The polyomavirus JC (JCV) infects 85% of healthy individuals, and its reactivation in a limited number of immunosuppressed people causes progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system. We hypothesized that JCV-specific cytotoxic T lymphocytes (CTLs) might control JCV replication in healthy individuals, blocking the evolution of PML. Using 51Cr release and tetramer staining assays, we show that 8 of 11 HLA-A*0201+ healthy subjects (73%) harbor detectable JCV-specific CD8+ CTLs that recognize one or two epitopes of JCV VP1 protein, the HLA-A*0201-restricted VP1p36 and VP1p100 epitopes. We determined that the frequency of JCV VP1 epitope-specific CTLs varied from less than 1/100,000 to 1/2,494 peripheral blood mononuclear cells. More individuals had JCV VP1-specific than cytomegalovirus-specific CTLs (8 of 11 subjects [73%] versus 2 of 10 subjects [20%, respectively]). These results show that a CD8+ T-cell response against JCV is commonly found in immunocompetent people and suggest that these cells might protect against the development of PML.
To assess the cellular immune response against another DNA virus which infects a majority of the adult population, we tested the PBMC of 2 of 10 individuals and that CTLS is a more sensitive technique than FBTS. The presence of functionally active effector cells in the positive (Fig. IA and B, panel c) but not in the negative (Fig. IA and B, panel d), sorted cell populations of both healthy individuals. The presence of CMV was performed in a clinical laboratory of our institution. We found that 3 of 11 subjects (27%) had anti-CMV IgG (subjects 6, 10, and 11). The serological analysis for CMV was performed by in vitro stimulation with the peptide VP1p36, we sought to determine if similar results would be seen with an anti-CMV IgG, was not tested for the presence of CMV-specific CTLs.

To determine the frequency of virus epitope-specific CTLs in the PBMC of these healthy individuals prior to any in vitro stimulation, we used the fresh blood tetramer staining (FBTS) assay and the CTL sorting (CTLS) technique, as previously described (7) (Table 2). With FBTS, the number of tetramer-positive cells was directly calculated and expressed as a number of virus epitope-specific CD8\(^+\) CTLs per PBMC in fresh blood. For the CTLs technique, 50 million PBMC were isolated from fresh blood, stained with a given phycoerythrin (PE)-conjugated tetramer, incubated with anti-PE microbeads (Miltenyi Biotec) and sorted with an AUTOMACS cell sorter (Miltenyi Biotec) into a tetramer-positive and a tetramer-negative fraction. This technique allowed us to detect very rare virus epitope-specific CTLs and calculate their frequency among unstimulated PBMC.

JCV VP1\(_{p36}\)-specific CTLs were detected by FBTS in the PBMC of 2 of 10 individuals. Based on this method, the frequency of these CTLs was 1/4,785 PBMC for one subject (subject 6) and 1/2,494 PBMC for the other subject (subject 9). We also determined the frequency of VP1\(_{p36}\)-specific CTLs by CTLS in subject 9 and found a frequency of 1/22,883 PBMC, 1 log lower than the frequency determined by FBTS. The CTLS method was used to evaluate the PBMC of two additional subjects (subjects 2 and 4) who had no detectable JCV VP1\(_{p36}\)-specific CTLs as determined by FBTS. A nonhomogenous quantity (Fig. 1A, panel a) and a minute quantity (Fig. 1B, panel a) of tetramer binding cells were detected in the positive fraction, compared to a negligible quantity detected in the negative fraction (Fig. 1A and B, panel b). These sorted cells were then stimulated with the VP1\(_{p36}\) peptide in the presence of irradiated autologous feeder cells, and rIL-2 (50 U/ml) was added after 72 h. An expansion of VP1\(_{p36}\) tetramer binding cells were readily detected after 11 to 14 days of stimulation in culture in the positive (Fig. 1A and B, panel e, but not in the negative (Fig. 1A and B, panel d), sorted cell populations of both healthy individuals. The presence of functionally active effector cells in the positive (Fig. 1A and B, panel e) but not the negative (Fig. 1A and B, panel f) sorted cell populations was furthermore demonstrated in a \(^{51}\)Cr release cell killing assay. The frequency of VP1\(_{p36}\)-specific CTLs could not be estimated with precision since the number of tetramer binding cells before in vitro stimulation was very low, being equal to or less than 1/100,000 PBMC. These results indicated that these cells were very rare in fresh blood from these two healthy individuals and that CTLS is a more sensitive technique than FBTS.

To rule out the possibility that VP1\(_{p36}\)-specific lymphocytes were being expanded de novo from the PBMC of healthy individuals by in vitro stimulation with the peptide VP1\(_{p36}\), we sought to determine if similar results would be seen with another well-characterized HLA-A*0201-restricted CTL epitope peptide to stimulate the expansion of CTLs. We stimulated the

### TABLE 1. Detection of JCV- and CMV-specific CTL in 11 healthy subjects

| Subject | Anti-JCV IgG titer | JCV VP1\(_{p36}\) F BTS | CCTS | \(^{51}\)Cr RA | JCV VP1\(_{p100}\) F BTS | CCTS | \(^{51}\)Cr RA | CMV pp65\(_{p495}\) F BTS | CCTS | \(^{51}\)Cr RA |
|---------|------------------|----------------|--------|----------|----------------|--------|----------|----------------|--------|----------|
| 1       | 1/16             | 0.6            | —      | 0.3      | —              | 0.6    | —        |
| 2       | 1/32             | —              | —      | —        | —              | —      | —        |
| 3       | 1/32             | 1.0            | —      | —        | —              | —      | —        |
| 4       | 1/32             | 4.2            | —      | —        | —              | —      | —        |
| 5       | 1/32             | —              | —      | —        | —              | —      | —        |
| 6       | 1/32             | 0.2            | 13.9   | 30       | —              | 10     | 19       |
| 7       | 1/32             | —              | 2.4    | —        | —              | —      | —        |
| 8       | 1/32             | 0.3            | —      | —        | —              | —      | —        |
| 9       | 1/32             | 0.3            | 17.7   | 32       | —              | 6.5    | 15       |
| 10      | 1/32             | —              | —      | —        | —              | —      | —        |
| 11      | 1/16             | NA             | —      | NA       | NA             | NA     | NA       |

a Results of tetramer staining assays are expressed as percentages of CD8\(^+\) T cells. Results of the \(^{51}\)Cr release assay are expressed as percentages of specific lysis of target cells by effector cells at an effector cell-to-target cell ratio of 20:1. Anti-CMV IgG was detected only in patients 6, 10, and 11. CTLS, cultured cell tetramer staining; \(^{51}\)Cr RA, \(^{51}\)Cr release assay performed with in vitro-stimulated PBMC; —, negative result; NA, not available.

To determine the frequency of virus epitope-specific CTLs in the PBMC of these healthy individuals prior to any in vitro stimulation, we used the fresh blood tetramer staining (FBTS) assay and the CTL sorting (CTLS) technique, as previously described (7) (Table 2). With FBTS, the number of tetramer-positive cells was directly calculated and expressed as a number of virus epitope-specific CD8\(^+\) CTLs per PBMC in fresh blood. For the CTLs technique, 50 million PBMC were isolated from fresh blood, stained with a given phycoerythrin (PE)-conjugated tetramer, incubated with anti-PE microbeads (Miltenyi Biotec) and sorted with an AUTOMACS cell sorter (Miltenyi Biotec) into a tetramer-positive and a tetramer-negative fraction. This technique allowed us to detect very rare virus epitope-specific CTLs and calculate their frequency among unstimulated PBMC.

JCV VP1\(_{p36}\)-specific CTLs were detected by FBTS in the PBMC of 2 of 10 individuals. Based on this method, the frequency of these CTLs was 1/4,785 PBMC for one subject (subject 6) and 1/2,494 PBMC for the other subject (subject 9). We also determined the frequency of VP1\(_{p36}\)-specific CTLs by CTLS in subject 9 and found a frequency of 1/22,883 PBMC, 1 log lower than the frequency determined by FBTS. The CTLS method was used to evaluate the PBMC of two additional subjects (subjects 2 and 4) who had no detectable JCV VP1\(_{p36}\)-specific CTLs as determined by FBTS. A nonhomogenous quantity (Fig. 1A, panel a) and a minute quantity (Fig. 1B, panel a) of tetramer binding cells were detected in the positive fraction, compared to a negligible quantity detected in the negative fraction (Fig. 1A and B, panel b). These sorted cells were then stimulated with the VP1\(_{p36}\) peptide in the presence of irradiated autologous feeder cells, and rIL-2 (50 U/ml) was added after 72 h. An expansion of VP1\(_{p36}\) tetramer binding cells were readily detected after 11 to 14 days of stimulation in culture in the positive (Fig. 1A and B, panel e, but not in the negative (Fig. 1A and B, panel d), sorted cell populations of both healthy individuals. The presence of functionally active effector cells in the positive (Fig. 1A and B, panel e) but not the negative (Fig. 1A and B, panel f) sorted cell populations was furthermore demonstrated in a \(^{51}\)Cr release cell killing assay. The frequency of VP1\(_{p36}\)-specific CTLs could not be estimated with precision since the number of tetramer binding cells before in vitro stimulation was very low, being equal to or less than 1/100,000 PBMC. These results indicated that these cells were very rare in fresh blood from these two healthy individuals and that CTLS is a more sensitive technique than FBTS.

To rule out the possibility that VP1\(_{p36}\)-specific lymphocytes were being expanded de novo from the PBMC of healthy individuals by in vitro stimulation with the peptide VP1\(_{p36}\), we sought to determine if similar results would be seen with another well-characterized HLA-A*0201-restricted CTL epitope peptide to stimulate the expansion of CTLs. We stimulated the

### TABLE 2. Determination of the frequency of virus epitope-specific CTL in fresh blood

| Subject | JCV VP1\(_{p36}\) F BTS | JCV VP1\(_{p100}\) F BTS | CMV pp65\(_{p495}\) F BTS |
|---------|----------------|----------------|----------------|
| 2       | 1/100,000     | —              | NA            |
| 4       | —              | —              | —              |
| 6       | 1/4,785       | NA             | 1/9,100       |
| 9       | 1/2,494       | 1/22,883       | 1/75,200      |
| 10      | NA            | NA             | 1/6,000       |

a Results of FBTS and CTLS are expressed as numbers of tetramer-positive cells/total numbers of PBMC; —, negative result; NA, not available.
PBMC of human immunodeficiency virus-negative (HIV−) subject 2 with the HIV Gag p77 peptide and evaluated the lymphocytes with the corresponding tetramer (18, 23). No tetramer binding cells were detected in the positive or negative lymphocyte fraction before or 2 weeks after in vitro stimulation with the HIV Gagp77 peptide (data not shown). Then, to rule out the possibility that HLA-A*0201/JCV VP1 p36 tetramer staining of the PBMC of healthy individuals was simply the result of a particularly high affinity of the VP1 p36 peptide for the HLA-A*0201 molecule, we compared the binding affinities of JCV VP1 p36, VP1p100, and HIV Gag p77 to the T2 cell line, which expresses only the HLA-A*0201 molecule (21). This study demonstrated that the binding affinities of these three peptides to the HLA-A*0201 molecule were similar (data not shown). All together, these results suggest that de novo expansion of VP1p36-specific CTLs in the PBMC of healthy individuals in vitro was highly unlikely. Finally, to examine whether JCV VP1 epitope-specific CTLs were able to recognize an epitope processed by cells expressing the entire VP1 protein, the PBMC of subject 4 were stimulated with VP1p36 in the presence of rIL-2 as described above. After 2 weeks, JCV VP1 p36 cells were sorted with the corresponding tetramer. These tetramer-positive cells were put back into culture in the presence of rIL-2 for an additional 2-week period and were used as effector cells in a 51Cr release assay (A and B, panels e and f). Tetr, tetramer; stim., stimulation; E:T ratio, effector cell/target cell ratio.

FIG. 1. Low frequencies of JCV VP1 p36-specific CTLs in two healthy individuals. Fifty million fresh PBMC of healthy subjects 2 and 4 were stained with the HLA-A*0201/JCV VP1 p36 PE-labeled tetramer and sorted with an AUTOMACS cell sorter with PE-labeled immunomagnetic beads. A positive (A and B, panel a) and a negative (A and B, panel b) fraction were collected and analyzed immediately after the cells were sorted by flow cytometry. Sorted cells were stimulated in vitro in the presence of VP1 p36 and feeder cells and stained with the VP1 p36 tetramer after 14 days (A, panels c and d) or 11 days (B, panels c and d). The percentage of all CD8+ T cells that bind the tetramer is indicated in each panel. These cells were then assessed for the presence of functionally active effector cells in a 51Cr release assay (A and B, panels e and f).
cells represented 0.2 and 1.8% of CD8αβ+ T cells or 1/9,100 and 1/6,000 PBMC, respectively. This result is similar to that reported in previous studies (2, 25). Therefore, we did not perform CTLs in these cases.

The underlying hypothesis of this study was that JCV-specific CTLs are present in the PBMC of healthy, immunocompetent subjects. Our results show that a majority of the JCV-infected healthy individuals studied (73%) had detectable JCV-specific CTLs in their blood. Studies of the cellular immune responses against CMV and Epstein-Barr virus, two other viruses that establish lifelong latent infections and cause severe disorders only in a minority of immunosuppressed individuals, have also shown a very good concordance rate between the results of serology and the detection of virus-specific CD8+ T cells. All subjects who were seropositive for CMV had detectable CMV-specific CD8+ T cells (9, 11, 25). Epstein-Barr virus-specific CD8+ T cells were also present in all seropositive healthy individuals at least 10 years after their seroconversion (3, 22).

Interestingly, more subjects had CTLs directed against JCV VP1p36 than against JCV VP1p100, and no subject had CTLs recognizing the latter epitope only. In addition, for those subjects who had a CTL response against both epitopes, JCV VP1p36 was always recognized by a greater number of CD8+ T cells than JCV VP1p100. These findings show that JCV VP1p36 is a more immunodominant epitope than JCV VP1p100 and might explain why we were unable to detect JCV VP1p100-specific CTLs in a limited number of healthy individuals in a previous study (13). This was confirmed by the determination of the frequency of JCV-specific CTLs prior to in vitro stimulation: JCV VP1p36-specific CTLs were found more often and in higher numbers in PBMC than JCV VP1p100-specific CTLs. The range of frequencies of VP1 peptide-specific CTLs in PBMC was relatively broad, from less than 1/100,000 to 1/2,494 PBMC. These values are similar to those reported for HIV+ patients with PML who had a favorable clinical outcome (7). Our results also indicate that CTLs is a more sensitive technique than FBTS to determine the frequency of JCV-specific CTLs. This conclusion is clearly illustrated by the facts that JCV VP1p36-specific CTLs could be detected in the PBMC of two subjects (subjects 2 and 4) and JCV VP1p100-specific CTLs could be detected in the PBMC of one subject (9) but that FBTS was negative in studies of all three subjects. However, when JCV-specific cells were frequent enough to be detected by FBTS (JCV VP1p36-specific CTLs in subject 9), the result was one log higher than with CTLs, reflecting the fact that CTLs underestimates the frequency of epitope-specific CTLs. Finally, the fact that JCV VP1p36-specific CTLs were able to recognize and destroy cells expressing the entire VP1 protein indicates that this epitope is indeed processed and presented on major histocompatibility complex I molecules by naturally infected cells. These data suggest that recognition of this epitope by CTLs might play a significant role in the containment of JCV in healthy individuals.

When we compared the frequencies of JCV-specific CTLs with those of CTLs specific to a well-known immunodominant epitope of CMV, we found that a greater number of individuals in our study harbored CTLs recognizing JCV VP1 epitopes than CMV pp65p26. This difference in the cellular immune responses against the two viruses correlates well with the results of the humoral immune response. Indeed, while all our study subjects had detectable anti-CMV IgG, only three of them had anti-CMV IgG. This phenomenon likely reflects a higher rate of infection by JCV than by CMV in our cohort. A possible explanation for this difference might be the young age of our subjects. More than 85% of adults in the beginning of their third decade are already infected by JCV, whereas the rate of infection by CMV is approximately 50% in the general population (9) and increases with age (12).

Do these JCV-specific CD8+ T cells play a role in preventing the development of PML in healthy individuals? In mice infected with CMV, the immune system contributes to preventing the onset of CMV-associated disease, and CD8+ T cells have been shown to be more critical than CD4+ and NK cells in this viral containment (19). The fact that CMV is a very slowly replicating virus provides time for the CMV-specific CD8+ T cells to recognize and lyse infected target cells before the formation of infectious virus (19). Interestingly, JCV is also a slow-growing virus (17). It is possible that virus-specific CD8+ T cells, even in low numbers, are able to prevent the spread of JCV. This viral control is quite effective in most immunosuppressed individuals, as reflected by the fact that only 0.07% of HIV+ patients with hematologic malignancies, 0.8% of liver transplant recipients, and 5.1% of AIDS patients develop PML (15, 20).

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