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Association of Prolonged Survival in HLA-A2+ Progressive Multifocal Leukoencephalopathy Patients with a CTL Response Specific for a Commonly Recognized JC Virus Epitope

Igor J. Koralnik,2✉ Renaud A. Du Pasquier,*† Marcelo J. Kuroda,† Jörn E. Schmitz,† Xin Dang,† Yue Zheng,† Michelle Lifton,† and Norman L. Letvin†

The role of JC virus (JCV)-specific CTL was explored in the immunopathogenesis of progressive multifocal leukoencephalopathy (PML). We identified a 9-aa epitope of the JCV capsid protein VP1, the VP1p100 peptide ILMWEAVTL, which is recognized by CTL of HLA-A2+ HIV+PML survivors. We then constructed an HLA-A*0201/VP1p100 tetrameric complex that allowed us to assess by flow cytometry the PBMC of 13 PML patients and 11 control subjects for the presence of JCV-specific CTL. VP1p100-specific CTL were detected by tetramer binding in VP1p100-stimulated PBMC of five of seven (71%) PML survivors and zero of six PML progressors (p = 0.02). Two of three HIV+ patients with a leukoencephalopathy resembling PML, but with no virologic evidence of JCV infection, also had detectable VP1p100-specific CTL in their PBMC. PBMC of eight HIV+ patients with other neurologic diseases and healthy control subjects had no detectable JCV-specific CTL. These data suggest that the JCV-specific cellular immune response may be important in the containment of PML, and the tetramer-staining assay may provide a useful prognostic tool in the clinical management of these patients. The Journal of Immunology, 2002, 168: 499–504.

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3Abbreviations used in this paper: JCV, JC virus; B-LCL, B-lymphoblastoid cell line; PML, progressive multifocal leukoencephalopathy.

Materials and Methods

Selection of study subjects

To measure the immune response against selected JCV nonamer peptides, a total of 24 HLA-A2+ study subjects was enrolled in this study, including 10 HIV+/PML patients, 3 HIV+/PML patients, 6 HIV+ patients, 5 healthy HIV+ control subjects. The diagnosis of PML was ascertained by clinical and neuroradiological criteria and confirmed by brain biopsy or by positive JCV PCR in the cerebrospinal fluid. Of the 10 HIV+/PML patients, 6 were survivors whose disease had improved or remained stable 2...
to 5.5 years after their initial diagnosis of PML. Three HIV+/PML patients had a progressive neurologic disease and a rapid fatal outcome in 7 wk to 5 mo after their diagnosis. One HIV+/PML patient had been diagnosed with PML 6 years before testing, and presented with clinical and radiological evidence of disease progression at the time of testing.

Of the three HIV+/PML patients, one who had a history of non-Hodgkin lymphoma was neurologically stable 8.5 years after her initial diagnosis. This patient had received Ara C for treatment of PML. The two other HIV+/PML patients had progressive disease and a fatal outcome 3–5 mo after the diagnosis of PML. One of them had a history of autoimmune CD30+ stem cell transplant for multiple myeloma, and the other had received a bone marrow transplant for treatment of acute myeloid leukemia. Of the six HIV+ patients without PML, three had a leukoencephalopathy that was clinically and radiologically consistent with PML, but with negative JCV PCR in the cerebrospinal fluid or brain biopsy specimen. The other three HIV+ patients had other neurological diseases, including a history of CMV polyradiculopathy, HIV encephalopathy, and thoracic polyradiculitis, respectively. All of these patients had negative JCV PCR in cerebrospinal fluid samples, and were survivors of their neurological diseases (Table I).

**Peptide selection**

A computer algorithm (http://bimas.dct.nih.gov/molbio/hla_bind/) was used to predict 9-aa peptides from JCV T, t, VP1, VP2, VP3, and agnoprotein for their likely ability to bind to the HLA-A*0201 molecule (15, 16). A total of 11 nonamer peptides from the T (n = 5), VP1 (n = 4), and VP2 (n = 2) proteins was selected and synthesized.

**Functional lysis assay**

PBMC from HLA-A2-positive individuals were isolated using a Ficoll-diatrizoate gradient. These PBMC were cultured in aliquots of 7 × 10^6 cells with pools of peptides derived from the T, VP1, and VP2 proteins, at a concentration of 1 μg/ml for each peptide. Cells were cultured in RPMI 1640 at a density of 3.5 × 10^5 PBMC/ml. After 72 h, an equal volume of RPMI/12% FCS containing 40 U/ml IL-2 was added to each culture well, and every 2 days thereafter half of the medium was changed. After 11–14 days, the peptide-stimulated cells were analyzed in a 51Cr release assay. EBV-transformed autologous B-lymphoblastoid cell lines (B-LCL) were used as target cells. Aliquots of 10^5 B-LCL were incubated overnight with either peptide pools or individual peptides at a concentration of 5 μg/ml. An OVA peptide (SIINFEKL) or the peptide p11B (ALSEGCTPYDIN) from the SIV was used as negative control peptides. After a 16-h incubation period, target cells were labeled with 100 μCi ^51Cr for 90 min. These cells were washed, and 10^5 cells were added to the effector cells as targets in 96-well U-bottom plates in a final volume of 200 μl/well. The assays were performed in duplicate. When a positive result was obtained using a peptide pool, the positive result was confirmed using individual 9-aa peptides.

**Construction of the tetrameric HLA-A*0201/VP1p100 complex**

The HLA-A*0201 protein was expressed in vitro from the plasmid HLA-A2/γ/β/Hir/BrA substrate peptide (17). The HLA-A*0201 protein was then refolded in vitro with human β2-microglobulin in the presence of the peptide VP1p100, as described (18). The HLA-A*0201/VP1p100 monomers were purified by gel filtration and biotinylated with the BirA enzyme (Avidity, Denver, CO). The biotinylated monomers were then mixed with PE-labeled streptavidin (Prozyme, San Leandro, CA) at a molar ratio of 4:1 to generate the HLA-A*0201/VP1p100 tetramers.

**Staining and phenotypic analysis of VP1p100-specific CD8+ T cells**

The mAbs used for this study were directly coupled to FITC, allophycocyanin, or PE-Texas Red. The following mAbs were used: anti-CD8α(SK1)-FITC (Becton Dickinson, San Jose, CA), anti-CD8α/β(28G15)-PE-Texas Red, and anti-CD3(UCHT1)-allophycocyanin (Beckman Coulter, Miami, FL). The PE-coupled tetrameric HLA-A*0201/VP1p100 and the three mAbs noted above were used in four-color flow cytometric analyses. Two hundred nanograms of the PE-coupled tetrameric HLA-A*0201/VP1p100 were used in conjunction with the directly labeled mAbs to stain 100 μl fresh whole blood or 5 × 10^6 lymphocytes that were cultured in vitro with the VP1p100 peptide. To remove RBCs, fresh blood samples were lysed using a Q-Prep Workstation (Beckman Coulter). The lysed samples were washed with PBS, and centrifuged for 3 min at 300 × g. Similarly, the stained cultured lymphocyte samples were washed in PBS and centrifuged for 3 min at 300 × g. The supernatants were decanted, and cells were resuspended in 0.5 ml PBS containing 1.5% paraformaldehyde. Samples were analyzed on a FACS Calibur Flow Cytometry System (Becton Dickinson). Data presentation was performed using WinMDI software version 2.7 (Joseph Trotter, La Jolla, CA) and Microsoft PowerPoint software version 97 (Microsoft, Redmond, WA).

**MHC class I typing**

The MHC class I alleles expressed by the study were determined using standard serologic tissue-typing procedures. In addition, molecular analyses to determine HLA-A*02 subtypes were performed on five subjects.

**DNA extraction from plasma**

Extraction of DNA from plasma samples was performed, as previously described (19). Four and a half milliliters of plasma were centrifuged at 2000 rpm for 5 min to remove remaining cells. Viral particles were collected by centrifugation of the supernatant at 214,000 × g, resuspended in Tris-NaCl-EDTA (TNE) buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA), lysed by adding SDS (1% final concentration), and incubated in the presence of 0.1 mg/ml proteinase K at 60°C for 1 h. DNA was then extracted with phenol/chloroform/isomyl alcohol, precipitated with ethanol, and resuspended in Tris-EDTA buffer.

**PCR amplification of JCV DNA**

PCR amplification of JCV DNA and detection of the amplified products were performed, as previously described (19), using the primer pair VP11/VP12, which flanks a 181-bp fragment of the VP1 gene (20). The PCR reaction was analyzed by electrophoresis on a 2% agarose gel and transferred onto nylon membranes by Southern blotting, and amplified products were detected by hybridization to the JCV-specific 32P end-labeled oligonucleotide probe IKVP1S, as described. The positive control JCV VP1 gene fragment was obtained as previously described (19). Using these conditions, we could reliably detect as few as 10 copies of JCV DNA. PCR oligonucleotide primer sequences: VP11, 5'-cagataccttgatgaagttctg-3' (nt 1662–1681); VP12, 5'-cattagaggcctagccctc-3' (nt 1842–1822); IKVP1S, 5'-ggagacttcctctgaagttc-3' (nt 1693–1717).

**Results**

We sought to develop a technical approach that would facilitate a simple and quantitatively precise measurement of JCV-specific

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**Table I. Detection of JCV VP1p100-specific effector cells in PBMC of PML patients and control subjects**

<table>
<thead>
<tr>
<th>Diagnosis*</th>
<th>Functional Lysis Assay</th>
<th>Fresh Blood Tetramer Staining</th>
<th>Cultured Cells Tetramer Staining</th>
</tr>
</thead>
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<td>HIV+/PML survivors (6)</td>
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<td>0/6</td>
<td>4/6</td>
</tr>
<tr>
<td>HIV+/PML survivors (1)</td>
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<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td>HIV+/PML progressors (4)</td>
<td>3/4</td>
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<tr>
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<td>0/2</td>
</tr>
<tr>
<td>HIV+/leukoencephalopathy (3)</td>
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<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>HIV+/OND (3)</td>
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</tr>
<tr>
<td>HIV controls (5)</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* PML, progressive multifocal leukoencephalopathy; OND, other neurologic diseases.
CTL in human PBMC. To this end, we explored the possibility that a CTL response might exist that recognizes a dominant JCV epitope presented to CD8+ T lymphocytes by a common MHC class I allele. A series of eleven 9-aa JCV peptides were synthesized that were predicted by computer algorithms to bind to the common MHC class I molecule A*0201.

PBMC from HLA-A2+ study subjects were stimulated with these peptides and assessed as effector cells in a 51Cr release assay using as target cells autologous B-LCL pulsed with each of these peptides. The JCV VP1p100-108 nonamer peptide ILMWEAVTL (VP1p100) was recognized by CTL from three of the six HLA-A2+ HIV+/PML survivors that were evaluated (Fig. 1). No VP1p100-specific CTL activity could be detected in the PBMC of one HIV−/PML survivor, six HIV+/PML patients who had progressive disease, two HIV+ patients without PML, and five normal control subjects. Interestingly, two HLA-A2+ HIV+ patients with leukoencephalopathy resembling PML by clinical and radiological criteria, but with negative JCV PCR in the cerebrospinal fluid or negative brain biopsy, had detectable CTL specific for this epitope (Fig. 1). These results suggested that VP1p100 was indeed an epitope recognized by CTL of HLA-A2+ PML survivors.

To confirm that CTL recognition of the VP1p100 epitope was HLA-A2 restricted, target cells that were fully MHC class I mismatched and target cells that shared only the A2 allele were selected from a panel of previously characterized B-LCL. Autologous B-LCL and these selected allogeneic B-LCL pulsed with VP1p100 were assessed as targets in a standard 51Cr release assay (Fig. 2). The autologous and A2+, but not the fully allogeneic target cells, were lysed by the A2+ effector cells. These experiments, therefore, confirmed that the VP1p100-specific CTL were HLA-A2 restricted in their target cell recognition.

Since our goal was to create an easily performed and quantitative assay for JCV-specific CTL, we used this knowledge of the VP1p100 epitope peptide presented to CTL by HLA-A*0201 to create a tetrameric HLA-A*0201/JCV peptide complex. Fresh whole blood and cultured lymphocyte specimens from 24 HLA-A2+ study subjects were stained with the tetrameric HLA-A*0201/VP1p100 complex, and analyzed by flow cytometry, gating on the CD8+CD3+ cells. These individuals were, for the most part, the same as those whose PBMC were assessed by the functional lysis assay. Two HIV+/PML progressors studied had rapidly progressive neurological disease that precluded obtaining PBMC for further functional testing, and B-LCL were not available from one HIV− individual with other neurological diseases (Table 1). No tetramer staining of CD8+ T cells from any of the fresh blood samples was observed. However, the lymphocytes of four of six HIV+/PML survivors had between 2.1% and 13.5% VP1p100-specific CD8+ T cells demonstrable after in vitro peptide stimulation. Tetramer binding was also detected to 1.1% of CD8+ T cells of one HIV+/PML survivor, and to 5.7% and 6% of CD8+ T cells, respectively, from two HIV+ individuals with leukoencephalopathy of unknown etiology (Fig. 3).

**FIGURE 1.** The JCV VP1p100 peptide is recognized by CTL from HLA-A2+ survivors of PML. Autologous target cells sensitized with the JCV VP1 peptide p100–108 ILMWEAVTL (VP1p100) were lysed by VP1p100-stimulated PBMC of three of six HLA-A2+ HIV-infected patients who were survivors of PML (a–f) and two HIV+ patients with JCV-negative leukoencephalopathy of unknown etiology (g and h), but not from VP1p100-stimulated PBMC of an HLA-A2+ HIV+/PML survivor (i), or an HIV+/PML patient with progressive disease (j). The percentage of specific lysis indicates the difference in specific 51Cr release between target cells pulsed with the VP1p100 peptide and those pulsed with the control peptide. E:T ratios are shown in the box in f.

**FIGURE 2.** Effector cell recognition of the JCV VP1p100 peptide is HLA-A2 restricted. Autologous (a), and HLA-A2-matched only (b) but not fully MHC class I-mismatched target cells (c) pulsed with VP1p100 were lysed by the PBMC of an HIV+/PML survivor.
To assess the correlation between the results of the functional lysis assay and the tetramer-staining assay, the percentage of tetramer-staining CD8+ T cells was compared with the percentage of specific lysis at an E:T ratio of 20:1 in the same cell population in 12 separate experiments performed on PBMC of seven patients who had detectable tetramer-staining CD8+ T cells (Fig. 4). A linear correlation between these values was seen, suggesting that the functional lysis assay and the tetramer-staining assay were measuring the same population of functionally active effector CTL.

To ascertain whether these results were reproducible, the functional lysis assay was repeated four times over a 1-year period using as effector cells VP1p100-stimulated PBMC from an HIV+/PML survivor. A mean percentage of specific lysis of 31 ± 6 was seen at an E:T of 20:1. The tetramer-staining assay was repeated three times on PBMC of this patient during the same time period, showing a mean percentage of tetramer-staining CD8+ T cells of 10.8 ± 2.4 (data not shown). Thus, these assays were quite reproducible.

JCV is rarely found in the peripheral blood of normal persons (19, 21–23). JC viremia occurs in the setting of immunosuppression, and JCV DNA has been detected in the blood of HIV+ patients with or without PML who have CD4+ cell counts below 200/μl (19, 21, 24–26). We sought to determine whether the presence of VP1p100-specific CTL was positively or negatively correlated with detectable JCV in the blood. In the 24 study subjects, JCV DNA could only be detected in plasma samples from 3 of 6 PML progressors (one HIV+ and two HIV- patients). Overall, PML progressors had lower peripheral blood CD4+ T cell counts (57 ± 68/μl) and higher HIV viral loads (67,800 ± 85,500 copies/ml) than PML survivors (CD4+ T cell counts 467 ± 248/μl, and HIV viral loads <50 copies/ml in five and 3694 copies/ml in one). Thus, the presence of VP1p100-specific CTL, as well as evidence of immune reconstitution demonstrated by high CD4+ T cell counts and suppression of HIV replication, correlated with undetectable JC viremia.

**Discussion**

Having defined an HLA-A*0201-restricted JCV CTL epitope and constructed an HLA-A*0201/JCV peptide tetramer complex, we were in a position to evaluate the frequency of JCV-specific CTL in the peripheral blood of various human populations. In fact, staining of fresh blood with the tetrameric HLA-A*0201/VP1p100 complex was negative in all study subjects, suggesting that the precursor frequency of VP1p100-specific CTL is less than 0.1% of CD8+ T cells, the limit of detection of this assay. However, PBMC from five of seven HIV+ and HIV- PML survivors and two of three HIV+ patients with leukoencephalopathy of unknown etiology demonstrated, after 2 wk of in vitro stimulation with the VP1p100 homamer, positive tetramer staining ranging from 1.1 to 13.5% of CD8+ T cells. These data indicate that the VP1p100 epitope is commonly recognized by CTL in these patients. Moreover, the tetramer-staining assay appears to be more sensitive than the functional lysis assay for detecting CTL specific for this epitope, since peptide-stimulated PBMC from two patients had undetectable cytolytic activity specific for the VP1p100 measured by a functional lysis assay, yet 2.1% and 1.1% of their VP1p100-stimulated CD8+ T cells, respectively, stained with the HLA-A*0201/JCV VP1p100 tetramer complex (Figs. 1 and 3, d and i). Finally, the VP1p100 epitope is located at nt position 1766–1792 on the JCV genome, which is conserved among JCV genotypes at the amino acid level (27).

**FIGURE 3.** Tetrameric HLA-A*0201/JCV VP1p100 complex binds to a population of VP1p100-stimulated CD8+ T lymphocytes in PBMC of HLA-A2+ survivors of PML. The percentage of all CD8+ T cells that bind this tetramer is indicated in each panel. Tetramer-positive cells were detected in VP1p100-stimulated CD8+ T lymphocytes of four of six HLA-A2+ HIV+/PML survivors (a–f), two HLA-A2+ HIV+ patients with JCV-negative leukoencephalopathy of unknown etiology (g and h), and one HLA-A2+ HIV-/PML survivor (i). Negligible tetramer binding is seen in the CD8+ T lymphocytes of an HLA-A2-, HIV+/PML patient with progressive disease (j). Study subjects represented in a–j are the same as those evaluated in the study shown in Fig. 1. Cells were gated on CD3+ CD8+ T lymphocytes. Results displayed in a–j represent staining with an anti-CD8αβ Ab and the tetrameric HLA-A*0201/JCV VP1p100 complex.

Importantly, PBMC from one HIV+/PML survivor and one HIV-/PML survivor had no demonstrable functional cytolytic activity specific for the VP1p100 (Fig. 1, d and i), yet 2.1 and 11.1% of their CD8+ T cells, respectively, were stained with the tetrameric HLA-A*0201/JCV VP1p100 tetramer complex (Fig. 3, d and i). PBMC from no study subject had demonstrable VP1p100-specific functional CTL and an absence of this tetramer staining.

**FIGURE 4.** Correlation between the functional lysis and tetramer-staining assays. A linear correlation is observed between the cytolytic activity of VP1p100-specific effector cells and percentage of VP1p100-stimulated cells staining with the HLA-A*0201/JCV VP1p100 tetramer complex: y = 0.33 × −1.2203; R^2, variance.
Since two of the seven PML survivors evaluated in this study did not have detectable JCV-specific CTL, we sought to determine how these individuals might differ from the other subjects in this cohort. Different HLA-A2 subtypes have been shown to be functionally distinct in their ability to bind and present peptide (28). In Caucasians, the A*0201 subtype is expressed in ~95% (29), and 49 other subtypes are expressed by the remainder of A2* individuals. The two HIV+/PML survivors who did not have demonstrable VP1\textsubscript{p100}-specific CD8+ T cells were assessed to determine whether they expressed A2 subtypes other than the A*0201. Molecular typing showed subtype A*0201 in one subject, and A*0206 in the other. The A*0206 molecule differs from A*0201 by only a single amino acid in the floor of the binding groove, and endogenous peptides eluted from this molecule have been shown to have a leucine in anchor positions 2 and 9, similar to those eluted from the A*0201 molecule (30). Therefore, we would expect the VP1\textsubscript{p100} peptide, which has a leucine in anchor positions 2 and 9, to be able to bind to the groove of the A*0206 molecule. Hence, the A*02 subtype of these two study subjects did not appear to be able to bind to the groove of the A*0206 molecule. The two HIV+/H11001 survivors who did not have detectable JCV-specific CTL in their PBMC.

Although PML is usually a rapidly fatal disease, approximately 10% of affected individuals survive more than 1 year (9). We were interested in determining whether the presence of a detectable cellular immune response against JCV in individuals with PML was associated with their clinical outcome. JCV-specific CTL were detectable in five of seven (71%) PML survivors, but none of the six PML patients with progressive neurologic disease (Fisher exact test, two-tailed,  \( p = 0.02 \)). This observation is consistent with the notion that this cellular immune response plays an important role in the prevention of disease progression (13). Therefore, the detection of JCV-specific CTL in PBMC of individuals with PML appears to be a good prognostic marker of disease evolution.

Two of three HIV+ patients with leukoencephalopathy of unknown etiology had detectable JCV-specific CTL in their PBMC. These two patients were also survivors of their neurologic disease, and were clinically stable 15–20 mo after their diagnosis. These two patients may indeed have PML, but with a JC viral load below 50 copies/ml cerebrospinal fluid, the level of detection of this assay (19). It is conceivable that the brain biopsy tissue sample came from an area of brain that was totally demyelinated and therefore devoid of JCV-infected oligodendrocytes. It is also possible that these patients were infected with an as yet undescribed polyoma-virus that shares CTL epitopes with JCV, yet is sufficiently distinct in sequence from JCV to evade detection by PCR using JCV-specific primers.

To determine whether the presence of JCV-specific CTL in PBMC was associated with the clearance of JCV from the blood, the plasma of all 24 study subjects was subjected to PCR amplification for the detection of JCV DNA. JCV DNA could only be detected in the plasma samples of three subjects, all of them individuals who were PML progressors. These results suggest that the presence of JCV-specific CTL in PBMC of patients with PML is associated with clearance of JCV from the blood. It should be noted that PML progressors also had lower CD4+ T cell counts and higher plasma HIV viral loads than PML survivors. Therefore, detectable JCV viremia in PML progressors may also reflect the depressed global immune status of these individuals.

JCV is found in renal tubular epithelial cells, and 30% of healthy individuals shed JCV in the urine (19, 31). JCV viremia, however, is usually not detectable in immunocompetent individuals (19, 21, 22). The mechanisms that contain JCV replication in normal individuals are not well understood. It is possible that JCV-specific CTL may prevent JCV spread in the bloodstream, but the CTL responses in these individuals might be below the level of detection of our assays, or they might be directed against other JCV epitopes.

The detection of JCV-specific CTL in survivors of PML suggests that these cells may be instrumental in the prevention of disease progression. The tetramer-staining assay may therefore have direct relevance as a prognostic tool in the clinical management of these patients.

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