529.
50. Kleihauer A, Grigoleit U, Hebart H, et al. Ex vivo generation of human cytomegalovirus-specific cytotoxic T cells by peptide-pulsed dendritic cells. Br J Haematol. 2001;113:231-239.
51. Stanier P, Taylor DL, Kitchen AD, Wales N, Tryhorn Y, Tyms AS. Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. BMJ. 1989;299:897-898.
52. Taylor-Wiedeman J, Sissons JG, Borysiewicz LK, Sinclair JH. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. J Gen Virol. 1991;72: 2059-2064.
53. Bevan IS, Walker MR, Daw RA. Detection of human cytomegalovirus DNA in peripheral blood leukocytes by the polymerase chain reaction. Transfusion. 1993;33:783-784.
54. Mendelson M, Monard S, Sissons P, Sinclair J. Detection of endogenous human cytomegalovirus in CD34+ bone marrow progenitors. J Gen Virol. 1996;77:3099-3102.
55. Taylor-Wiedeman J, Sissons P, Sinclair J. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. J Virol. 1994;68:1597-1604.
56. Alexander-Miller MA, Leggatt GR, Berzofsky JA. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc Natl Acad Sci U S A. 1996;93: 4102-4107.
57. Blattman JN, Antia R, Sourdive DJ, et al. Estimating the precursor frequency of naïve antigen-specific CD8 T cells. J Exp Med. 2002;195:657-664.

MAJOR ARTICLE

Differences between T Cell–Type and Natural Killer Cell–Type Chronic Active Epstein-Barr Virus Infection

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Infections of T cells and natural killer (NK) cells play a central role in the pathogenesis of chronic active Epstein-Barr virus (CAEBV) infection. To characterize the virologic and cytokine profiles of T cell–type and NK cell–type infection, 39 patients with CAEBV infection were analyzed. Patients with T cell–type infection had higher titers of immunoglobulin G against early and late EBV antigens, suggesting lytic cycle infection. However, the pattern of EBV gene expression was latency type II; *BZLF1*, which is a hallmark of lytic cycle infection, could not be detected in any patients, regardless of infection type. Patients with CAEBV infection had high concentrations of proinflammatory, T helper cell type 1, and anti-inflammatory cytokines. The cytokine profile in patients with NK cell–type infection was similar to that in patients with T cell–type infection, but the concentration of IL-13 was high in patients with NK cell–type infection. These findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

Epstein-Barr virus (EBV) is a ubiquitous virus that infects most individuals by early adulthood. Primary EBV infection is usually asymptomatic but sometimes progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [1, 2]. EBV infection can be chronic in apparently immunocompetent hosts [3, 4]. Chronic active EBV (CAEBV) infection is characterized by chronic or recurrent mononucleosis-like infectious symptoms, such as fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, and hypersensitivity to mosquito bites [3–5]. Patients with CAEBV infection have an unusual pattern of EBV-related antibodies and high viral loads in peripheral blood [3–7]. CAEBV infection is associated with high mortality and morbidity.

Recent studies have indicated that the clonal expansion of EBV-infected T cells and natural killer (NK) cells plays a central role in the pathogenesis of CAEBV infection [5, 8–11]. In a previous study, we found that patients with CAEBV infection fall into 2 clinically distinct groups, on the basis of whether the infected cells in their peripheral blood were mainly T cells or NK cells [5]. T cell–type infection is characterized by fever and high titers of EBV-related antibodies, whereas NK cell–type infection is characterized by hypersensitivity to mosquito bites and high titers of IgE. Furthermore, patients with T cell–type infection have significantly poorer outcomes [5, 12]. EBV-infected T cells might become activated and release inflammatory cytokines, such as interferon (IFN)–γ or tumor necrosis factor (TNF)–α, resulting in severe inflammation and fever [13, 14]. However, it is still not known why these 2 manifestations of the disease have different symptoms and courses.

The purpose of the present study was to gain a better

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understanding the pathogenesis of CAEBV infection by characterizing the virologic profiles of T cell–type and NK cell–type infection and identifying the differences between them. We analyzed 20 patients with T cell–type infection and 19 patients with NK cell–type infection. The 2 types of CAEBV infection were compared in both virologic and immunologic analyses, including an analysis of cytokine profiles.

PATIENTS, MATERIALS, AND METHODS

Patients. Thirty-nine patients with CAEBV infection were enrolled in the present study. Informed consent was obtained from all of the patients or their parents. Of the 39 patients, 24 had been included in our previous study of the clinical characteristics of CAEBV infection [5]. All of the patients met the following diagnostic criteria [5]: (1) they had EBV-related illness or symptoms for >6 months (including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, or hypersensitivity to mosquito bites); (2) they had increased quantities of EBV in either affected tissue or peripheral blood (the quantity of EBV was defined as increased when ≥1 of the following criteria was met: EBV DNA was detected in either affected tissue or peripheral blood by Southern-blot hybridization, EBV-encoded small RNA 1 [EBER-1]–positive cells were detected in either affected tissue or peripheral blood [15], or $>10^{2.5}$ copies/μg DNA was detected in peripheral-blood mononuclear cells [PBMCs] [15]); and (3) they did not manifest evidence of any previous immunologic abnormalities or of any other recent infection that might explain the condition (all of the patients examined were negative for antibody against HIV).

Cells. For the EBV gene-expression experiment, a lymphoblastoid cell line (LCL) that was transformed with B95-8 virus was used as a positive control, and BJAB, an EBV-negative B cell line, was used as a negative control.

Samples. Samples were collected at the time of diagnosis or before the administration of immunosuppressive therapy, such as chemotherapy or hematopoietic stem-cell transplantation. EDTA-treated peripheral blood collected from patients was centrifuged and separated into plasma and cell fractions; the cell fractions were separated into PBMCs on Ficoll-Paque density gradients (Pharmacia Biotech).

Titers of anti–EBV nuclear antigen (EBNA) antibodies were measured by use of an anticomplement immunofluorescence method. Titers of anti–viral capsid antigen (VCA) and anti–early antigen–diffuse restricted (EA-DR) IgG were measured by use of an immunofluorescence method. These titers were measured in all patients.

DNA was extracted from either 2×10^6 PBMCs or 200 μL of plasma by use of a QIAamp blood kit (Qiagen). To differentiate between free EBV DNA molecules and virions or nucleocapsids, selected plasma samples were examined for EBV content by digestion with deoxyribonuclease (RQ1 RNase-free DNase; Promega) for 30 min at 37°C [16, 17]. As controls, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatants of LCL and BJAB cultures were used. LCL was treated with *n*-butyrate and phorbol 12-myristate 13-asetate, to induce lytic cycle infection, and the culture supernatants were used as a control that contained enveloped virions.

For patients from whom fresh samples were available ($n = 19$), RNA was extracted from 2×10^6 PBMCs by use of an RNA extraction kit (QIAamp RNA Blood Mini Kit; Qiagen). cDNA was synthesized by use of Superscript reverse transcriptase II (Gibco Life Technology), as described elsewhere [18].

Determination of EBV-infected cells. To determine which cells were infected with EBV, PBMCs were fractionated into $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, and $CD56^+$ cells by use of an immunobead method (DynaBeads; Dynal A/S) [5]. For some patients with T cell–type infection from whom a sufficient quantity of PBMCs was obtained, fractionation into $CD4^+$ and $CD8^+$ cells was also performed. The fractionated cells were analyzed by either quantitative polymerase chain reaction (PCR) or in situ hybridization with the EBER-1 probe [15]. Patients were defined as having T cell–type infection when $CD3^+$ cells either were the main cells giving a positive hybridization signal with EBER-1 or contained more EBV DNA than other cells in the blood sample [5]. Patients were defined as having NK cell–type infection when $CD16^+$ or $CD56^+$ cells were the main ones containing EBV [5]. Repeated tests were performed for some patients, and similar results were obtained; in the present study, representative results are shown.

For some patients, infected cells were identified in biopsied or autopsied tissues, such as lymph nodes, liver, and spleen. Double labeling by use of in situ hybridization with the EBER-1 probe and immunostaining with surface marker antibody were performed as described elsewhere [19].

Clonality of EBV. The clonality of EBV was determined by Southern blotting with a terminal-repeat probe, as described elsewhere [20, 21]. PBMC-extracted genomic DNA was digested with *Bam*HI, subjected to gel electrophoresis, transferred to a nylon membrane, hybridized with a ^{32}P-labeled *Xho*I fragment from the terminal region of EBV, and visualized by autoradiography.

Quantification of EBV DNA. Both PBMCs and plasma from all of the patients were assayed for viral load. A real-time quantitative PCR assay with a fluorogenic probe was performed as described elsewhere [15, 22]. As a positive standard for quantification, pGEM-BALF5 was used [15]. The quantity of EBV DNA was calculated as the number of copies per microgram or per milliliter of plasma.

Amplification of EBV-specific RNA transcripts by PCR. To detect latent gene expression (*EBNA1*, *EBNA2*, and *LMP1* [latent member protein]), nested PCR was performed essentially as described elsewhere [23]. For *EBNA1*, 3 different primer

sets were used, to determine promoter usage. Approximately 50 ng of total RNA (converted to cDNA) was used as template. The amplified products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light. To detect *BZLF1*, which encodes a transactivator protein and is expressed in lytic cycle infection, nested PCR was performed with the primers described by Prang et al. [24]. To detect the gene for gp350/220, which is also expressed in lytic cycle infection, nested PCR was performed with the outer primers described by Kelleher et al. [25] and newly selected inner primers (5′-CATCACCGGTGACACCAAGT-3′ and 5′-TGCTGGCGAACTGGTGGACA-3′). To detect a housekeeping gene, the human glyceraldehyde-3-phosphate dehydrogenase gene was amplified by single PCR; the sequences of the primer pair were 5′-GAAGGTGAAGGTCGGAGT-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. All of the primer pairs used in the present study were designed to span introns, to avoid amplification of genomic DNA.

Quantification of cytokine gene transcription by use of a real-time PCR assay. Cytokine gene transcription was quantified by use of a real-time PCR assay, as described elsewhere [18]. Transcription of the genes for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p35, IL15, IFN-γ, and TNF-α was assessed by use of a TaqMan Cytokine Gene Expression Plate I (Applied Biosystems). A predeveloped primer/probe (Assays-On-Demand Gene Expression Products; Applied Biosystems) was used to measure IL-13 separately. Each well contained TaqMan primers and probes for assaying human cytokine mRNA and rRNA as an endogenous control. Approximately 25 ng of total RNA (converted to cDNA) was used to quantify the expression of each cytokine gene. All of the assays were conducted in duplicate. To calculate the relative expression of a cytokine gene in cells, the value for the expression of a cytokine gene was divided by that for the internal control rRNA, by use of the comparative threshold cycle method described by the manufacturer (P/N 4306744; Applied Biosystems) [18, 26].

Determination of plasma cytokine concentration by use of ELISA. Plasma cytokine concentrations were determined in all of the patients. Plasma concentrations of IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ were determined by use of sandwich-type ELISA kits (R&D Systems); these assays were conducted in accordance with the manufacturer's instructions [18, 27]. Sample values were determined from a standard curve. The minimum detectable concentrations of IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ were 1, 10, 3.9, 5, 32, and 8 pg/mL, respectively.

Statistical analyses. Statistical analyses were conducted by use of StatView software (version 5.0; SAS Institute). Fisher's exact test or the χ^2 test was used to compare differences in clinical measurements. The Mann-Whitney *U* test was used for statistical comparisons of laboratory data, viral load, cytokine gene expression, and plasma cytokine concentrations. $P < .05$ was considered to be statistically significant.

RESULTS

Characteristics of T cell–type and NK cell–type CAEBV infection. Of the 39 patients with CAEBV infection, 20 had infections that were defined as T cell type, and 19 had infections that were defined as NK cell type. The differences in clinical and laboratory measurements between the 2 types of CAEBV infections are shown in table 1. The patients with T cell–type infection had a poorer prognosis (death rate, 60%); 12 of them died, with the causes of death including hepatic failure ($n = 4$), malignant lymphoma ($n = 2$), and cardiac complications ($n = 2$) (for death by other causes, $n = 4$). Three patients received hematopoietic stem-cell transplantation; 2 are still alive, and 1 relapsed and died shortly afterward. T cell–type infection was strongly characterized by high fever and anemia; other characteristics of T cell–type infection were hepatomegaly and lymphadenopathy, although the statistical significance was marginal. In contrast, the patients with NK cell–type infection had a better prognosis (death rate, 26%); 5 of them died, with the causes of death including complications related to hematopoietic stem-cell transplantation ($n = 3$), sepsis ($n = 1$), and interstitial pneumonia ($n = 1$). Seven patients received hematopoietic stem-cell transplantation; 4 are still alive, and 3 died of transplantation-related complications. NK cell–type infection was characterized by large granular lymphocytosis, hypersensitivity to mosquito bites, and a high IgE concentration. These observed differences between the 2 types of CAEBV infection are in agreement with the results of our previous study [5].

Determination of EBV-infected cells. For 28 patients, fractionation of PBMCs followed by quantitative PCR was used to determine the cell types that were infected (table 2). Using this method, we determined that 15 patients had T cell–type infection, because $CD3^+$ cells contained more EBV DNA than did the other cell populations. In 3 of these patients, mainly $CD4^+$ T cells were infected; in 2 of these patients, mainly $CD8^+$ T cells were infected. In contrast, mainly $CD16^+$ cells (not $CD3^+$ cells) were infected in 13 patients, who were therefore determined to have NK cell–type infection (table 2). In some patients, both $CD16^+$ and $CD19^+$ cells contained more EBV DNA than did unfractionated cells, suggesting that both NK and B cells were infected with EBV.

For the remaining 11 patients, in situ hybridization was used to determine which cell types were infected. For 3 patients with NK cell–type infection, infected cells were identified by fractionating PBMCs and then performing EBER-1 in situ hybridization. EBER-1 was detected in 15%, 25%, and 60% of the $CD56^+$ cells in these 3 patients, indicating that most of the infected cells were NK cells (as described elsewhere [28]). For the other 8 patients, tissue samples were used to identify in-

Table 1. Differences in clinical and laboratory measurements between T cell–type and NK cell–type chronic active Epstein-Barr virus (EBV) infection.

	T cell–type infection (n = 20)	NK cell–type infection (n = 19)	P
Sex, M:F, no.	10:10	11:8	.43
Age at onset, mean ± SD, years	12.6 ± 9.5	8.0 ± 4.9	.19
Death rate, %	**60**	26	**.03**
Time to death from onset, mean ± SD, years	2.9 ± 1.3	5.4 ± 3.8	.27
Symptoms			
Fever >1 day/week, %	**81**	38	**.01**
Hepatomegaly, %	79	53	.09
Lymphadenopathy, %	58	32	.096
Large granular lymphocytosis, %	11	**58**	**.003**
Hypersensitivity to mosquito bites, %	11	**68**	**.0002**
Laboratory data			
WBC count, mean ± SD, cells/μL	5200 ± 5500	6100 ± 3200	.11
Hemoglobin concentration, mean ± SD, g/dL	**10.7 ± 1.2**	12.1 ± 1.9	**.03**
Platelet count, mean ± SD, 10^4 cells/μL	21.4 ± 7.6	19.2 ± 8.5	.52
IgG level, mean ± SD, mg/dL	1990 ± 1440	1590 ± 450	.36
IgE level, mean ± SD, mg/dL	190 ± 220	**5650 ± 6470**	**.002**
EBV-related antibody			
Anti-VCA IgG level, GMT	**2010**	310	**.001**
Anti–EA-DR IgG level, GMT	**610**	70	**.007**
Anti-EBNA level, GMT	27	45	.12
Viral load			
PBMCs, mean ± SD, copies/μg	$10^{3.9 \pm 0.7}$	$10^{4.4 \pm 0.6}$	**.03**
Plasma, mean ± SD, copies/mL	$10^{2.8 \pm 1.1}$	$10^{2.5 \pm 2.0}$	.87

NOTE. Values in boldface indicate statistically significant results. Either Fisher's exact test or the χ^2 test was used to compare symptoms; the Mann-Whitney U test was used to compare laboratory data and viral load. EA-DR, early antigen–diffuse restricted; EBNA, EBV nuclear antigens; F, female; GMT, geometric mean titer; M, male; PBMCs, peripheral-blood mononuclear cells; VCA, viral capsid antigen; WBC, white blood cell.

fected cells. Double labeling with EBER-1 and surface markers showed that, in 5 patients, most EBER-1–positive cells were $CD3^+$, indicating that they had T cell–type infection. In 3 patients, most EBER-1–positive cells were $CD16^+$ (not $CD3^+$), indicating that they had NK cell–type infection. Some of these results have been described elsewhere [29].

Virologic analyses. EBV-related antibody titers were compared between the 2 types of CAEBV infection. The patients with T cell–type infection had significantly higher titers of anti-VCA and anti–EA-DR IgG (table 1). The anti-EBNA antibody titers were comparable between the 2 types of CAEBV infection. The higher titers of antibody against early and late EBV antigens (i.e., anti–EA-DR IgG and anti-VCA IgG) but not against latent antigen (i.e., anti-EBNA antibody) suggested the possibility of lytic cycle infection in T cells.

To examine whether lytic cycle infection existed in EBV-infected T or NK cells, for 19 patients from whom fresh samples were available (for T cell–type infection, n = 11; for NK cell–type infection, n = 8), reverse-transcription PCR was used to examine EBV gene expression in PBMCs. *BZLF1*, which encodes a transactivator protein and is a hallmark of lytic cycle infection [1], was not detected in any of the 19 patients (table 3); another lytic gene, for gp350/220, was also not detected. *EBNA1*, *LMP1*, and *LMP2A* were detected in PBMCs from nearly one-half of the 19 patients, indicating that they had the latency type II pattern. The Qp promoter, but not the Cp/Wp promoter, was used for *EBNA1* transcription. Representative results are shown in figure 1. Because it was possible that lytic cycle infection was present at sites other than PBMCs, EBV gene expression was examined in autopsy or biopsy samples from 2 patients with T cell–type infection. The *BZLF1* and gp350/220 genes were not detected in the livers, lymph nodes, or spleens of these patients (data not shown). The pattern of EBV gene expression in these tissue samples was also latency type II.

Next, the viral load in peripheral blood was investigated by use of real-time PCR. The viral load in PBMCs was higher in patients with NK cell–type infection (table 1). Interestingly, the viral load in plasma was similar between the patients with each type. To examine the viral load in plasma, plasma samples from selected patients were digested with deoxyribonuclease before extraction of DNA. As a preliminary experiment, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatant of an LCL culture containing enveloped virions were tested. The control plasmid DNA was sensitive to deoxy-

Table 2. Determination of Epstein-Barr virus (EBV)–infected cells in peripheral-blood mononuclear cells.

Type of chronic active EBV infection, patient	EBV DNA, copies/μg						Mainly infected cells	Clonality of cells
	Fractionated cells					Unfractionated cells		
	CD3+	CD4+	CD8+	CD16+	CD19+			
T cell								
1	**220,000**	760	**230,000**	100,000	760	210,000	T (CD8+)	Monoclonal
2	**90,000**	ND	ND	17,000	15,000	24,000	T	ND
3	**43,000**	**21,000**	1500	**12,500**	2400	8600	T (CD4+)	Monoclonal
4	**23,000**	ND	ND	1100	1600	14,000	T	Monoclonal
5	**18,000**	ND	ND	6200	0	7300	T	ND
6	**17,000**	ND	ND	4300	5200	16,000	T	ND
7	**12,000**	ND	ND	5700	2700	11,000	T	ND
8[a]	12,000	4500	2500	6500	5000	51,000	T	Monoclonal
9	**11,600**	ND	ND	2700	2000	6100	T	Monoclonal
10	10,000	27,000	4900	1900	1200	42,000	T (CD4+)	Oligoclonal
11	**7600**	ND	ND	80	260	6600	T	ND
12	**6600**	ND	ND	0	900	3900	T	Polyclonal
13	**3600**	ND	ND	840	950	3200	T	ND
14	**400**	**720**	90	250	20	340	T (CD4+)	Monoclonal
15	160	10	**220**	0	20	200	T (CD8+)	Polyclonal
NK cell								
1	120,000	ND	ND	**400,000**	**470,000**	210,000	NK, B	Monoclonal
2	21,000	ND	ND	**170,000**	3200	110,000	NK	Monoclonal
3	7400	ND	ND	**89,000**	17,000	78,000	NK	ND
4	11,000	ND	ND	**86,000**	18,000	75,000	NK	Monoclonal
5	10,000	ND	ND	**54,000**	23,000	36,000	NK	Monoclonal
6	3300	ND	ND	**35,000**	1900	20,000	NK	Monoclonal
7	7600	ND	ND	25,000	1700	28,000	NK	ND
8[a]	1800	ND	ND	**16,000**	**9200**	7000	NK, B	Oligoclonal
9	300	ND	ND	**15,000**	0	2000	NK	ND
10	200	ND	ND	15,000	1800	31,000	NK	ND
11	1600	ND	ND	4500	570	8100	NK	Monoclonal
12	50	ND	ND	**4300**	110	1600	NK	Oligoclonal
13	0	ND	ND	**2700**	**1300**	820	NK, B	ND

NOTE. Values in boldface indicate that EBV DNA was concentrated after fractionation. ND, not done.

[a] Infected cells were confirmed by double labeling of tissue samples.

ribonuclease (percentage of reduction of EBV DNA after digestion, 99.9%), but the LCL supernatant was resistant to the enzyme (percentage of reduction of EBV DNA after digestion, 55.7%). Five plasma samples from each group of patients were tested. After deoxyribonuclease digestion, the percentages of reduction of EBV DNA were 100%, 92.8%, 96.8%, 97.3%, and 100% in the samples from the patients with T cell–type infection and 99.8%, 98.3%, 100%, 100%, and 100% in the samples from the patients with NK cell–type infection. Thus, plasma from both groups of patients was sensitive to deoxyribonuclease, indicating that most of the EBV DNA in plasma, rather than consisting of enveloped virions, consisted of free EBV DNA molecules, which were likely derived from dead or damaged cells.

The clonality of EBV was analyzed by use of Southern blotting. The majority of both T cell–type and NK cell–type infections were monoclonal (table 2). There was a trend in that those patients with polyclonal or oligoclonal proliferation had lower viral loads, although there was no difference between the 2 types of CAEBV infection.

Cytokine profiles. Differences in the symptoms or immunologic responses between the 2 types of CAEBV infection might be due to the differences in the cytokine production profiles of either EBV-infected cells themselves or inflammatory cells. The plasma concentrations of cytokines (IL-1β, IL-4, IL-10, IL-12, IL-13, and IFN-γ) were estimated and compared between the 2 types of CAEBV infection. IL-1β, IL-10, and IFN-γ, none of which are detected in healthy individuals, were detected in plasma from many patients with CAEBV infection (figure 2); there were no significant differences between the T cell–type and NK cell–type infections. IL-13, which also is not detected in healthy individuals, was frequently detected in the patients with NK cell–type infection, and the concentration was higher than that in the patients with T cell–type infection. The patients who developed

Table 3. Summary of Epstein-Barr virus (EBV) gene expression in peripheral-blood mononuclear cells.

Type of chronic active EBV infection	*EBNA1*[a]			*EBNA2*	*LMP1*	*LMP2A*	*BZLF1*	gp350/220	*GAPDH*
	Common	Qp	Cp/Wp						
T cell (n = 11)	4	1	0	0	4	3	0	0	11
NK cell (n = 8)	4	3	0	0	4	4	0	0	8

NOTE. Data are no. of samples positive for the indicated gene. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

[a] For *EBNA1*, 3 different primer sets were used to determine promoter usage.

hemophagocytic syndrome had high concentrations of inflammatory cytokines, such as IL-1β and IFN-γ.

Next, the transcription of cytokine genes in PBMCs was investigated in the 19 patients from whom fresh samples were available. Assays were performed for IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p35, IL-13, IL-15, IFN-γ, and TNF-α. Transcription of the genes for IL-1α, IL-1β, IL-10, IL-13, IL-12p35, IL-15, TNF-α, and IFN-γ was high in the patients with CAEBV infection, whereas transcription of the genes for IL-4 and IL-5 was undetectable in most patients. There were no statistical differences in the transcription of these cytokine genes between the 2 types of CAEBV infection, including for IL-13.

DISCUSSION

It has been shown that infections of T and NK cells play a central role in the pathogenesis of CAEBV infection. A recent national survey in Japan showed that the majority of patients with CAEBV infection had infections that belonged to either the T cell type or the NK cell type; only 2 of 82 patients had an infection that mainly involved B cells [12]. EBV-infected T cell or NK cell lines have been established from patients with CAEBV infection [20, 21]. In the present study, we identified 20 patients with CAEBV infection in whom mainly T cells were infected and 19 patients with CAEBV infection in whom mainly NK cells were infected. Recently, a small-scale study reported that, during acute EBV-associated hemophagocytic lymphohistiocytosis, EBV infection was predominant in $CD8^+$ cells, but that, in patients with CAEBV infection, EBV infection was predominant in non-$CD8^+$ cells [30]. However, the results of the present study indicated that, in some of our patients with CAEBV infection, EBV infection was predominant in $CD8^+$ T cells.

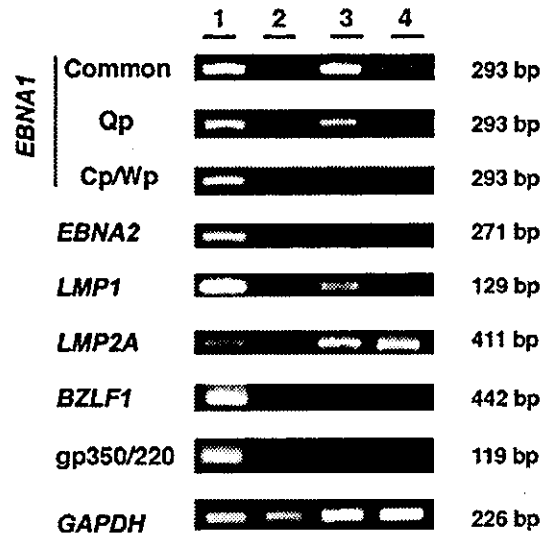

Figure 1. Expression of Epstein-Barr virus (EBV) genes in peripheral blood. *Lane 1:* EBV-positive lymphoblastoid cell line. *Lane 2:* BJAB, an EBV-negative B cell line. *Lane 3:* Peripheral blood from a patient with T cell–type chronic active EBV (CAEBV) infection. *Lane 4:* Peripheral blood from a patient with NK cell–type CAEBV infection. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

To determine which cells were infected with EBV, we used immunomagnetic cell isolation to fractionate PBMCs, followed by quantitative PCR. This method is very rapid and convenient, but its disadvantage is the relatively poor purity of the selected cells. EBV-infected cells can contaminate uninfected cell fractions. Furthermore, the real-time PCR assay is so sensitive that the contaminating EBV genome can be detected; therefore, this method can determine only the cell population that is mainly infected. The low levels of EBV DNA seen in other cell populations do not always mean that they are infected with EBV; however, the results of the present study showed that, in some of our patients, both NK cells and B cells were infected with EBV (table 2). Perhaps >1 cell lineage harbors EBV in some patients with CAEBV infection. Electric cell sorting followed by EBER-1 in situ hybridization is a more accurate method for determining EBV-infected cell lineages. Using this method, Kasahara et al. found that, in some patients with CAEBV infection, different cell lineages were infected [30].

Originally, CAEBV infection was characterized by an unusual pattern of EBV-related antibodies, such as high titers of anti-VCA and anti–EA-DR IgG or the absence of anti-EBNA antibody [3, 4, 6, 31], although recent observations indicate that high titers of these EBV-related antibodies are not necessary for CAEBV infection to be diagnosed [5]. In patients with CAEBV infection—and especially in patients with T cell–type infection who have high titers of EBV-related antibody—high