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Chronic Herpesvirus Reactivation Occurs in Aging

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Abstract

The aged immune system is characterized by clonal expansions of CD8+ T cells of which a substantial portion are directed against Epstein-Barr virus (EBV) and cytomegalovirus (CMV). It is unknown if these expansions represent increased viral reactivation or simply reflect an accumulation over time. We investigated herpesvirus reactivation in young and old subjects co-infected with CMV and EBV. Using molecular and serological techniques, we found significant increases in both the frequency and magnitude of EBV and CMV reactivation in elderly subjects. CMV DNA was frequently detected in the urine of elderly subjects; EBV load in peripheral blood was also significantly increased. Notably, EBV DNA in plasma was detected in a majority of the elderly subjects which was supported by frequent transcription of late structural genes. Furthermore, CD8+ T cells specific for EBV structural antigens were detected in samples from the elderly. Samples from our younger control group were negative for EBV DNA in plasma, CMV DNA in urine, expression of structural transcripts, and lacked CD8+ T cells specific for EBV structural antigens. These findings indicate that the aged immune system is no longer able to control EBV and CMV reactivation that could now be characterized as chronic instead of latent.

Keywords

aging; herpesvirus; Epstein-Barr Virus; Cytomegalovirus

Introduction

During aging there is a progressive accumulation of senescent CD8+ T cells which lack CD28, a costimulatory molecule critical to the outcome of antigen recognition and signal transduction induced by the T-cell receptor (Lenschow et al., 1996). This expanded $CD8^+$ $CD28^-$ T cell subset has been shown to be closely associated with Cytomegalovirus (CMV) infection (Wang et al., 1995;Looney et al., 1999;Khan et al., 2002;Wikby et al., 2002). CMV is typically acquired asymptomatically during childhood. Subsequently, CMV persists in the host for life and remains latent in myeloid-progenitor cells and possibly endothelial cells (Bruggeman, 1993;Hahn et al., 1998). The frequency of CMV-specific T cells can reach 25% or more of the CD8+ pool and are often present as oligoclonal expansions (Gillespie et al., 2000;Khan et al.,

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2002;Lang et al., 2002). It is thought that such large expansions directed against a single virus may limit immune responses to other pathogens (Pawelec et al., 2004).

In light of this, it was recently reported that immune responses to Epstein-Barr virus (EBV) were significantly impaired in CMV-seropositive elderly adults (Khan et al., 2004). EBV, another herpesvirus, infects greater than 90% of the adult population and is associated with numerous diseases such as Burkitt's lymphoma, oral hairy leukoplakia, and Hodgkin's disease (Okano et al., 1988;Walling et al., 1992). Control over EBV reactivation is mediated by cytotoxic T-lymphocytes (Khanna et al., 1995), and EBV-specific T cells in the elderly have been demonstrated to have impaired effector function (Ouyang et al., 2003). Notably, increased numbers of CD8+CD28- T cells along with low CD4+ T cells and poor proliferative responses were found to predict higher mortality in the elderly (Ferguson et al., 1995;Pawelec et al., 2001). However, it is not known whether the increased numbers of herpesvirus-specific T cell reflect increased viral reactivation during aging or whether their accumulation simply represents the duration of infection (Pawelec, 2005).

Little is know about CMV and EBV reactivation in the elderly. Increased anti-viral antibodies to both CMV and EBV have been detected in the elderly (Glaser et al., 1985;Musiani et al., 1988;Weymouth et al., 1990) suggesting that impaired cellular responses may result in subsequent upregulation of viral protein expression and increased viral replication. However, direct evidence is lacking. Studies conducted to date have failed to detect CMV DNA in blood from elderly subjects (Khan et al., 2004;Vescovini et al., 2004), although for EBV the frequency of spontaneous lymphoblastoid cell lines (LCLs) has been shown to be significantly greater in the elderly (Rangan and Armatis, 1991). In addition, the prevalence of EBV DNA in peripheral blood from elderly subjects was also unexpected high (Vescovini et al., 2004).

In this study, we describe how the frequency and magnitude of herpesvirus reactivation is affected by the aging process. Our results indicate that chronic reactivation of both CMV and EBV occurs in older adults. These data give new insight regarding EBV and CMV reactivation in aging and provide an explanation for the age-related increases in virus-specific CD8+ T cells.

Methods

Subjects

Elderly donors were recruited from the Sealy Center on Aging Volunteer Registry at the University of Texas Medical Branch (UTMB) at Galveston. This is a registry of healthy volunteers, living in the Houston-Galveston area, who are over 55 years of age and are willing to participate in basic research or clinical studies. Blood samples were collected every 2 months over a 6 month period from 11 healthy subjects (ages between 66 and 83; mean 76 ± 5) without known immune-compromising medical conditions and not taking any medications with known immune modulating activities. Healthy young adults (ages 25-55), also living in the Houston-Galveston area, were also recruited. Because we could only collect blood and urine samples one time from the control group, a greater number (n=31) of subjects were enrolled. All blood samples and first morning urine voids were collected between 7-9 am; urine was collected, stored, and analyzed under identical conditions. The UTMB Institutional Review Board approved this investigation, and informed consent was obtained from all participants.

Measurement of anti-viral antibodies

Anti-viral antibody titers were determined by indirect immunofluorescence as previously described (Stowe et al., 2000). Commercially prepared substrate slides and control sera (Microgen Laboratories, La Marque, TX, and Bion Enterprises, Park Ridge, IL) were used for

determining IgG antibody titers to EBV-viral capsid antigen (VCA), -early antigen (EA), EBV-nuclear antigen (EBNA), and CMV. All specimens were batch analyzed and read blind-coded.

Cytokine flow cytometry assay

Intracellular cytokine staining assays were performed as described elsewhere (Crucian et al., 2001;Komanduri et al., 2001). Peripheral blood mononuclear cells (PBMCs), isolated from heparinized whole blood by density-gradient centrifugation, were immediately stimulated with HLA-A*0201-restricted epitopic peptides (10 μg/ml/peptide) or controls (PBS). The following peptides and sequences (in parenthesis) were used: EBV lytic cycle: 1) BMLF-1 (GLCTLVAML), 2) gp110 (ILIYNGWYA), 3) gp350 (VLQWASLAV); EBV latent cycle: 1) EBNA-3A (SVRDRLARL), 2) LMP-2A (CLGGLLTMV). CMV pp65 peptides corresponding to residues 495-503 (NLVPMVATV) were also used to stimulate CD8+ T cells. Costimulatory monoclonal antibodies (mAbs) – CD28 and CD49d mAbs (1 ug/ml each) (BD Biosciences) – were added to each tube including the negative (PBS) controls. The tubes were vortexed and incubated for 6 h at 37°C with 5% CO_2, with the addition of Brefeldin A (10 ug/ml) for the last 5 h. Following stimulation, cells were washed in PBS, incubated for 5 min at 37°C in 0.02% EDTA and washed again. Cells were then incubated for 10 minutes in FACSPerm solution (BD Biosciences), washed, and stained with anti-IFN-γ (PE), anti-CD8 (PerCP), and anti-CD69 (APC) antibodies. Samples were then fixed in 1% paraformaldehyde and analyzed using a FACSCalibur cytometer and CellQuest software (BD Biosciences). Flow cytometry data was analyzed and presented using Flow Jo Software (Tree Star, San Carlos, CA); 50,000 events were analyzed for each sample.

Nucleic Acid Extraction and Real-Time Quantitative PCR

To determine viral load and EBV gene expression, B cells were isolated from fresh PBMCs using magnetic beads (CD19 beads, Dynal Inc., Lake Success, NY) and immediately snap-frozen and stored at -80°C. RNA was isolated from 1×10^6 B-cells using TRIzol (Invitrogen Corp., Carlsbad, CA) with glycogen (Roche, Pleasanton, CA). RT-PCR amplification for EBV gene expression was carried out as described below.

For DNA extraction, ethanol was added to the lower organic phase and the suspension was centrifuged 5 minutes at 2,000×g. One hundred microliters of 0.1 M NaCitrate in 10 % ethanol was added to the pellet, incubated for 30 minutes at room temperature, and centrifuged. This step was repeated three times. After centrifugation, the DNA pellet was washed by 75 % ethanol and resuspended in 500 μl TE buffer. Twenty-five microliters of 10% SDS and 25 μl Proteinase K (10 mg/ml stock) was added and placed at 37°C overnight. DNA was extracted by phenol/chloroform treatment, and precipitated by 3M potassium acetate with 95% ethanol at -80°C for 30 minutes. The DNA was pelleted by centrifugation, washed, and resuspended in distilled water.

Three milliliters of each urine sample (for CMV) or 1 ml of plasma (for EBV) was concentrated to 140 uL and 200 uL, respectively, by centrifugation using a Microsep concentrator 100K (Pall Filtron Corp., Northborough, MA). Extraction of genomic/viral DNA from concentrated urine was performed using the QIAamp Viral RNA Kit (Qiagen Inc., Santa Clarita, CA); DNA from concentrated plasma was isolated using the QIAamp 96 DNA blood kit (Qiagen). EBV DNA was quantitated by real-time PCR using an ABI 7700 sequence detector or a Strategene MX3005P thermocycler as described (Kimura et al., 1999). CMV DNA was quantitated using the same methodology but with primers that targeted the immediate early gene (Tanaka et al., 2000). The limits of detection for the real-time PCR assays were 2-10 copies.

RT-PCR Amplification

To determine viral gene transcription, specimen RNA was treated with DNase and was reverse transcribed into cDNA by use of oligo-dT and EBER1-specific primers and avian myeloblastosis virus reverse trancriptase (Promega, Madison, WI). Each cDNA specimen was amplified by nested PCR using primer sets specific for 9 EBV genes: EBER-1, EBNA-1 Qp, LMP-1, EBNA-1 Cp/Wp, EBNA-2, BZLF-1, SM, EBNA-1 Fp, and gp220. Except for EBER-1, all primers were designed from gene exon sequences flanking introns that are spliced out of the mRNA transcript during translational expression of the gene (Walling et al., 2001;Walling et al., 2003). EBER-1 cDNA amplifications were performed in parallel with amplification of RNA not treated with reverse transcriptase as a control for DNA contamination of the RNA.

Forty cycles of amplification were performed each for the initial reaction and the nested reaction using Vent DNA polymerase (New England Biolabs, Ipswich, MA) and a Strategene Robo-Cycler. Standard techniques were employed to prevent and detect *in vitro* contamination of the PCR reactions. Amplified products were identified by size, by use of agarose-gel electrophoresis with ethidium-bromide. Specificity of the amplified products was demonstrated by Southern blot hybridization to a [^{32}P]-labeled oligonucleotide probe specific for sequences internal to those used for amplification.

Statistical Analysis

Statistical analysis was performed using SigmaStat software v2.03 (SPSS, Chicago, IL). Since the method of doubling dilutions was used to obtain antibody titer results, base 2 logarithmic conversions were used to reduce variance for statistical comparisons. Intergroup comparisons were performed using the Mann-Whitney *U* test. Where expressed, results are mean ± SE and *P* values less than 0.05 were considered significant.

Results

EBV and CMV Antibody Titers

All of the subjects that enrolled in this study were seropositive for EBV and CMV indicating past exposure. As shown in Table 1, significant differences were observed for EBV EA and CMV in the elderly as compared to younger subjects. Titers for EBV VCA ranged from 40 to 2560 in the elderly (*n* =35 samples) and from 40 to 640 in younger healthy adults (*n* =31 samples). For EBV EA, titers ranged from <10 to 640 in the elderly and from <10 to 40 in the younger group. For EBNA, titers ranged from <4 to 64 for both the elderly and young. For CMV, titers ranged from 160 to >640 in the elderly and 10 to 160 in the younger group.

Herpesvirus-specific CD8 T cells

Of the 11 elderly subjects recruited, 8 were HLA-A*0201+ (73%). Seventeen PBMC samples were collected longitudinally from these 8 subjects and stimulated with either EBV- and CMV-specific peptides or negative controls, and then analyzed for $CD8^+CD69^+$ and intracellular IFN-γ production. Positive responses were judged on clear CD69 expression and little/no non-specific staining. Fig. 1 shows the highest frequencies of EBV- and CMV-specific CD8+ T cells of selected elderly donors. The highest frequencies of aged CD8+ T cells directed against a single peptide were those specific for pp65 (13.2%). This was followed by responses to BMLF, LMP-2A, and EBNA-3A (4.6%, 3.8%, and 2.6%, respectively). In our younger group of HLA-A*0201+ subjects (*n* =19), pp65-responses also dominated with the highest frequency being 2.6%. For EBV-responses in the younger group, the largest response for a single peptide was for EBNA-3A followed by BMLF although the mean frequency for BMLF was significantly higher than any other EBV-encoded protein. A summary of the responses to EBV

and CMV peptides for elderly subjects and younger control subjects is shown in Table 2. Overall, significantly higher responses were observed for EBV and CMV antigens in our elderly subjects as compared to the younger group.

Viral Load

In order to quantitate viral load in peripheral blood, we performed real-time quantitative PCR on B cells isolated from peripheral blood. In samples (n =28) from our elderly subjects, EBV DNA copies ranged from <50 to 1,943,650 genomes per 10^6 B cells (mean 175,101 ± 90,710). For our younger subjects (n =24 samples), the mean copy number was mean 15 ± 9 per 10^6 B cells (range undetectable to 228 genomes) which was near the limits of the PCR assay. Overall, the viral load in the elderly was significantly higher (P <0.001) than the younger subjects.

EBV DNA was also quantitated in plasma. Unexpectedly, we found EBV DNA in plasma samples from 7 of 11 (55%) elderly subjects; no EBV DNA was detected in plasma from any of our 31 younger subjects. Thirteen of 35 plasma samples (37%) from elderly subjects were EBV DNA-positive; the number of EBV genome copies in positive plasma ranged from 10-400 copies/mL. The presence of EBV DNA in plasma was significantly greater in the elderly group (P <0.001; Fisher Exact Test).

Because CMV DNA was not detected in any of the PBMC samples (data not shown), we extracted DNA from urine since this is an alternative specimen for diagnosing CMV infections. CMV DNA was frequently detected in urine (17 of 30 samples; 57%) from elderly subjects. Copies per milliliter ranged from below detection to 20,300 (mean 2026 ± 902). Overall, 10 of 11 (91%) elderly individuals shed CMV DNA in their urine during the 6-month period. In contrast, CMV DNA was not detected in any urine sample from our younger control subjects. The presence of CMV DNA was significantly greater in the elderly group (P <0.001).

EBV RNA Analysis

Nine viral genes were selected for RT-PCR amplification of RNA samples. The pattern of EBV gene expression may be used to determine the presence of latency I-III (EBER-1, EBNA-1 Qp and Cp/Wp, LMP-1, EBNA-2), immediate-early replicative (BZLF-1), early replicative (SM, EBNA-1 Fp), and late replicative (gp220) transcripts. The accumulated data for all subject's samples are shown in Table 3. All elderly samples demonstrated high expression of EBER-1, a non-polyadenylated viral RNA that is transcribed at high levels in EBV-infected B lymphocytes, which was in agreement with our findings of high EBV DNA copy number in peripheral blood B lymphocytes. Expression of gp220 was almost universally detected (9 of 11 subjects; 82%); gp220 transcripts were found in 15 of 28 samples (54%). Expression of LMP-1 was the next most frequently expressed gene, with detectable expression in samples from 8 of 11 subjects (73%); 10 of 28 samples (36%) were LMP-1-positive. Expression of EBNA-1 (Cp/W/p; Fp; and Qp) was commonly observed as well. BZLF-1, EBNA-2, and SM were less frequently detected; one subject's sample was positive for both SM and EBNA-2, while BZLF-1 was expressed in another subject's sample.

In contrast, EBV lytic and latent gene expression was infrequent in our younger group (Table 4). β-actin mRNA was amplified in all 24 samples (100%) confirming the extraction of high-quality RNA from the samples. EBER-1 was detected in 19 of 24 samples (70.3%). Samples from subjects 12, 13, 21, and 24 were Qp-positive. Another two samples were Fp-positive (subjects 23 and 24), while samples from subjects 14 and 11 were LMP-1- and BZLF-1-positive, respectively. Overall, seven samples from our younger control subjects were positive for EBV lytic or latent gene expression.

Discussion

Several important and novel findings have resulted from this study. First, reactivation of CMV frequently occurs in aging as demonstrated by increased anti-CMV antibodies and direct detection of CMV DNA in urine. Prior studies looking at CMV in blood have yielded negative results (Khan et al., 2004;Vescovini et al., 2004). However, CMV can be detected in the urine of newborns congenitally infected with CMV (Demmler et al., 1988), and PCR examination of urine has been determined to be suitable for the detection of CMV in immunosuppressed patients (Xu et al., 1993). Accordingly, PCR was used to directly detect CMV in urine which revealed an unexpectedly high frequency of shedding in aged subjects, whereas none of the urine samples from our younger subjects were CMV DNA-positive. The results from the younger subjects are consistent with those from our prior study where CMV DNA was found in only 1 of 81 urine samples including multiple samples from 11 subjects (Mehta et al., 2000). Since detection of CMV in blood from elderly subjects has been elusive, the results presented here indicate that although CMV frequently reactivates in aging it is subclinical in nature.

Second, EBV chronically reactivates in aging as well. The increased EBV anti-EA antibodies are in agreement with our prior study (Glaser et al., 1985). Although the elevation of anti-VCA antibodies did not reach significance in this study, our prior study that included a greater number of subjects did show that anti-VCA antibodies are significantly greater in the elderly (Glaser et al., 1985). These results suggested that either the frequency or magnitude of EBV reactivation resulted in increased production of viral proteins and perhaps increased viral replication. This notion was further supported by our results demonstrating the presence of EBV-specific T cells that were specific to both latent and lytic epitopes. Prior studies of younger EBV-seropositive adults have demonstrated a predominance of CD8+ T cells directed against early lytic epitopes (Tan et al., 1999;Saulquin et al., 2000), whereas in older subjects EBV-specific T cells directed against latent epitopes (e.g., EBNA 3A, LMP-2A) can be detected and in some instances predominate (Khan et al., 2004;Vescovini et al., 2004). We not only found detectable responses to EBNA-3A and LMP-2A but also T cells reactive to the EBV structural antigens gp350 and gp110. These results suggest that EBV-infected cells are no longer expressing the restricted set of viral genes that define latency, but that they are also expressing the full latent and lytic gene patterns.

To address this issue, we analyzed peripheral blood B cells using molecular techniques which revealed frequent transcription of gp220 in our aged samples (Note: EBV glycoprotein gp220/350 is the major glycoprotein associated with the EBV envelope, and the 220kD protein is the result of RNA splicing). In peripheral blood taken from young donors, viral transcription has primarily been limited to LMP-2A and EBNA-1 (Tierney et al., 1994;Chen et al., 1995;Miyashita et al., 1995). Expression of gp350 has been recently reported in patients with infectious mononucleosis and transplant recipients but not in peripheral blood of healthy subjects (Hopwood et al., 2002). An unexpectedly high frequency of LMP-1 expression was also found. LMP-1 transcripts are typically restricted to patients with EBV-related diseases such as posttransplant recipients (Qu et al., 2000;Hopwood et al., 2002) and have not been found in young healthy subjects (Tierney et al., 1994;Chen et al., 1995;Miyashita et al., 1995;Hopwood et al., 2002). Notably, LMP-1 acts as an oncogene, activating cellular transcription factors of the NF-kB and AP-1 family and upregulating cellular adhesion molecules, cytokine production, and B cell proliferation (Rowe et al., 1998). We also observed co-expression of EBNA-2 and LMP-1 in a single blood specimen (Subject 10). The expression of these two transcripts is representative of an immunoblastic phenotype (i.e., latency pattern III) characterized by upregulation of adhesion molecules (e.g., CD23, ICAM-1, LFA-1) (Rowe et al., 1998). Accordingly, future studies will include measurements to determine if the immunoblastic phenotype is present in aged peripheral blood B cells.

The increased EBV lytic and latent gene expression suggests that greater viral load would be present in peripheral blood of aged subjects, and PCR analysis confirmed this hypothesis. They also suggest that productive virus replication (i.e., linear viral genomes) is occurring. To investigate this further, we analyzed plasma for the presence of EBV DNA. Vescovini and coworkers (Vescovini et al., 2004) found only one positive sample. However, we found EBV DNA in a much larger number of plasma samples. We attribute this to the higher sensitivity of our approach, in which we concentrated a relatively larger volume of plasma prior to extracting DNA that revealed an unusually high percentage of aged subjects (55%) with EBV DNA in their plasma. Notably, EBV DNA has been frequently detected in the plasma of patients with EBV-associated diseases (Gan et al., 1994;Yamamoto et al., 1995;Berger et al., 2001), and the presence of EBV DNA in plasma has proven to have prognostic significance (Lechowicz et al., 2002).

Overall, these results support the concept that the expansion of virus-specific CD8+ T cells is due to increased herpesvirus reactivation and replication associated with aging. However, herpesviruses can also be reactivated as a result of stress from academic exams as well as spaceflight (Stowe et al., 2001;Glaser and Kiecolt-Glaser, 2005), and we have found increased numbers of virus-specific CD8+ T cells concomitant with the increases in herpesvirus reactivation in astronauts (R.P. Stowe, unpublished data). Notably, stress in inherent in the aging process (e.g., bereavement, caregiving, etc.) and has been shown to greatly impair immune responses (Kiecolt-Glaser et al., 1991;Kiecolt-Glaser et al., 1996). Thus, it is also likely that periodic viral reactivation due to stress contributes to the increased pool of herpesvirus-specific T cells over time. Further studies are needed to understand the effects of increased herpesvirus reactivation and its role in healthy aging.

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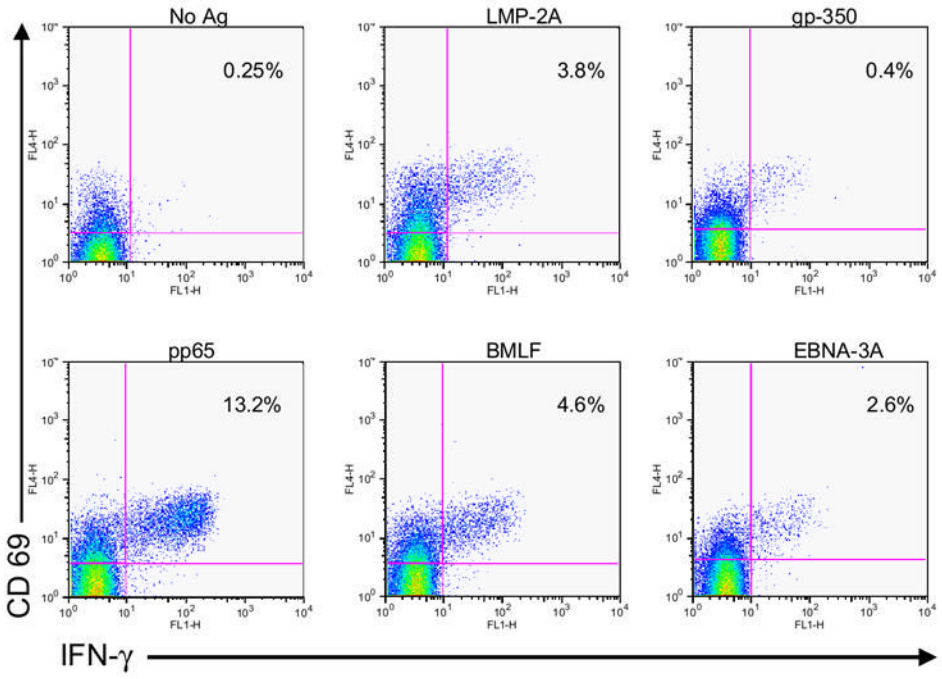

Figure 1.

Frequency of herpesvirus-specific CD8 T cells in healthy elderly subjects. Fifty thousand cells were included in each analysis. The frequency of CD8+ T cells shown indicate the percentage of CD69 and IFN-γ-positive cells after pulsing with A*0201-restricted peptides to EBV (lytic and latent proteins) and CMV (pp65).

Table 1

Age-related changes in antiviral antibody titers[a]

	Aging	Young	*P* value
EBV-VCA	7.0 ± 0.3	6.2 ± 0.2	0.154
EBV-EA	4.5 ± 0.3	3.0 ± 0.2	<0.001
EBNA	4.0 ± 0.3	3.5 ± 0.4	0.253
CMV	9.2 ± 0.3	5.2 ± 0.3	<0.001

[a] Log_2-antibody titers of EBV-viral capsid antigen (VCA), EBV-early antigen (EA), EBV-nuclear antigen (EBNA), and cytomegalovirus (CMV).

Table 2

Comparison of the frequency of herpesvirus-specific CD 8^+ T-cells in peripheral blood

Peptide	Elderly Mean Frequency (range)	Young Mean Frequency (range)	*P* value
BMLF	0.50 (0-4.60)	0.20 (0-0.50)	0.014
EBNA-3A	0.40 (0-2.60)	0.08 (0-1.0)	0.005
gp110	0.10 (0-0.40)	bd[a]	0.019
gp350	0.10 (0-0.40)	bd	0.018
LMP-2A	0.50 (0-3.80)	bd	0.001
pp65	1.10 (0-13.2)	0.20 (0-2.60)	0.001

[a] below detection

Table 3

Summary of Nested RT-PCR Analysis of EBV Gene Expression[a] in Aging

Subject	EBER-1	Qp	Cp/Wp	LMP-1	EBNA-2	BZLF-1	SM	Fp	gp220	EBV DNA in plasma[b]
1	+++	+	+	+				+	+++	
2	+++		+	+				+	++	+
3	+++	+	+	+				+	+++	
4	+++	+		+		+		+	++	
5	+++	+							+	+
6	+++	+	+	+				+	+++	+
7	+++			+				+		+
8	+++	+							+++	
9	+++			+				+	+	+
10	+++			+	+++		+++	+	+++	+
11	+++		+							+

Note: accumulated data for multiple (2-3) timepoints for each elderly subject.

[a] Legend (+++ = highly expressed; ++ = moderately expressed; + = low expression)

[b] + = EBV DNA present

Table 4

Summary of EBV Gene Expression in Healthy Young Adults[a]

Subject	Actin	EBER-1	Qp	Cp/ Wp	LMP1	EBNA-2	BZLF-1	SM	Fp	gp220
1	+++									
2	+++	+++								
3	+	+								
4	+++	+								
5	+++									
6	+++	+++								
7	+++	+								
8	+	+								
9	+	+++								
10	+++									
11	+++	+					+			
12	+	+	+							
13	+++	+++	+							
14	+++	+			+					
15	+++	+								
16	+++	+								
17	+++	+++								
18	+++									
19	+++									
20	+++	+++								
21	+++	+	+							
22	+++	+++								
23	+++	+++							+	
24	+++	+++	+						+	

[a] Legend (+++ = highly expressed; ++ = moderately expressed; + = low expression).