Virological and Immunological Characteristics of Human Cytomegalovirus Infection Associated With Alzheimer Disease

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Serum, cerebrospinal fluid (CSF), and cryopreserved lymphocytes from subjects in the Rush Alzheimer’s Disease Center Religious Orders Study were analyzed for associations between cytomegalovirus (CMV) infection and clinical and pathological markers of Alzheimer disease. CMV antibody levels were associated with neurofibrillary tangles (NFTs). CSF interferon γ was only detected in seropositive subjects and was significantly associated with NFTs. The percentage of senescent T cells (CD4+ or CD8+CD28−CD57+) was significantly higher for CMV-seropositive as compared to CMV-seronegative subjects and was marginally associated with the pathologic diagnosis of Alzheimer disease (CD4+) or amyloid-β (CD8+). Immunocytochemical analysis showed induction of amyloid-β in human foreskin fibroblasts (HFFs) infected with each of 3 clinical CMV strains. In the same subjects, there was no association of herpes simplex virus type 1 (HSV-1) antibody levels with CMV antibody levels or clinical or pathological markers of Alzheimer disease. HSV-1 infection of HFFs did not induce amyloid-β. These data support an association between CMV and the development of Alzheimer disease.

Keywords. Alzheimer’s disease; cytomegalovirus; interferon-gamma; CD28-/CD57+ T cells; amyloid-β.

The majority of human cytomegalovirus (CMV) infections in immunocompetent persons are mild or asymptomatic. However, the infection is lifelong, with the virus maintained in a state of latency or low-level persistence. CMV seropositivity within the population ranges from 20%–100%, depending on age and socioeconomic status [1–3]. Symptomatic disease occurs most often under conditions of immunodeficiency, such as in patients with human immunodeficiency virus infection with or without AIDS, transplant recipients, and congenitally infected infants [4–6]. However, there is evidence that in normal immunocompetent persons, CMV reactivates periodically from latency without overt symptoms of disease. Under the conditions of immunocompetency, the virus is rarely detected in the blood, but reactivation can be detected occasionally in fluids from other body compartments, including urine, saliva, semen, cervicovaginal secretions, and breast milk [7–11]. These reactivations may lead to the higher percentage of CMV-specific T cells seen with increasing age [12–14]. In addition, age-related phenotypic and functional changes have been associated with CD4+ and CD8+ CMV-specific T-cell subsets. There is decreased expression of the costimulatory molecules CD27 and CD28 with an increased expression of CD57, a phenotype associated with terminally differentiated and senescent cells known to secrete proinflammatory cytokines such as interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) [15–18]. These cytokines have been reported to induce the deposition of amyloid-β peptides (Aβ), one of the hallmarks of Alzheimer disease pathology, which has led to the hypothesis that inflammation plays a significant role in the progression of Alzheimer disease [12, 19–22]. Because
CMV is known to infect the central nervous system (CNS), the proinflammatory response to CMV infection in the CNS could be a contributing factor in the development of chronic neurodegenerative diseases such as Alzheimer disease [15, 23]. Recent studies have also suggested a role for herpes simplex virus type 1 (HSV-1) in Alzheimer disease, because HSV-1 infects the CNS and, like CMV, causes a latent infection that is repeatedly reactivated [24–28]. These studies have reported detection of HSV-1 DNA with accumulation of Alzheimer disease–associated abnormal proteins in the CNS [24, 26–28]. The main objective of the current study was to determine whether immunological and virological features of CMV infection are associated with specific clinical and pathological measures of Alzheimer disease. CMV-seropositive and -seronegative cohorts were identified. Blood and CSF samples from the subjects in each cohort were analyzed for CMV-specific T-cell responses, T-cell phenotypes, and proinflammatory cytokines. Among CMV-seropositive subjects, the association of these immunological responses with markers of Alzheimer disease pathology were evaluated. Finally, the induction of Aβ in human cell cultures infected with CMV was assessed. For comparative purposes, the association of HSV-1 infection with these same measures was also determined.

## METHODS

### Source of Study Specimens

Specimens were obtained from subjects from the Religious Orders Study involving >1150 Catholic clergy at >40 sites across the United States. This is a longitudinal clinicopathological study of aging and dementia approved by the Rush University Medical Center Institutional Review Board. Participants without a known history of dementia were enrolled. Informed consent was obtained for annual clinical examinations, acquisition and analysis of blood samples, and an Anatomical Gift Act to donate brain and cerebrospinal fluid (CSF) at the time of death. Clinical and neuropathological evaluation focused on Alzheimer disease and other age-related conditions [29, 30]. The follow-up rate exceeds 95%, and the autopsy rate exceeds 90%.

### Alzheimer Disease Pathology

Tissue from 1 brain hemisphere was fixed in paraformaldehyde and paraffin embedded. Bielschowsky-silver-stained sections were reviewed to obtain a modified NIA-Reagan Alzheimer disease pathological diagnosis without regard for clinical diagnosis [29]. Specific and sensitive measures of Alzheimer disease pathology were obtained using immunohistochemical detection of Aβ (1:50; Clone 6F/3D; DAKO) plaques and paired helical filament (PHF)–tau (1:2000, clone AT8; Thermo Scientific Pierce) neurofibrillary tangles (NFTs). The amyloid burden was estimated using computer-assisted sampling (Stereo Investigator 9.0) and image analysis across 5 cortical regions (hippocampus, midfrontal, inferior temporal, inferior parietal, and occipital cortices). NFTs were also sampled and counted in the same regions to obtain the average cortical density of PHF-tau NFTs [31].

### Subject Selection

A total of 254 serum specimens from deceased subjects were screened for CMV immunoglobulin G (IgG) levels. CMV-seropositive subjects were selected to provide an even distribution among the following 3 diagnostic categories proximate to death, using accepted criteria as previously reported [29]: no cognitive impairment, mild cognitive impairment, and probable Alzheimer disease. Selection also required the availability of post mortem CSF and cryopreserved peripheral blood mononuclear cell (PBMC) specimens. Fifty-eight subjects were identified for the seropositive cohort (Table 1).

Thirty-nine seronegative subjects were selected for comparison (Table 1) on the basis of the availability of PBMC and CSF specimens. Diagnostic criteria were not used because the pool of seronegative subjects was much smaller than that of the seropositive subjects. However, the resulting distribution of diagnostic categories did not differ significantly between seronegative and seropositive subjects (P = .571; Table 1).

### Quantitation of CMV and HSV-1 Antibodies

CMV IgG antibody levels were measured in serum and CSF, using a cytomegalovirus-specific enzyme-linked immunosorbent assay (ELISA; GenWay Biotech, San Diego, CA). The OD was read at 450 nm, using a DuPont Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA). Quantitative results were

| Table 1. Comparison of Profiles of 58 Cytomegalovirus (CMV)–Seropositive and 39 -Seronegative Cohorts |
|-----------------|-----------------|-----------------|---|
| Characteristic  | CMV Seropositive | CMV Seronegative | P  |
| Age at death, y | 88.74 ± 7.51     | 84.41 ± 5.27    | .001 |
| Age at last evaluation | 88.18 ± 7.56 | 83.74 ± 5.24 | .001 |
| Sex             | Male 18          | 18              | .025 |
|                 | Female 40         | 21              |     |
| Clinical diagnosis | No cognitive impairment | 21 | 17 | .571 |
|                 | Mild cognitive impairment | 19 | 9 |     |
|                 | Probable Alzheimer disease | 18 | 13 | .092 |
|                 | HSV-1 status     |                 |     |
|                 | Seropositive 42  | 20*             |     |
|                 | Seronegative 16  | 17*             |     |

Data are no. of subjects or mean ± SD. Abbreviation: HSV-1, herpes simplex virus type 1.
* Data were missing for 2 subjects.
obtained using kit calibrators and recorded in international units per milliliter.

HSV-1 IgG in serum was measured semiquantitatively, using an HSV-1–specific IgG ELISA (GenWay Biotech). The OD was read at 450 nm, using the DuPont Kinetic Microplate Reader. Results were recorded as the ratio of the OD of the sample to the mean OD of the kit cutoff control. Ratio values of <1.0 were considered negative.

**Serum and CSF Cytokines**

Cytokine levels from serum and CSF were quantitated using the following human ELISA kits from Invitrogen (Carlsbad, CA): interferon γ (IFN-γ), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α). IP-10 levels were determined using the Human CXCL10/IP-10 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Assays were performed using kit protocols. Results were reported as picograms per milliliter.

**Flow Cytometry**

Cryopreserved PBMCs were incubated overnight at 37°C. Viability was determined by trypan blue staining. For seropositive subjects, approximately 2×10^6 viable cells were stimulated with a pool of 15-mer overlapping CMV pp65 peptides (BD Pharmingen, strain AD169; 1.75 µg/mL) with costimulatory antibodies CD28/CD49d and brefeldin A for 6 hours. Viable (Aqua Live/Dead; Invitrogen, Eugene, OR) CD3+CD4+ and CD3+CD8+ T cells were analyzed for intracellular expression of IFN-γ and TNF-α in response to the CMV peptides. A second tube of unstimulated cells was used as a negative control for CMV pp65 and for immunophenotyping of T cells. The following antibodies were used along with the Cytofix/Cytoperm (BD) kit for flow cytometric analysis: anti-CD3-Pacific Blue, anti-CD45RA-APC, anti-CCR7-PE-Cy7, and anti-TNF-α AF700 (BD Pharmingen, San Diego, CA); anti-CD8-APC-H7, anti-CD28-PerCP-Cy5.5, and anti-IFN-γ FITC (Becton Dickinson, BD, San Jose, CA); anti-CD4-PE-Texas Red (Life Technologies, Grand Island, NY); and anti-CD57-PE (Miltenyi, Cambridge, MA). Cells from seronegative subjects were also analyzed for immunophenotype by flow cytometry.

**Detection of Aβ in CMV- and HSV-1–Infected Cells**

Human foreskin fibroblast (HFF) monolayers were infected with low-passage CMV clinical strains (BI-1, BI-4, or BI-6) and cultured for 5–6 days. These strains were obtained from unrelated transplant recipients (using a protocol approved by the Rush University Medical Center Institutional Review Board), passage <10 times, and determined to be mycoplasma free. Cell-free stocks were generated by water lysis of infected monolayers. BI-4 and BI-6 remain phenotypically cell associated, while BI-1 exhibits cell-free infectivity (Figure 1). Additional HFF monolayers were infected with HSV-1 (F strain) and cultured for 24 or 48 hours. Monolayers were fixed in PIPES-formaldehyde and treated with H₂O₂ (3% in methanol) to quench endogenous peroxidases, followed by addition of FcR blocking reagent (Miltenyi Biotec, Auburn, CA) and, finally, another block of 5% horse serum. The following primary antibodies were used for individual monolayers: (1) mouse anti-human Aβ, clone 6F/3D, (Dako North America, Carpinteria, CA); (2) mouse IgG1 isotype control; (3) mouse cytomegalovirus monoclonal antibody (DDG9 and CCH2; ThermoScientific/Pierce); and (4) mouse anti-VP5 (6F10; HSV-1; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody was horse biotinylated anti-mouse IgG (H + L; Vector Laboratories, Burlingame, CA). Binding of the secondary antibody was detected by immunoperoxidase staining, using the Vectastain Elite ABC kit (standard) with diaminobenzidine substrate (Vector Laboratories) plus nickel. Stained monolayers were visualized using a Nikon Eclipse Ti-S inverted light microscope equipped with a Nikon Digital Sight DS-Fi1 color camera. Multiple images of the cytopathic effect of each virus stained with either anti-human Aβ or mouse IgG isotype control antibodies were analyzed using ImageJ 1.46r software (available at: http://imagej.nih.gov/ij/ [accessed 20 May 13]).

**Statistical Analysis**

The mean pixel transmission determined by ImageJ analysis for Aβ and IgG isotype control images obtained from the same experiment for each virus were compared using t tests and confirmed by use of Wilcoxon rank sum tests. Immunological and pathological data were summarized as box plots, dot plots, and descriptive statistics. Measures for the 3 diagnostic groups (ie, no cognitive impairment, mild cognitive impairment, and Alzheimer disease) were compared using Kruskal-Wallis tests. Associations between quantitative and ordinal measures were tested using Spearman rank correlation coefficients. Statistical significance was initially set at an α level of 0.05. For a more conservative assessment, a Bonferroni correction was used for the 4 diagnostic and pathological comparisons, which set statistical significance at an α level of 0.0125.

**RESULTS**

**Serum IgG**

Of the 254 serum specimens analyzed for quantitative CMV IgG levels, 192 (76%) were CMV-antibody positive, and 62 (24%) were CMV-antibody negative. The prevalence of CMV seronegativity was somewhat higher than the expected level for the age of the subjects (Table 1), compared with the general population [32]. The selected study cohort consisted of 97 subjects: 58 seropositive and 39 seronegative (Table 1).

CMV serum IgG levels ranged from 1.4 to 18.2 IU/mL (mean, 10.2 IU/mL; Table 2). There was a significant association of serum CMV IgG levels with the density of NFT and a marginal association with amyloid load (Table 3).
HSV-1 IgG levels were measured semiquantitatively in the serum samples of both CMV-seropositive and -seronegative subjects (Table 1). These levels were analyzed for association with clinical diagnosis and Alzheimer disease pathology. In contrast to CMV, no associations of HSV-1 IgG levels were found with clinical or pathological indices (Table 4).

**Quantitation of Serum Cytokines**

The levels of the proinflammatory cytokines IL-6, TNF-α, and IFN-γ were measured in serum (Table 2). Very few samples from either CMV-seropositive or -seronegative subjects had IL-6 or IFN-γ values above the lower cutoff for the respective ELISAs, while 20 seropositive serum samples had detectable TNF-α. The seronegative samples were not tested for TNF-α. There was no association with clinical diagnosis or Alzheimer disease pathology for any of the serum cytokines in either patient group.

**Quantitation of CSF Cytokines**

CSF specimens were analyzed for IL-6, TNF-α, IFN-γ, and IP-10. Among CMV-seropositive subjects, only 5 CSF specimens had detectable TNF-α levels. In contrast, >80% of specimens from seropositive subjects had IL-6, IFN-γ, and IP-10 levels above the assay lower cutoff value. The CSF IL-6 levels were not associated with clinical diagnosis or Alzheimer disease pathology. However, CSF IFN-γ levels from seropositive

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**Figure 1.** Induction of amyloid-β (Aβ) in human foreskin fibroblasts infected with cytomegalovirus (CMV) or herpes simplex virus type 1 (HSV-1). Isotype control is mouse immunoglobulin G (IgG) 1. **A,** CMV strain BI-1. **B,** CMV strain BI-4. **C,** CMV strain BI-6. CMV-infected monolayers were stained with anti-CMV antibody to immediate early/early antigen 6 days after inoculation. **D,** HSV-1 F strain, 24 hours after inoculation. **E,** HSV-1 F strain, 48 hours after inoculation. HSV-1–infected monolayers were stained with anti-HSV-1 antibody to VP5 antigen.
Table 2. Comparison of Cytokine Levels and T-Cell Phenotypes in Cytomegalovirus (CMV)–Seronegative and -Seropositive Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>CMV Seropositive</th>
<th></th>
<th>CMV Seronegative</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Number detected/Number tested</td>
<td>Mean ± SD</td>
<td>Number detected/Number tested</td>
<td></td>
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<tr>
<td>Serum level</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CMV IgG, IU/mL</td>
<td>10.20 ± 3.78</td>
<td>59/58</td>
<td>Not relevant</td>
<td>Not relevant</td>
<td></td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>27.72 ± 16.57</td>
<td>20/58</td>
<td>Not tested</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>25.71 ± 19.18</td>
<td>5/58</td>
<td>12.67 ± 4.04</td>
<td>3/39</td>
<td></td>
</tr>
<tr>
<td>IFN-γ, pg/mL</td>
<td>73.42 ± 17.27</td>
<td>2/58</td>
<td>0</td>
<td>0/39</td>
<td></td>
</tr>
<tr>
<td>CSF level</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>CMV IgG, IU/mL</td>
<td>4.59 ± 5.34</td>
<td>11/44</td>
<td>Not relevant</td>
<td>Not relevant</td>
<td></td>
</tr>
<tr>
<td>TNF-α, pg/mL</td>
<td>1649.80 ± 3603.58</td>
<td>5/58</td>
<td>Not tested</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>187.35 ± 180.54</td>
<td>55/58</td>
<td>106.04 ± 154.76</td>
<td>23/23</td>
<td>.047</td>
</tr>
<tr>
<td>IFN-γ, pg/mL</td>
<td>85.29 ± 46.26</td>
<td>47/58</td>
<td>0</td>
<td>0/23</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IP-10, pg/mL</td>
<td>376.37 ± 416.56</td>
<td>40/40</td>
<td>237.15 ± 284.73</td>
<td>22/22</td>
<td>.1223</td>
</tr>
<tr>
<td>CMV pp65 CD4+ T cells, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IFN-γ expression</td>
<td>0.38 ± 0.91</td>
<td>58/58</td>
<td>Not relevant</td>
<td>Not relevant</td>
<td></td>
</tr>
<tr>
<td>TNF-α expression</td>
<td>0.59 ± 1.15</td>
<td>58/58</td>
<td>Not relevant</td>
<td>Not relevant</td>
<td></td>
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<tr>
<td>CMV pp65 CD8+ T cells, %</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>IFN-γ expression</td>
<td>0.97 ± 0.99</td>
<td>58/58</td>
<td>Not relevant</td>
<td>Not relevant</td>
<td></td>
</tr>
<tr>
<td>TNF-α expression</td>
<td>1.06 ± 1.04</td>
<td>58/58</td>
<td>Not relevant</td>
<td>Not relevant</td>
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<tr>
<td>T-cell phenotype, %</td>
<td></td>
<td></td>
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<tr>
<td>CD4+CD28+CD57−</td>
<td>78.79 ± 10.41</td>
<td>39/58</td>
<td>96.83 ± 1.90</td>
<td>35/39</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CD4+CD28−CD57+</td>
<td>3.13 ± 3.96</td>
<td>39/58</td>
<td>0.20 ± 0.28</td>
<td>35/39</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CD8+CD28+CD57−</td>
<td>42.42 ± 17.14</td>
<td>39/58</td>
<td>69.65 ± 20.71</td>
<td>35/39</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CD8+CD28−CD57+</td>
<td>26.21 ± 15.91</td>
<td>39/58</td>
<td>15.10 ± 14.21</td>
<td>35/39</td>
<td>.0013</td>
</tr>
</tbody>
</table>

Abbreviations: CSF, cerebrospinal fluid; IFN-γ, interferon γ; IgG, immunoglobulin G; IL-6, interleukin 6; TNF-α, tumor necrosis factor α.

Table 3. Spearman Correlation Between Cytomegalovirus (CMV)–Related Clinical Data and Clinical Diagnosis and Markers of Alzheimer Disease (AD) Pathology Among CMV-Seropositive Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Clinical Diagnosis</th>
<th>AD Pathologic Diagnosis</th>
<th>Neurofibrillary tangle Density</th>
<th>Amyloid Load</th>
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<tr>
<td></td>
<td>ρ</td>
<td>P</td>
<td>ρ</td>
<td>P</td>
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<tr>
<td>Serum CMV IgG level</td>
<td>. . .</td>
<td>&gt;.075</td>
<td>. . .</td>
<td>&gt;.075</td>
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<tr>
<td>IFN-γ expression</td>
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<tr>
<td>CMV pp65 CD4+ T cells</td>
<td>. . .</td>
<td>&gt;.075</td>
<td>- . . .</td>
<td>&gt;.075</td>
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<tr>
<td>CMV pp65 CD8+ T cells</td>
<td>. . .</td>
<td>&gt;.075</td>
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<td>&gt;.075</td>
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<tr>
<td>T-cell phenotype</td>
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</tr>
<tr>
<td>CD4+CD28−CD57+</td>
<td>. . .</td>
<td>&gt;.075</td>
<td>. . .</td>
<td>&gt;.075</td>
</tr>
<tr>
<td>CD8+CD28−CD57+</td>
<td>. . .</td>
<td>&gt;.075</td>
<td>- . . .</td>
<td>&gt;.075</td>
</tr>
<tr>
<td>CSF level</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IL-6</td>
<td>. . .</td>
<td>&gt;.075</td>
<td>. . .</td>
<td>&gt;.075</td>
</tr>
<tr>
<td>IP-10</td>
<td>. . .</td>
<td>&gt;.075</td>
<td>. . .</td>
<td>&gt;.075</td>
</tr>
</tbody>
</table>

Statistical significance was initially set at an α level of 0.05. For a more conservative assessment, a Bonferroni correction was used, which set statistical significance at α level of 0.0125.

Abbreviations: CSF, cerebrospinal fluid; IFN-γ, interferon γ; IgG, immunoglobulin G; IL-6, interleukin 6; PHF, paired helical filament.

a Based on NIA-Reagan criteria.
b Using antibodies to PHF-tau.
c Using antibodies to amyloid beta.
subjects were marginally associated with clinical diagnosis and significantly associated with NFT density (Table 3).

CSF samples from only 23 of the 39 seronegative subjects were available for cytokine analysis. All of these CSF samples had detectable IL-6 and IP-10 (Table 2). Although the mean quantitative levels of CSF IL-6 and IP-10 were higher for seropositive as compared to seronegative subjects, the differences were not significant between the 2 groups. Among seropositive subjects, there was no association between CSF IL-6 levels and any of the markers of Alzheimer disease pathology and only a marginal association between CSF IP-10 and clinical diagnosis (Table 3).

Over 80% of CSF samples from seropositive subjects had detectable IFN-γ (mean level, 85.3 pg/mL), but no CSF samples from CMV-seronegative subjects had detectable IFN-γ. Replicate ELISAs performed on 3 different days gave similar negative results for the seronegative subjects, which confirmed that there was a highly significant difference between CSF IFN-γ levels in seropositive as compared to seronegative subjects (Table 2).

CMV-Specific T cells
Cryopreserved PBMCs from CMV-seropositive subjects were analyzed by flow cytometry to determine the percentages of CMV-specific CD4+ and CD8+ T cells by intracellular expression of IFN-γ and TNF-α in response to the CMV-specific antigen, pp65. Cells positive for both cytokines were detected in CD8+ and CD4+ T-cell subsets in all samples; however, mean percentages (Table 2) were lower than reported elsewhere [7, 15, 17, 18, 36, 38, 39]. One possible reason for the lower detectable response rate was the use of only a single CMV-specific antigen in the assay. Peptides from additional CMV antigens such as glycoprotein B and immediate early antigen would be expected to enhance the detection of CMV-specific T cells [36–38]. The association of the CD4+ T-cell response to pp65 antigen with the pathological diagnosis of Alzheimer disease (NIA-Reagan criteria) approached significance (Table 3), while the CD8+

T-cell response to pp65 was not associated with either clinical or pathological markers of Alzheimer disease.

T-Cell Phenotypes
The viability of stored PBMC samples was reduced in some cases. Consequently, only 40 of 58 seropositive subjects and 36 of 39 seronegative subjects had sufficient viable lymphocytes for flow cytometric phenotypic analysis. T-cell phenotypes were associated with CMV serostatus. (Table 2). For both CD4+ and CD8+ T cells, the percentage of CD28+/CD57− cells was significantly higher in the CMV-seronegative cohort, while the percentage of CD28−CD57+ (senescent) cells was significantly higher in the CMV-seropositive cohort. These results support previous observations of an increase in the percentage of highly differentiated effector T cells that is both CMV and age dependent [7, 15, 17, 18, 36, 38, 39]. Among CMV-seropositive subjects, there were marginal associations of the percentage of CD8+CD28−CD57+ T cells with the pathological diagnosis of Alzheimer disease by NIA-Reagan criteria and the percentage of CD28−CD57+ CD4+ T cells with the accumulation of Aβ (Table 3).

Immunocytochemistry of Cells Infected With CMV and HSV
It was previously reported that HSV-1 infection of cells in culture led to intracellular accumulation of Aβ [28, 40]. To investigate the comparable ability of CMV to induce accumulation of this Alzheimer disease pathological hallmark, HFFs were infected with either clinical strains of CMV or HSV-1 (F strain). HFFs were chosen because they are human cells known to be permissive for both CMV and HSV infection. CMV clinical strains BI-4 and BI-6 are cell associated, while BI-1 produces extracellular virus. For all 3 strains Aβ was only induced in cells showing the typical CMV cytopathic effect (Figure 1A–C). The results of quantitative analysis of anti-Aβ antibody staining as compared to IgG isotype control antibody staining, using Image J software, were highly significant (P < .001). Anti-HSV

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**Table 4. Spearman Correlation Between Herpes Simplex Virus Type 1 (HSV-1) Immunoglobulin G (IgG) Level and Clinical Diagnosis and Markers of Alzheimer Disease (AD) Pathology**

<table>
<thead>
<tr>
<th>HSV-1 IgG Level</th>
<th>Clinical Diagnosis</th>
<th>AD Pathologic Diagnosisa</th>
<th>Neurofibrillary tangle Densityb</th>
<th>Amyloid Loadc</th>
<th>CSF IFN-γ Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>P</td>
<td>p</td>
<td>P</td>
<td>p</td>
</tr>
<tr>
<td>Overall cohort (n = 95d)</td>
<td>−0.028</td>
<td>.788</td>
<td>−0.085</td>
<td>.413</td>
<td>0.027</td>
</tr>
<tr>
<td>CMV-seropositive subjects (n = 62)</td>
<td>0.067</td>
<td>.632</td>
<td>−0.167</td>
<td>.233</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Abbreviations: CMV, cytomegalovirus; CSF, cerebrospinal fluid; PHF, paired helical filament.

a Based on NIA-Reagan criteria.

b Using antibodies to PHF-tau.

c Using antibodies to amyloid beta.

d Data were missing for 2 CMV-seronegative subjects.
VP5 (major capsid) antibody staining showed that HSV-1 infection had spread throughout the monolayers at 24 and 48 hours after inoculation. However, Aβ did not appear to be induced at either time point (Figure 1D and 1E).

**DISCUSSION**

In this study of deceased and autopsied subjects from a clinical-pathological community cohort, we found associations of CMV-related immunologic and virologic characteristics with Alzheimer disease neuropathology and additional trends toward associations with clinical diagnosis. Direct links between CMV and Alzheimer disease pathology include (1) significant association of CMV-specific serum IgG antibody levels with NFTs; (2) CD4+ T-cell response to the CMV pp65 antigen with the Alzheimer disease pathological diagnosis (by NIA-Reagan criteria; Table 3), which approached significance; and (3) highly significant induction of Aβ, by CMV infection of HFF.

Indirect links between CMV infection and Alzheimer disease were also observed. Most importantly, there was a highly significant association of CSF IFN-γ with CMV serostatus. More than 80% of CSF samples from the seropositive cohort had measurable IFN-γ, while no IFN-γ was detected in any of the CSF samples from the seronegative subjects. Among seropositive subjects, CSF IFN-γ levels were associated with NFTs. These results show that CMV seropositivity is associated with CSF IFN-γ level, which in turn is associated with a pathological marker of Alzheimer disease.

T-cell phenotypes provide another indirect link between CMV infection and Alzheimer disease pathology. Percentages of CD4+ and CD8+ T cells with the CD28−CD57− phenotype were significantly lower among CMV-seropositive as compared to seronegative subjects, while percentages of CD4+ and CD8+ T cells with the CD28−CD57+ phenotype were significantly higher among seropositive as compared to seronegative subjects (Table 2). These results are in agreement with those reported by others [12, 34, 36, 39, 41]. Our study further demonstrated marginal associations of CD8+CD28−CD57+ T cells with a pathological diagnosis of Alzheimer disease (by NIA-Reagan criteria) and CD4+CD28−CD57+ T cells with accumulation of Aβ (Table 3). Thus, CMV seropositivity is associated with the CD28−CD57+ CD4+ T-cell phenotype and the CD28−CD57+ CD8+ T-cell phenotype, which in turn are associated with the hallmarks of Alzheimer disease neuropathology or pathological diagnosis of Alzheimer disease. CD28−CD57+ cells have been reported to produce IFN-γ [15, 16]. The higher percentage of these terminally differentiated cells observed in the CMV-seropositive subjects in the present study may be linked to the CSF IFN-γ levels found exclusively in this cohort.

HSV-1, which belongs to the same herpesvirus family as CMV, is a reported risk factor for Alzheimer disease because it is neurotropic, it causes encephalitis, and HSV-1 DNA has been detected in amyloid plaques [42]. Induction of Aβ by HSV-1 in cell culture has been reported [28, 43]. However, there is evidence that HSV-1 infection does not produce the same effects in vivo that have been observed with CMV. A study by Aiello et al looked at CMV and HSV-1 antibody levels and cognitive decline [3]. Subjects with higher CMV antibody levels showed higher rates of cognitive decline, but there was no similar association with HSV-1 antibody levels. Another study looked at the levels of T-cell subsets in CMV- and HSV-1-seropositive persons [17]. CMV seropositivity was highly associated with lower percentages of naive T cells (CD45RA+CCR7+CD27+CD28+) and an increased percentage of highly differentiated effector memory cells (CD45RA−/−CCR7−CD27−CD28−). In contrast, HSV-1 seropositivity did not have a significant effect on T-cell subset distribution. Our data did not show a correlation of HSV-1 antibody levels with any of the clinical or pathological hallmarks of Alzheimer disease. There also was no apparent induction of Aβ in HFF even when 100% of the monolayer exhibited the typical HSV-1 cytopathic effect. However, it is possible that the observed differences in induction of Aβ are dependent on virus strain and/or cell type.

There have been a number of studies of the effect of CMV on the distribution of T-cell subsets and correlation of these subsets with immunosenescence and immunopathology. In some cases, production of proinflammatory cytokines was linked with CMV seropositivity [7, 14, 33, 36, 39, 44, 45], and proinflammatory cytokines have been implicated in Alzheimer disease [19–21]. However, the relationship of potential lifelong CMV reactivation with age-related immunosenescence and mortality remains controversial [46]. The variable results from these studies may reflect the role of host genetic factors, as reported in studies of CMV in other disease states, as well as in immunocompetent persons. For example, CMV DNA has been detected in a large percentage of glioblastomas; however, the incidence of the disease is low, compared with the incidence of the virus [47]. In a report of a small group of individuals with increased longevity, there was no CMV-related alteration in T-cell subsets leading to immunosenescence, and the risk of mortality was 30% lower than that for the general population [45]. These findings suggest that it is the manner in which the immune system responds to the virus rather than the viral infection that is most important in determining the long-term outcome.

A limitation of the current study is the relatively small number of subjects. As a result, we have reduced the level for determining significance. Even under these more conservative criteria (α = 0.0125), the associations of CMV seropositivity with CSF IFN-γ and CMV serum IgG levels with Alzheimer disease pathology remain highly significant. There are also a number of associations between CMV seropositivity and pathological markers of Alzheimer disease that have P values of < .05 but do not reach significance under the more stringent criteria. By comparison, no association between HSV-1 seropositivity and...
any immunological or pathological marker of Alzheimer disease even approached significance.

The positive associations that we have found between CMV infection, the immune response to CMV, and ultimately Alzheimer disease are intriguing and suggest that a larger longitudinal study of the relationship of CMV with Alzheimer disease is warranted. If a definitive link between CMV infection and Alzheimer disease can be established, it is possible that anti-CMV therapy could be of benefit in controlling CMV reactivation. There is recent evidence to support this therapeutic approach. Antiviral agents were reported to inhibit the production of Aβ in HSV-1–infected cells [28]. Antiviral control of CMV in other diseases has been reported to reduce immune activation and subsequent disease progression [11, 44]. A recent report of an elderly mouse model showed reversal of immune senescence, using acyclovir antiviral therapy for murine CMV [48]. However, the significance for human CMV remains to be established because the 2 viruses share very little sequence homology and because human CMV is much less sensitive to acyclovir.

Currently, long-term antiviral therapy for patients with asymptomatic CMV infection is not feasible because of the toxicity of the limited number of approved drugs and the potential for drug resistance. This underscores the need for new effective antiviral treatments for CMV infection that have minimal side effects and, more importantly, the need for a vaccine to prevent CMV infection.

Notes

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