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Chronic herpesvirus reactivation occurs in aging

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11 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.

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25 1. 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; Loooney et al., 1999; Khan et al., 2002; Wikby et al., 2002). CMV is typically acquired asymptotically 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., 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).

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55 Notably, increased numbers of CD8+CD28– T cells along
56 with low CD4+ T cells and poor proliferative responses
57 were found to predict higher mortality in the elderly (Fer-
58 guson et al., 1995; Pawelec et al., 2001). However, it is not
59 known whether the increased numbers of herpesvirus-spe-
60 cific T cell reflect increased viral reactivation during aging
61 or whether their accumulation simply represents the dura-
62 tion of infection (Pawelec, 2005).

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

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

86 2. Methods

87 2.1. Subjects

88 Elderly donors were recruited from the Sealy Center
89 on Aging Volunteer Registry at the University of Texas
90 Medical Branch (UTMB) at Galveston. This is a registry
91 of healthy volunteers, living in the Houston–Galveston
92 area, who are over 55 years of age and are willing to
93 participate in basic research or clinical studies. Blood
94 samples were collected every 2 months over a 6 month
95 period from 11 healthy subjects (ages between 66 and
96 83; mean 76 ± 5) without known immune-compromising
97 medical conditions and not taking any medications with
98 known immune modulating activities. Healthy young
99 adults (ages 25–55), also living in the Houston–Galveston
100 area, were also recruited. Because we could only collect
101 blood and urine samples one time from the control
102 group, a greater number ($n = 31$) of subjects were
103 enrolled. All blood samples and first morning urine voids
104 were collected between 7 and 9 am; urine was collected,
105 stored, and analyzed under identical conditions. The
106 UTMB Institutional Review Board approved this investi-
107 gation, and informed consent was obtained from all
108 participants.

2.2. Measurement of anti-viral antibodies 109

110 Anti-viral antibody titers were determined by indirect
111 immunofluorescence as previously described (Stowe et al.,
112 2000). Commercially prepared substrate slides and control
113 sera (Microgen Laboratories, La Marque, TX, and Bion
114 Enterprises, Park Ridge, IL) were used for determining
115 IgG antibody titers to EBV–viral capsid antigen (VCA),
116 early antigen (EA), EBV–nuclear antigen (EBNA), and
117 CMV. All specimens were batch analyzed and read blind-
118 coded.

2.3. Cytokine flow cytometry assay 119

120 Intracellular cytokine staining assays were performed as
121 described elsewhere (Crucian et al., 2001; Komanduri et al.,
122 2001). Peripheral blood mononuclear cells (PBMCs), iso-
123 lated from heparinized whole blood by density–gradient
124 centrifugation, were immediately stimulated with HLA-
125 A*0201-restricted epitopic peptides (10 $\mu\text{g}/\text{ml}/\text{peptide}$) or
126 controls (PBS). The following peptides and sequences (in
127 parenthesis) were used: EBV lytic cycle: (1) BMLF-1
128 (GLCTLVAML), (2) gp110 (ILYNGWYA), (3) gp350
129 (VLQWASLAV); EBV latent cycle: (1) EBNA-3A
130 (SVRDRLARL), (2) LMP-2A (CLGGLLTMV). CMV
131 pp65 peptides corresponding to residues 495–503
132 (NLVPMVATV) were also used to stimulate CD8+ T cells.
133 Costimulatory monoclonal antibodies (mAbs) – CD28 and
134 CD49d mAbs (1 $\mu\text{g}/\text{ml}$ each) (BD Biosciences) – were add-
135 ed to each tube including the negative (PBS) controls. The
136 tubes were vortexed and incubated for 6 h at 37 °C with 5%
137 CO₂, with the addition of Brefeldin A (10 $\mu\text{g}/\text{ml}$) for the
138 last 5 h. Following stimulation, cells were washed in PBS,
139 incubated for 5 min at 37 °C in 0.02% EDTA and washed
140 again. Cells were then incubated for 10 min in FACSPerm
141 solution (BD Biosciences), washed, and stained with anti-
142 IFN- γ (PE), anti-CD8 (PerCP), and anti-CD69 (APC)
143 antibodies. Samples were then fixed in 1% paraformalde-
144 hyde and analyzed using a FACSCalibur cytometer and
145 CellQuest software (BD Biosciences). Flow cytometry data
146 was analyzed and presented using Flow Jo Software (Tree
147 Star, San Carlos, CA); 50,000 events were analyzed for
148 each sample.

2.4. Nucleic acid extraction and real-time quantitative PCR 149

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

158 For DNA extraction, ethanol was added to the lower
159 organic phase and the suspension was centrifuged 5 min
160 at 2000g. One hundred microliters of 0.1 M Na Citrate in

161 10% ethanol was added to the pellet, incubated for 30 min
162 at room temperature, and centrifuged. This step was
163 repeated three times. After centrifugation, the DNA pellet
164 was washed by 75% ethanol and resuspended in 500 μ L TE
165 buffer. Twenty-five microliters of 10% SDS and 25 μ L Pro-
166 teinase K (10 mg/ml stock) was added and placed at 37 $^{\circ}$ C
167 overnight. DNA was extracted by phenol/chloroform
168 treatment, and precipitated by 3 M potassium acetate with
169 95% ethanol at -80° C for 30 min. The DNA was pelleted
170 by centrifugation, washed, and resuspended in distilled
171 water.

172 Three milliliters of each urine sample (for CMV) or
173 1 ml of plasma (for EBV) was concentrated between
174 140 and 200 μ L, respectively, by centrifugation using a
175 Microsep concentrator 100 K (Pall Filtron Corp., North-
176 borough, MA). Extraction of genomic/viral DNA from
177 concentrated urine was performed using the QIAamp
178 Viral RNA Kit (Qiagen Inc., Santa Clarita, CA); DNA
179 from concentrated plasma was isolated using the QIA-
180 amp 96 DNA blood kit (Qiagen). EBV DNA was quan-
181 titated by real-time PCR using an ABI 7700 sequence
182 detector or a Stratagene MX3005 P thermocycler as
183 described (Kimura et al., 1999). CMV DNA was quanti-
184 tated using the same methodology but with primers that
185 targeted the immediate early gene (Tanaka et al., 2000).
186 The limits of detection for the real-time PCR assays were
187 2–10 copies.

188 2.5. RT-PCR amplification

189 To determine viral gene transcription, specimen RNA
190 was treated with DNase and was reverse transcribed into
191 cDNA by use of oligo-dT and EBER1-specific primers
192 and avian myeloblastosis virus reverse transcriptase (Pro-
193 mega, Madison, WI). Each cDNA specimen was ampli-
194 fied by nested PCR using primer sets specific for 9
195 EBV genes: EBER-1, EBNA-1 Qp, LMP-1, EBNA-1
196 Cp/Wp, EBNA-2, BZLF-1, SM, EBNA-1 Fp, and
197 gp220. Except for EBER-1, all primers were designed
198 from gene exon sequences flanking introns that are
199 spliced out of the mRNA transcript during translational
200 expression of the gene (Walling et al., 2001; Walling
201 et al., 2003). EBER-1 cDNA amplifications were per-
202 formed in parallel with amplification of RNA not treated
203 with reverse transcriptase as a control for DNA contam-
204 ination of the RNA.

205 Forty cycles of amplification were performed each for
206 the initial reaction and the nested reaction using Vent
207 DNA polymerase (New England Biolabs, Ipswich, MA)
208 and a Stratagene Robo-Cycler. Standard techniques were
209 employed to prevent and detect *in vitro* contamination of
210 the PCR reactions. Amplified products were identified by
211 size, by use of agarose-gel electrophoresis with ethidium-
212 bromide. Specificity of the amplified products was demon-
213 strated by Southern blot hybridization to a [32 P]-labeled
214 oligonucleotide probe specific for sequences internal to
215 those used for amplification.

216 2.6. Statistical analysis

217 Statistical analysis was performed using SigmaStat soft-
218 ware v2.03 (SPSS, Chicago, IL). Since the method of dou-
219 bling dilutions was used to obtain antibody titer results,
220 base 2 logarithmic conversions were used to reduce vari-
221 ance for statistical comparisons. Intergroup comparisons
222 were performed using the Mann-Whitney *U* test. Where
223 expressed, results are means \pm SE and *P* values less than
224 0.05 were considered significant.

225 3. Results

226 3.1. EBV and CMV antibody titers

227 All of the subjects that enrolled in this study were sero-
228 positive for EBV and CMV indicating past exposure. As
229 shown in Table 1, significant differences were observed
230 for EBV EA and CMV in the elderly as compared to youn-
231 ger subjects. Titers for EBV VCA ranged from 40 to 2560
232 in the elderly ($n = 35$ samples) and from 40 to 640 in youn-
233 ger healthy adults ($n = 31$ samples). For EBV EA, titers
234 ranged from <10 to 640 in the elderly and from <10 to
235 40 in the younger group. For EBNA, titers ranged from
236 <4 to 64 for both the elderly and young. For CMV, titers
237 ranged from 160 to >640 in the elderly and 10–160 in the
238 younger group.

239 3.2. Herpesvirus-specific CD8 T cells

240 Of the 11 elderly subjects recruited, 8 were HLA-
241 A*0201+ (73%). Seventeen PBMC samples were collected
242 longitudinally from these 8 subjects and stimulated with
243 either EBV- and CMV-specific peptides or negative con-
244 trols, and then analyzed for CD8+CD69+ and intracellu-
245 lar IFN- γ production. Positive responses were judged on
246 clear CD69 expression and little/no non-specific staining.
247 Fig. 1 shows the highest frequencies of EBV- and
248 CMV-specific CD8+ T cells of selected elderly donors.
249 The highest frequencies of aged CD8+ T cells directed
250 against a single peptide were those specific for pp65
251 (13.2%). This was followed by responses to BMLF,
252 LMP-2A, and EBNA-3A (4.6%, 3.8%, and 2.6%, respec-
253 tively). In our younger group of HLA-A*0201+ subjects
254 ($n = 19$), pp65-responses also dominated with the highest
255 frequency being 2.6%. For EBV-responses in the younger

Table 1
Age-related changes in antiviral antibody titers^a

	Aging	Young	<i>P</i> value
EBV-A	7.0 \pm 0.3	6.2 \pm 0.2	0.154
EBV-EA	4.5 \pm 0.3	3.0 \pm 0.2	<0.001
EBNA	4.0 \pm 0.3	3.5 \pm 0.4	0.253
CMV	9.2 \pm 0.3	5.2 \pm 0.3	<0.001

^a Log₂-antibody titers of EBV-viral capsid antigen (VCA), EBV-early antigen (EA), EBV-nuclear antigen (EBNA), and cytomegalovirus (CMV).

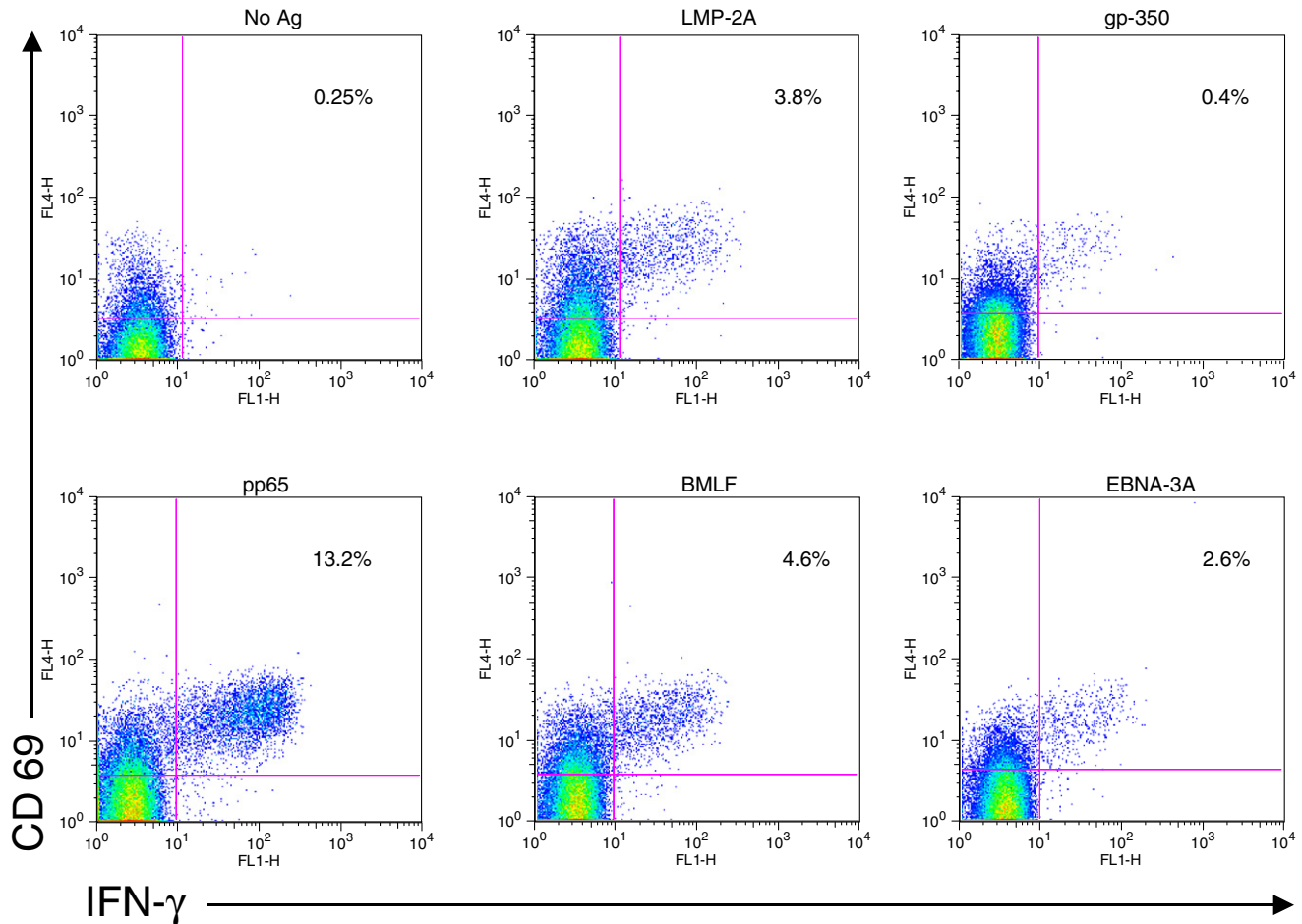


Fig. 1. Frequency of herpesvirus-specific CD8⁺ T cells in healthy elderly subjects. Fifty thousand cells were included in each analysis. The frequency of CD8⁺ T cells shown indicate the percentage of CD69 and IFN- γ -positive cells after pulsing with A*0201-restricted peptides to EBV (lytic and latent proteins) and CMV (pp65).

256 group, the largest response for a single peptide was for
 257 EBNA-3A followed by BMLF although the mean frequen-
 258 cy for BMLF was significantly higher than any other EBV-
 259 encoded protein. A summary of the responses to EBV and
 260 CMV peptides for elderly subjects and younger control
 261 subjects is shown in Table 2. Overall, significantly higher
 262 responses were observed for EBV and CMV antigens in
 263 our elderly subjects as compared to the younger group.

Table 2
 Comparison of the frequency of herpesvirus-specific CD8⁺ T cells in peripheral blood

Peptide	Elderly	Young	P value
	Mean frequency (range)	Mean frequency (range)	
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.

3.3. Viral load

264

In order to quantitate viral load in peripheral blood, 265
 we performed real-time quantitative PCR on B cells 266
 isolated from peripheral blood. In samples ($n = 28$) from 267
 our elderly subjects, EBV DNA copies ranged from 268
 <50 to 1,943,650 genomes per 10^6 B cells (mean 269
 $175,101 \pm 90,710$). For our younger subjects ($n = 24$ 270
 samples), the mean copy number was mean 15 ± 9 per 10^6 271
 B cells (range undetectable to 228 genomes) which was near 272
 the limits of the PCR assay. Overall, the viral load in the 273
 elderly was significantly higher ($P < 0.001$) than the younger 274
 subjects. 275

EBV DNA was also quantitated in plasma. Unexpectedly, 276
 we found EBV DNA in plasma samples from 7 of 11 277
 (55%) elderly subjects; no EBV DNA was detected in plasma 278
 from any of our 31 younger subjects. Thirteen of 35 279
 plasma samples (37%) from elderly subjects were EBV 280
 DNA-positive; the number of EBV genome copies in posi- 281
 tive plasma ranged from 10–400 copies/mL. The presence 282
 of EBV DNA in plasma was significantly greater in the 283
 elderly group ($P < 0.001$; Fisher Exact Test). 284

285 Because CMV DNA was not detected in any of the
 286 PBMC samples (data not shown), we extracted DNA from
 287 urine since this is an alternative specimen for diagnosing
 288 CMV infections. CMV DNA was frequently detected in
 289 urine (17 of 30 samples; 57%) from elderly subjects. Copies
 290 per milliliter ranged from below detection to 20,300 (mean
 291 2026 ± 902). Overall, 10 of 11 (91%) elderly individuals
 292 shed CMV DNA in their urine during the 6-month period.
 293 In contrast, CMV DNA was not detected in any urine sam-
 294 ple from our younger control subjects. The presence of
 295 CMV DNA was significantly greater in the elderly group
 296 ($P < 0.001$).

297 3.3. EBV RNA analysis

298 Nine viral genes were selected for RT-PCR amplifica-
 299 tion of RNA samples. The pattern of EBV gene expression
 300 may be used to determine the presence of latency I-III
 301 (EBER-1, EBNA-1 Qp and Cp/Wp, LMP-1, EBNA-2),
 302 immediate early replicative (BZLF-1), early replicative
 303 (SM, EBNA-1 Fp), and late replicative (gp220) transcripts.
 304 The accumulated data for all subject's samples are shown
 305 in Table 3. All elderly samples demonstrated high expres-
 306 sion of EBER-1, a non-polyadenylated viral RNA that is
 307 transcribed at high levels in EBV-infected B lymphocytes,
 308 which was in agreement with our findings of high EBV
 309 DNA copy number in peripheral blood B lymphocytes.
 310 Expression of gp220 was almost universally detected (9 of
 311 11 subjects; 82%); gp220 transcripts were found in 15 of
 312 28 samples (54%). Expression of LMP-1 was the next most
 313 frequently expressed gene, with detectable expression in
 314 samples from 8 of 11 subjects (73%); 10 of 28 samples
 315 (36%) were LMP-1-positive. Expression of EBNA-1 (Cp/
 316 W/p; Fp; and Qp) was commonly observed as well.
 317 BZLF-1, EBNA-2, and SM were less frequently detected;
 318 one subject's sample was positive for both SM and
 319 EBNA-2, while BZLF-1 was expressed in another subject's
 320 sample.

321 In contrast, EBV lytic and latent gene expression was
 322 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.

4. Discussion

332 Several important and novel findings have resulted from
 333 this study. First, reactivation of CMV frequently occurs in
 334 aging as demonstrated by increased anti-CMV antibodies
 335 and direct detection of CMV DNA in urine. Prior studies
 336 looking at CMV in blood have yielded negative results
 337 (Khan et al., 2004; Vescovini et al., 2004). However,
 338 CMV can be detected in the urine of newborns congenitally
 339 infected with CMV (Demmler et al., 1988), and PCR exam-
 340 ination of urine has been determined to be suitable for the
 341 detection of CMV in immunosuppressed patients (Xu
 342 et al., 1993). Accordingly, PCR was used to directly detect
 343 CMV in urine which revealed an unexpectedly high fre-
 344 quency of shedding in aged subjects, whereas none of the
 345 urine samples from our younger subjects were CMV
 346 DNA-positive. The results from the younger subjects are
 347 consistent with those from our prior study where CMV
 348 DNA was found in only 1 of 81 urine samples including
 349 multiple samples from 11 subjects (Mehta et al., 2000).
 350 Since detection of CMV in blood from elderly subjects
 351 has been elusive, the results presented here indicate that
 352 although CMV frequently reactivates in aging it is subclin-
 353 ical in nature.

354 Second, EBV chronically reactivates in aging as well.
 355 The increased EBV anti-EA antibodies are in agreement
 356 with our prior study (Glaser et al., 1985). Although the
 357 elevation of anti-VCA antibodies did not reach significance
 358 in this study, our prior study that included a greater num-
 359 ber of subjects did show that anti-VCA antibodies are
 360

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).

361 significantly greater in the elderly (Glaser et al., 1985).
 362 These results suggested that either the frequency or magni-
 363 tude of EBV reactivation resulted in increased production
 364 of viral proteins and perhaps increased viral replication.
 365 This notion was further supported by our results demon-
 366 strating the presence of EBV-specific T cells that were
 367 specific to both latent and lytic epitopes. Prior studies of
 368 younger EBV-seropositive adults have demonstrated a
 369 predominance of CD8+ T cells directed against early lytic
 370 epitopes (Tan et al., 1999; Saulquin et al., 2000), whereas in
 371 older subjects EBV-specific T cells directed against latent
 372 epitopes (e.g., EBNA 3A, LMP-2A) can be detected
 373 and in some instances predominate (Khan et al., 2004;
 374 Vescovini et al., 2004). We not only found detectable
 375 responses to EBNA-3A and LMP-2A but also T cells reac-
 376 tive to the EBV structural antigens gp350 and gp110. These
 377 results suggest that EBV-infected cells are no longer
 378 expressing the restricted set of viral genes that define laten-
 379 cy, but that they are also expressing the full latent and lytic
 380 gene patterns.

381 To address this issue, we analyzed peripheral blood B
 382 cells using molecular techniques which revealed frequent
 383 transcription of gp220 in our aged samples (Note: EBV gly-
 384 coprotein gp220/350 is the major glycoprotein associated
 385 with the EBV envelope, and the 220 kDa protein is the
 386 result of RNA splicing). In peripheral blood taken from
 387 young donors, viral transcription has primarily been limit-
 388 ed to LMP-2A and EBNA-1 (Tierney et al., 1994; Chen
 389 et al., 1995; Miyashita et al., 1995). Expression of gp350
 390 has been recently reported in patients with infectious
 391 mononucleosis and transplant recipients but not in periph-

eral 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, activat-
 ing cellular transcription factors of the NF- κ B and AP-1
 family and upregulating cellular adhesion molecules, cyto-
 kine 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 expres-
 sion of these two transcripts is representative of an immu-
 noblastic phenotype (i.e., latency pattern III) character-
 ized 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 immunoblas-
 tic phenotype is present in aged peripheral blood B cells.

The increased EBV lytic and latent gene expression sug-
 gests 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 replica-
 tion (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 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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