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Research Article

High frequency of human cytomegalovirus (HCMV)-specific CD8+ T cells detected in a healthy CMV-seropositive donor

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Abstract. Human cytomegalovirus (HCMV) persists after infection but is controlled by cellular immune responses, particularly by CD8+ T cells. If infected individuals are immunosuppressed, HCMV can be reactivated. Upon testing the blood of healthy donors with human lymphocyte antigen tetramers, we found one individual with about 50% of his CD8+ T cells being specific for the immunodominant pp65 epitope NLVPMVATV. Over a period of 2 years the high level of HCMV-specific T cells was maintained, and no HCMV DNA could be detected. At one timepoint, however, HCMV-specific DNA was detected, while 65% of CD8+ T cells were specific for HCMV. When virus was detectable, a lower percentage of HCMV-specific CD8+ T cells showed interferon γ (IFN-γ) production after peptide stimulation in vitro. These data suggest that HCMV reactivation may also occur in immunocompetent persons, accompanied by the presence of HCMV-specific CD8+ T cells which are not producing IFNγ, and therefore potentially anergic or in vivo exhausted.

Key words. Human cytomegalovirus (HCMV); cytotoxic T cells (CTL); T cell frequency; T cell phenotype; epitope; HLA-A *0201.

Introduction

Human cytomegalovirus (HCMV) is a DNA virus from the β herpes virus group. After infection, this virus persists lifelong in immune privileged tissues without causing any clinical problems. Only under immunosuppression, occurring for example after bone marrow transplantation, during human immunodeficiency virus (HIV) infection, or leukemia, are infected individuals at risk to experience HCMV reactivation. Especially in recipients of bone marrow transplantation, HCMV reactivation can cause pneumonia, multiorgan damage, and lead to death [1–3].

Besides natural killer (NK) cells, cytotoxic CD8+ T lymphocytes (CTLs) play a major role in defense against HCMV [4, 5]. In human lymphocyte antigen (HLA)-A2+ individuals, CD8+ T cells against HCMV are mainly directed against the immunodominant CTL epitope NLVPMVATV derived from the pp65 protein [6] and can be quantitated with tetramers in the blood of healthy donors after HCMV infection at a frequency between 0.1 and 5% [7]. It is well known that patients who receive a bone marrow transplant and are suffering from HCMV reactivation have a lack of functional HCMV-specific CTLs [8–10]. While screening a large panel of HLA-A2-expressing,

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HCMV-positive healthy donors, we found one interesting case of a high frequency of HCMV-specific $CD8^+$ T cells, as described below.

Material and methods

Case report

Eight blood samples (Nov 98, Apr 99, Oct 99, Mar 00, Jun 00, Dec 00, Feb 01, May 01) of a 50-year-old male HCMV-antibody positive, HLA-A2 expressing donor with no history of illness or immune suppression were screened for NLVPMVATV-specific $CD8^+$ T cells and for CMV-specific DNA. The donor had not received a bone marrow transplant, was HIV antibody negative, had no diabetes mellitus, no bone marrow suppression, and no malignant cells were found in his blood. Control individuals were HLA-A2 positive, healthy blood donors. Blood of one 49-year-old healthy donor was taken for phenotyping.

Functional analysis and FACS staining

Chromium release assay, intracellular IFN-γ staining was performed as described [11, 12]. For intracellular IFN-γ staining, cells were stimulated with NLVPMVATV; the nonimmunogenic HLA-A2 ligand YLLPAIVHI (from autologous RNA helicase, $p68_{168-176}$) served as negative control. Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Weiterstadt, Germany) following the Fmoc/tBu strategy [11]. Their identity and purity were confirmed by matrix-assisted laser desorption ionization – time-of-flight mass spectrometry (G2025A, Hewlett-Packard, Waldbronn, Germany) and high-pressure liquid chromatography (Varian star, Zinsser, München, Germany). After 12 h of peptide incubation, cells were stained with CD8 tricolor (1:100, Caltag Laboratories, Burlingame, CA). Afterwards, cells were fixed and permeabilized (Cytofix/Cytoperm kit, Pharmingen, San Diego, CA) for 20 min and stained with phycoerythrin-labelled anti-human IFN-γ antibody (1:100, Pharmingen).

HLA-A*0201 tetramers folded around the peptide NLVPMVATV ($pp65_{495-503}$) were produced as described previously [12]. Cells were incubated at 4 °C with 10 µg/ ml of tetrameric complexes. After 15 min of incubation, CD8 tricolor and one of the fluorescein isothiocyanate-labelled antibodies specific for HLA-DR, CD28, CD44, CD45RO, CD45RA, CD57, CD62L (Immunotech), or CD38 (Pharmingen), respectively, were added. The samples were incubated on ice for a further 25 min. After washing, cells were fixed with PBS containing 2% formaldehyde. Triple-color analysis was performed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany).

CMV detection

DNA extraction and nested polymerase chain reaction (PCR) from plasma and leukocytes was performed as described previously using primer sequences from the fourth exon of the major immediate early gene as well as from the UL97 region [3, 13]. Late HCMV pp67 messenger RNA (mRNA) was analyzed from leukocyte samples using the Boom extraction method and nucleic acid sequence-based amplification (NASBA), and enhanced chemoluminescence for detection (NucliSens CMV pp67, Organon Teknika, Boxtel, the Netherlands).

Results and discussion

Over a period of more than 2 years (Nov 98–May 01), eight different blood samples of a HLA-A2 positive, healthy donor were taken. All blood samples were stained with CD8 antibody and the HCMV-specific NLVPMVATV/HLA-A2 tetramer. The frequency of tetramer-positive $CD8^+$ T cells in all eight samples ranged from 26 to 64% (table 1). This CMV-specific population was poly-

Table 1. Percentage of CMV-specific (CMV TE+) and CMV-negative (CMV TE–) CD8 T cells of PBL (first two lines), percentage of CMV-specific cells of $CD8^+$ T cells (3rd and 4th lines), percentage of marker-expressing cells of tetramer-positive cells (5th–12th lines).

	Nov 98	Apr 99	Oct 99	Mar 00	Jun 00	Dec 00	Feb 01	May 01	Control
CMV TE– $CD8^+$ in % of PBL	20	22	26	24	26	17	16	22	16
CMV TE+ $CD8^+$ in % of PBL	19	20	9	9	21	11	28	20	1
CMV TE+ $CD8^+$ in % of $CD8^+$	49	48	26	28	45	40	64	47	4
IFN-γ^+ after stimulation in % of $CD8^+$	ND	ND	20	22	32	33	16	29	4
CD45RA of CMV TE+	ND	96	92	88	ND	97	96	96	39
CD45RO of CMV TE+	ND	3	5	7	2	3	1	3	63
HLA DR of CMV TE+	ND	ND	ND	28	ND	ND	5	ND	14
CD38 of CMV TE+	ND	ND	ND	0	ND	ND	0	ND	41
CD57 of CMV TE+	ND	ND	ND	85	ND	ND	90	ND	68
CD62L of CMV TE+	ND	ND	ND	9	ND	ND	1	ND	6
CD44 of CMV TE+	ND	ND	ND	100	ND	ND	100	ND	100
CD28 of CMV TE+	ND	ND	ND	6	ND	ND	8	ND	2

Control: PBL derived from another CMV seropositive donor as described in material and methods, ND, not determined.

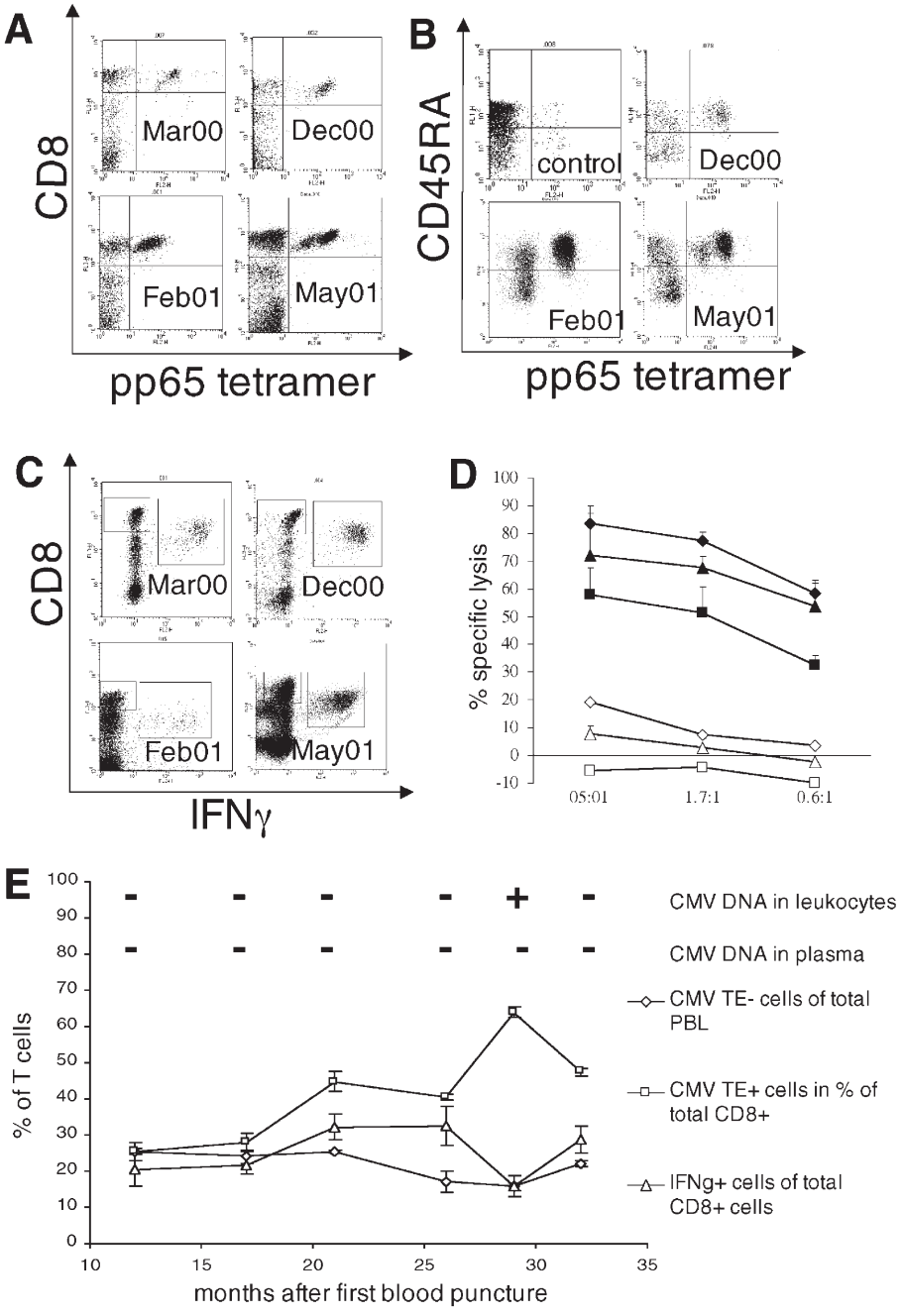

Figure 1. (*A*) High frequencies of HCMV-specific CD8+ T cells as determined by FACS staining of blood samples from March 00, December 00, February 01 and May 01. PBMC were stained with CD8 tricolor (y-axis) and NLVPMVATV tetramer (x-axis). Dot plots show cells in PBL gate determined by forward scatter and side scatter. Percentages are given for the upper right quadrant. (*B*) Tetramer-positive cells express high levels of CD45RA. CD8+ cells at different timepoints or from a control donor, stained with NLVPMVATV tetramer and CD45RA. Percentages are given for the upper right quadrant. (*C*) Comparison of the amount of INF-γ-producing cells. PBMC were stained for CD8 and intracellular IFN-γ after stimulation with NLVPMVATV for 12 h. After stimulation with irrelevant peptide (YLLPAIVHI), less than 0.3% of all CD8+ T cells were IFN-γ positive (data not shown). Percentage gives IFN-γ-positive cells of total CD8 T cells (high CD8 expressing). (*D*) Chromium release assay of clones derived from tetramer sort of March 00. Black symbols are target cells loaded with CMV peptide: white symbols are target cells loaded with irrelevant peptide. Target cells were T2 cells (diamonds), autologous PHA blasts (triangles) and allogeneic PHA blasts (squares). (*E*) Summary of tetramer staining over time course, shown in relation to IFN-γ production (values are given as mean ±SEM) and detection of HCMV-specific DNA. The minus indicates no HCMV-specific DNA detected; plus indicates HCMV-specific DNA detected.

clonal: it expressed 7 different Vβ chains of 16 tested (data not shown). In February 01, 64% of all CD8$^+$ cells and 28% of all peripheral blood lymphocytes (PBLs) were specific for NLVPMVATV (fig. 1, table 1).

All blood samples were tested for HCMV DNA. Interestingly, only one sample (Feb 01) was positive for HCMV DNA in leukocytes, while plasma was negative (fig. 1D). However, late HCMV pp67 mRNA was not detectable in leukocytes. This most likely indicates HCMV reactivation.

It was an unexpected finding that the elevated frequency of CMV-specific CD8$^+$ T cells was associated with CMV reactivation. Screening several blood samples of the donor for CD45RA and CD45RO, we found that CMV-specific CD8$^+$ T cells represented a CD45RA$^+$ phenotype (fig. 1B, table 1). We screened the sample of March 00 and February 01 for further phenotype markers. In both samples most of the HCMV-specific T cells expressed CD57 but not CD28, a phenotype commonly associated with terminal T cell differentiation [14]. HCMV-specific CD8$^+$ T cells expressed a normal phenotype for CD62L and CD44 compared with a control donor, but they did not express CD38 (table 1). In March 00, HLA-DR was expressed on 28% of tetramer-positive cells, while in February 01, only 4% of tetramer-positive cells expressed HLA-DR (table 1). Altogether, the different cell surface markers analyzed suggested a memory status of the HCMV-specific T cells. In our assays in healthy CMV-positive donors, more than 50% of HCMV-specific T cells express CD45RO (fig. 1B), but other groups have reported large differences in CD45 isoform expression [15]. Recently, anergic melanocyte-specific T cells have been described which expressed CD45RA and CD57 but not CD45RO and CD28 [16, 17], just like the T cells described here. Nevertheless, this phenotype has also been assigned to cytolytic effector T cells [17, 18].

When we analyzed the functionality of the HCMV-specific T cells using intracellular cytokine staining, between 20 and 33% of CD8$^+$ T cells in five samples other than February 01 were able to produce IFN-γ upon stimulation with the immunodominant epitope NLVPMVATV. CD8$^+$ T cells derived from such populations were also able to lyse peptide-loaded target cells (fig. 1B). In contrast, the sample from February 01 contained only 16% of IFN-γ-producing CD8$^+$ T cells (fig. 1C, D).

Thus, HCMV reactivation may be correlated to a large number of nonreactive T cells that can be stained by tetramers but do not produce IFN-γ. The situation seems similar to a 'stunned' phenotype that has recently been reported for temporarily nonreactive CD8$^+$ cells in HCV patients [19]. Such a certain kind of CD8$^+$ T cell dysfunction has also been documented for other persistent virus infections [20–22]. However, in our case 16% of CD8$^+$ T cells are still functional. HCMV reactivation with detectable HCMV-specific DNA in lymphocytes and granulocytes is usually seen only in immunosuppressed donors [1, 2, 23] and is associated with a lack of functional HCMV-specific CD8$^+$ T cells [24].

In this communication we describe a healthy donor with CMV reactivation which is accompanied by an increase in HCMV-specific T cells, most of which do not produce IFN-γ in vitro, and therefore might be anergic or in vivo exhausted. The reasons for this temporary lack of functionality is unknown. We conclude that HCMV reactivation may occur not only in immunosuppressed persons but also in immunocompetent individuals. This case confirms that HCMV is an infection which needs persistent control by functional cytotoxic T cells to avoid virus expansion.

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