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Lymphoma Diagnosis and Plasma Epstein-Barr Virus Load during Vicriviroc Therapy: Results of the AIDS Clinical Trials Group A5211

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Abstract

Background—Lack of functional CCR5 increases the severity of certain viral infections, including West Nile virus and tickborne encephalitis. In a phase II trial of the investigational CCR5 antagonist vicriviroc (AIDS Clinical Trials Group protocol A5211), 4 lymphomas occurred in study patients who received vicriviroc. Because of the known association between unregulated Epstein-Barr virus (EBV) replication and lymphoma in immunocompromised patients, we evaluated whether vicriviroc exposure was associated with lymphoma EBV antigen positivity and/or had an effect on plasma levels of EBV DNA.

Methods—Clinical findings for all 4 patients enrolled in the A5211 study who developed lymphoma (2 Hodgkin and 2 non-Hodgkin) were reviewed, and tumor specimens were assessed for evidence

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of ongoing EBV replication. Longitudinal plasma samples from 116 patients in the A5211 study were analyzed, and EBV DNA was quantified by real-time polymerase chain reaction.

Results—Plasma EBV DNA was not detected in the 2 patients with non-Hodgkin lymphoma; both patients with Hodgkin lymphoma who had samples tested had EBV DNA levels <3200 copies/mL. One patient with Hodgkin lymphoma had a lymph node core biopsy specimen that was strongly positive for EBV; the other 3 lymphomas were histochemically EBV negative. None of the 116 patients with available samples experienced sustained increases in plasma EBV levels.

Conclusions—CCR5 antagonism by vicriviroc treatment in treatment-experienced patients was not associated with reactivation of EBV infection.

Ninety percent to 95% of the adult human population carries Epstein-Barr virus (EBV) as a chronic latent infection; the vast majority of these patients experience no serious sequelae. Healthy adults experience episodes of transient EBV viremia, with EBV DNA levels in whole blood usually <2000 copies/mL [1]. In HIV-infected patients, EBV has been associated with the development of AIDS-related diffuse large cell Hodgkin and Burkitt lymphomas [2]. The CCR5 antagonist maraviroc is approved for treatment-experienced patients with HIV infection [3,4], and several other agents are in various stages of clinical development [5,6]. Functional inactivation of CCR5 because of a homozygous 32 amino acid deletion of this protein *(CCR5Δ32/CCR5Δ32)* is generally well tolerated. However, studies of CCR5 knockout mice and epidemiological data from human *CCR5Δ32* homozygotes suggest that this deletion may increase the susceptibility to and severity of certain infections, including tickborne encephalitis and West Nile virus [7–13].

The AIDS Clinical Trials Group (ACTG) protocol A5211 was a randomized, placebo-controlled phase IIb study that assessed the safety and efficacy of the investigational CCR5 antagonist vicriviroc (VCV) in 118 treatment-experienced patients [14]. Among the 118 enrolled participants, 4 of 90 VCV recipients and none of 28 control subjects developed lymphomas within the first 13 months of VCV treatment. In light of the known link between EBV and lymphomas, we explored whether VCV treatment affected the level of EBV viremia over time.

PATIENTS, MATERIALS, AND METHODS

Patients

ACTG A5211 was a 48-week study of VCV (5, 10, or 15 mg once daily) versus placebo plus optimized background therapy in 118 HIV-1–infected, treatment-experienced patients (Clinical Trials registration NCT00082498) [14]. Plasma HIV-1 RNA levels were determined using the Amplicor HIV-1 ultrasensitive assay (Roche Molecular Systems), and coreceptor tropism was determined using the Trofile assay (Monogram Biosciences.) An independent Study Monitoring Committee periodically reviewed the study. A separate rollover study provided VCV to patients after 48 weeks.

Lymphoma histology and immunohistochemistry

Biopsy specimens from 4 patients from 4 study sites, including left axillary lymph node, left supraclavicular lymph node, right cervical lymph node, and left inguinal lymph node specimens, were examined during diagnostic examination at the originating institution, including testing for appropriate immunophenotypic markers. The results were then reviewed centrally by 1 pathologist. Hematoxylin and eosin–stained sections from all patients were reviewed; the lesions were categorized on the basis of World Health Organization classification [15].

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Additional phenotypic markers were assessed during central review of the specific cases as part of this study. The additional antibodies evaluated in this study included CD15 (Becton-Dickinson Immunocytometry Systems), CD30 (clone BerH2), CD45 (leukocyte common antigen; clone 2B11/PD7), BCL2 (clone 124), Ki-67 (clone MIB-1; DAKO Cytomation), CD10 (Novoastra-Vision Biosystems), and LANA (clone LN3; Advanced Biotechnologies). In situ hybridization for EBV was performed using an EBER probe (Vision Biosystems).

Immunohistochemical staining was performed on the Bond Max Autostainer (Vision Biosystems). Formalin-fixed, paraffin-embedded tissue sections were deparaffinized, and endogenous peroxidase was inactivated. For each antibody, antigen retrieval was performed using either the Bond Epitope Retrieval Solution 1 or the Bond Epitope Retrieval Solution 2 at 99–100°C for 20–30 min. After retrieval, the sections were incubated with the primary antibody for 25 min, followed by a postprimary step for 15 min and then the polymer for 25 min (Bond Polymer Detection System; Vision Biosystems). Colorimetric development was performed with diaminobenzidine (Vision Bio-systems). In situ hybridization for EBV was performed using the EBV Probe ISH Kit (Novocastra Laboratories) according to the manufacturer's instructions.

Real-time PCR

Available plasma samples from 116 patients were tested at study entry (week 0) and at weeks 2, 16, and 48. For 2 patients, 1 of whom was randomized to receive 10 mg of VCV and 1 of whom was randomized to receive 5 mg of VCV, no plasma samples were available. All 116 patients did not have samples available at all time points. All patients with lymphoma had samples available for analysis from baseline and from at least 1 additional time point. DNA was extracted from stored plasma samples with use of the QIAamp DNA blood kit (Qiagen), and EBV DNA was quantified by a real-time PCR assay designed to amplify a 135–base pair segment of BNRF1 [16]. All clinical samples were run in duplicate. Each assay run contained a negative control, a positive control derived from a commercially available standard with known EBV virion number (Advanced Biotechnology), and a standard dilution curve. The standard curve with a dilution range of 1×10^{0}–1×10^{7} copies/mL was generated and run in duplicate with use of a plasmid that contained the BamHI C fragment of B95–8 EBV cloned into the BamHI site of pBR322. The assay detected down to 1 copy/reaction but did so with a loss of linearity and with decreased sensitivity. For this reason, we assigned a cutoff threshold of 20 copies/reaction; when corrected for, the input volume resulted in a detection threshold of 600 copies/mL of plasma. In 3 of 3 positive control subject samples with confirmatory commercially available positive EBV DNA results from whole blood, we detected EBV DNA in plasma by our real-time assay.

RESULTS

Lymphomas in ACTG A5211

As of 8 April 2008, the data cutoff date for the analysis of ACTG A5211 long-term outcome, there were 295.3 total patient-years of follow-up. This included follow-up time while participants were receiving placebo and follow-up time while participants received and did not receive the study drug. Four cases of lymphoma occurred in patients receiving VCV, including 1 case of recurrent Hodgkin lymphoma (HL), 1 case of non-Hodgkin lymphoma (NHL) in a patient with previously treated HL, and 2 de novo cases of lymphoma (1 HL and 1 NHL); this resulted in a lymphoma rate of 1.35 cases per 100 patient-years. No lymphomas occurred in placebo recipients. At their review 5 months after completion of study enrollment, the independent Study Monitoring Committee responsible for ongoing review of ACTG A5211 concluded that, although there was biologic plausibility, it was not possible to draw any conclusions about a causal relationship between VCV and malignancy because of the small

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number of participants and the advanced stage of HIV-1 infection in the individuals enrolled in the study. The committee recommended that patients be unblinded and continue study follow-up. Subsequently, the US Food and Drug Administration required that any patient exposed to a CCR5 antagonist as part of a clinical trial should be followed up for at least 5 years for the development of malignancy.

Baseline and serial plasma HIV-1 RNA levels, CD4 cell counts, and viral coreceptor use in the 4 patients who developed lymphomas are shown in figure 1. Case 1 involved a 51-year-old man with a history of HL (during 1999) in remission who was randomized to receive 10 mg of VCV daily. Eight weeks after enrollment in the study, he presented with back and bilateral hip pain. CT revealed lymphadenopathy involving the left supraclavicular, hilar, mediastinal, peripancreatic, retroperitoneal, and pelvic lymph nodes; there was no evidence of extranodal involvement. The patient received a diagnosis of recurrent HL on the basis of examination of a supraclavicular lymph node needle core biopsy specimen ~9 weeks after starting VCV therapy. The patient declined treatment after diagnosis and died 5 months later.

Case 2 occurred in a 47-year-old man with HL in remission who was randomized to receive 15 mg VCV daily. The initial lymphoma was diagnosed in 2002, for which he received 6 cycles of doxorubicin, bleomycin, vinblastine, and dacarbazine. Six months after enrollment in the study, the patient presented with a 7 × 8–cm left axillary mass that was diagnosed as diffuse large B cell NHL (stage IIIB) on the basis of examination of a cervical node biopsy specimen; no extranodal disease was noted. He received 5 cycles of cyclophosphamide, doxorubicin, and etoposide, which induced remission. Eight months after his diffuse large B cell NHL diagnosis, the patient received a diagnosis of recurrent disease and declined further treatment. The patient died 1 week later.

Case 3 occurred in a 49-year-old man with no history of lymphoma who was randomized to receive 5 mg of VCV daily. After 10 months, the dose was increased to 15 mg, and the patient continued to receive this increased dose through a rollover study. Thirteen months after the initiation of VCV therapy, he presented with weight loss, abdominal pain, and lymphadenopathy, and he received a diagnosis of mixed cellularity HL (stage IVA) on the basis of examination of lymph node and bone marrow biopsy specimens. Abdominopelvic CT findings were consistent with splenic involvement. The patient received 6 cycles of dose-modified doxorubicin, bleomycin, vinblastine, and dacarbazine but died 7 months after diagnosis.

Case 4 occurred in a 44-year-old man with no history of lymphoma who was randomized to receive 5 mg of VCV daily and continued to receive that dose for 6 months. The dose was increased to 15 mg after virologic failure, in accordance with a protocol amendment. Twelve days later, the patient presented with fevers, chills, drenching sweats, and axillary and inguinal lymphadenopathy. CT and positron emission tomography revealed diffuse enlarged, hypermetabolic lymph nodes and evidence of pulmonary, hepatosplenic, and omental involvement. He was diagnosed with diffuse large cell lymphoma (stage IV) on the basis of examination of lymph node and bone marrow biopsy specimens. He was treated with rituxan and cyclophosphamide, adriamycin, vincristine, and prednisone but died 5 months later.

Morphologically, cases 1 and 3 involved Reed-Sternberg cells and variants in a mixed inflammatory cell background (figure 2). Immunostaining revealed that the Reed-Sternberg cells and variants in all cases of HL were CD45 negative and CD15 and CD30 positive. All tumors, with the exception of the tumor from patient 1, were also CD20 positive. In situ hybridization showed that the Reed-Sternberg cells and variants were positive for EBV; however, results of immunostaining with an antibody to ORF73 (LANA) of Kaposi sarcoma–associated herpesvirus were negative. Examination of the lymph node biopsy specimen from

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patient 4 revealed sheets of malignant-appearing large lymphoid cells. Immunophenotypically, the cells were CD10 positive (indicative of germinal center cell origin) and BCL-2 positive and focally exhibited a high proliferation rate based on Ki67 expression (95%). The tumor cells were negative for both EBV and Kaposi sarcoma–associated herpesvirus on the basis of in situ hybridization and immunostaining, respectively. In patient 2, who also received a diagnosis of diffuse large cell lymphoma, the infiltrate was somewhat more polymorphic, but mitotic figures and apoptotic debris were easily identified. The cells were relatively round and more variable in size. Immunostaining revealed that the tumor cells were not infected with Kaposi sarcoma–associated herpesvirus on the basis of lack of immunoreactivity for LANA; although the tumor cells were negative for EBV, in situ hybridization revealed the presence of a few scattered small nontumor EBER-positive cells.

Analysis of samples from ACTG A5211

The real-time PCR assay used to detect EBV DNA in plasma was both accurate and reproducible. A standard curve generated with triplicate measurements demonstrated an r^2 value of 0.9977, with intra-and interassay coefficients of variation of 0.37%–2.03% (3–6 replicates) across a nominal EBV DNA range of $1 \times 10^{1.3–7}$ copies (data not shown). Of the 116 patients analyzed, 110 had EBV DNA PCR results from both study entry and follow-up. Six patients had missing baseline EBV samples—including 1 patient who was randomized to receive placebo and 5 who were randomized to receive VCV—and were excluded from further analyses. For the 110 patients with baseline EBV data, the mean plasma HIV-1 RNA level was 4.6 $\log_{10}$ copies/mL (range, 2.6–6.5 $\log_{10}$ copies/mL), and the mean CD4 cell count was 179 cells/mm^3 (range, 5–1133 cells/mm^3) at baseline. Fifteen patients had EBV DNA levels >600 copies/mL at any time point: 4 (15%) of 27 patients from the placebo arm and 11 (13%) of 83 from the VCV arms. Six (5.5%) of 110 patients, all randomized to the VCV arms, had detectable EBV DNA at baseline (mean EBV DNA level, 2857 copies/mL; range, 632–5688 copies/mL) (table 1). In 5 (83%) of these 6 patients, plasma EBV DNA levels decreased to <600 copies/mL by the last time point available for analysis. Among the 104 patients with an EBV DNA level below the limit of quantification at study entry and with at least 1 result available during follow-up, 5 (6%) of 77 patients in the VCV arms and 4 (15%) of 27 patients in the placebo arm developed EBV levels >600 copies/mL at some point during the study ($P = .23$, by Fisher's exact test).

EBV DNA was not detected in 1 patient treated with VCV who developed gastric adenocarcinoma (data not shown). Two of the 4 patients who developed lymphoma had detectable EBV DNA: one had detectable plasma EBV DNA at the entry visit that decreased to <600 copies/mL by week 2, and the other had undetectable plasma EBV DNA at study entry but subsequently developed detectable low-level EBV viremia (table 2). The time of onset of lymphoma relative to study entry varied from 9 to 54 weeks. A correlation between EBV positivity in tumor biopsy samples and early detectable plasma EBV viremia was seen only in case 3. No plasma samples were available for analysis near the time closest to the lymphoma diagnosis.

DISCUSSION

Because of the increased risk of severe neuroinvasive West Nile virus disease and mortality among persons homozygous for the *CCR5Δ32* allele, we investigated whether the CCR5 antagonist VCV was associated with reactivation of EBV, a known oncogenic virus. Changes in EBV replication would be especially relevant, given the well-documented increased risk of EBV-associated lymphomas in patients with advanced HIV infection. For the 4 patients in ACTG A5211 who developed lymphoma, no clear pattern of tumor EBV positivity, VCV dose, VCV exposure, or time to diagnosis from study entry was identified. These patients were

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already at a high risk for lymphoma (both NHL and HL) because of their underlying HIV-1 infection [17,18], particularly those who had evidence of EBV infection. However, both NHL biopsy specimens were EBV negative by in situ hybridization. Lymphoid tissue samples from the 2 patients with HL had EBV-positive Reed-Sternberg cells, but it has been well-documented that the Reed-Sternberg cells in HIV-related HL are typically EBV positive [19,20]. Furthermore, all patients were negative for Kaposi sarcoma–associated herpesvirus [21]. The incidence of HL is greater among patients with moderate immunosuppression (CD4 cell count, 225–249 cells/mm^3) than it is among those with severe immunosuppression (CD4 cell count, 0–24 cells/mm^3) [22]. An increase in CD4 cell count (seen in case 1 in our study) may be required to provide the milieu necessary for the growth and propagation of the Reed-Sternberg cells.

We did not find an association between VCV therapy and plasma EBV DNA levels in our analysis of these very treatment-experienced patients with advanced HIV disease. This finding was true for the study population as a whole, as well as for the patients who received a diagnosis of lymphoma. Patients who developed lymphoma had either low EBV DNA levels (<3200 copies/mL) or undetectable levels at the time points available for analysis. The highest EBV DNA level detected in plasma from any patient was 5688 copies/mL. By contrast, patients with infectious mononucleosis, posttransplantation lymphoproliferative disease, or AIDS–related NHL are reported to have mean plasma EBV DNA levels of 6,400, 540,000, and 1,000,000 copies/mL, respectively [23,24]. The interval from collection of the last plasma samples available for analysis to the onset of lymphoma, however, precludes us from ruling out an increase in EBV levels at the time of diagnosis.

Circulating EBV DNA can be detected in ~20% of asymptomatic HIV-infected patients receiving antiretroviral therapy [25]. Varying methods and standards for EBV DNA detection, however, make direct comparisons of EBV levels across studies problematic. Although HIV-infected patients are more likely to have detectable plasma EBV DNA than are HIV-uninfected control subjects, the absolute level of EBV DNA is not predictive of progression to AIDS-related NHL [26,27]. EBV DNA can be detected in plasma from the vast majority of patients with EBV-associated Hodgkin disease; antineoplastic therapy typically reduces circulating levels of EBV DNA [28,29]. Initiation of antiretroviral therapy does not appear to affect EBV DNA levels in a consistent manner, at least in PBMCs [25,26,30,31].

The occurrence of malignancies in patients in ACTG A5211 who received VCV raised concerns about the oncogenic potential of CCR5 antagonists and VCV specifically. The incidence of AIDS-related lymphomas among patients in ACTG A5211 who received VCV was 1.35 cases per 100 patient-years. No lymphoma diagnoses were made for placebo recipients. The observed differences in lymphoma rates between treated patients and control subjects could have been affected by the small total number of participants in the study, the 3-fold greater number of VCV recipients, and their significantly longer follow- up period, compared with placebo recipients. The overall lymphoma rate includes 2 patients with treated HL (thought to be in remission); patients with treated HL are clearly at risk for late-onset relapse and have a markedly increased risk of subsequent NHL [32–35]. The absolute lymphoma rate in ACTG A5211 is not dissimilar to historical AIDS-related lymphoma rates [36]. Persons enrolled in the EuroSIDA cohorts from the post-HAART era, who had CD4 cell counts of 100–200 cells/mm^3 and median plasma viral loads similar to those in participants in ACTG A5211, experienced 0.5–1.0 NHL events per 100 patient years [37]. The post-HAART era incidence of systemic AIDS-related lymphoma in patients with CD4 cell counts of 100–199 cells/mm^3 in a French cohort was 0.73 cases per 100 patient-years [38]. Historically, *CCR5Δ32* heterozygotes have had lower rates of AIDS-related lymphomas [39].

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The technical limitations of our study deserve mention. Measurement of EBV DNA levels in whole blood has greater sensitivity than does measurement in plasma, may more accurately reflect total EBV burden for a given patient, and may not correlate with plasma levels of EBV DNA [40]. It is possible, therefore, that a subtle increase in PBMC-associated EBV DNA level could have been missed in our analysis. EBV status was not determined prior to treatment randomization, nor were participants randomized on the basis of use of concurrent medications with possible anti-EBV activity (e.g., acyclovir and its derivatives).

The occurrence of lymphomas in ACTG A5211 does not appear to be related to an increased plasma EBV burden associated with VCV exposure, and a causal association of VCV with lymphoma is uncertain. Other mechanisms by which CCR5 blockade could contribute to the development of lymphoma (e.g., by interfering with tumor surveillance) could be proposed, but a study of VCV in 116 treatment-naive patients found no increased incidence of lymphoma [41,42]. Moreover, trials of maraviroc, in which >1000 persons received a CCR5 antagonist, have not shown an increased rate of lymphoma [3]. These findings have tempered concerns about a more general class-specific effect of CCR5 antagonism. Ongoing phase III clinical trials of VCV and continued long-term follow-up of patients treated with CCR5 antagonists, including evaluation of malignancies, will provide additional safety data on this novel class of antiretroviral drugs.

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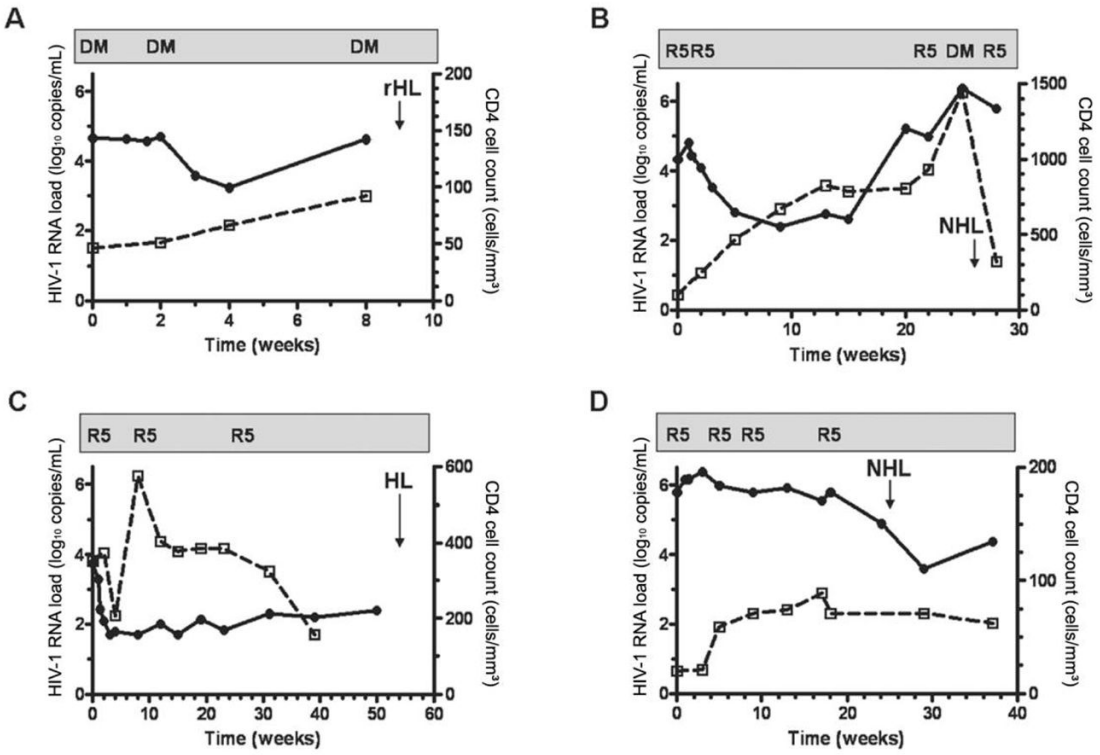

Figure 1.

Serial plasma viral loads, CD4 cell counts, and viral coreceptor use in 4 patients who developed lymphoma while receiving vicriviroc (VCV) during AIDS Clinical Trials Group A5211. *A,* Case 1 (Hodgkin lymphoma [HL]). *B,* Case 2 (non-Hodgkin lymphoma [NHL]). *C,* Case 3 (HL). *D,* Case 4 (NHL). HIV RNA levels are represented by closed circles, and CD4 cell counts are represented by open squares. Vertical arrows denote the time of lymphoma diagnosis. For patients 1 and 3, the lymphoma diagnosis occurred after the last available CD4 T cell count and plasma HIV-1 RNA load. Patient 1 had a screening coreceptor use assay (Trofile) performed 2 weeks prior to study entry that demonstrated CCR5-using virus only. At study entry and at all subsequent time points analyzed, however, dual-mixed virus was detected. Protocol-defined virologic failure was met when patients did not achieve a confirmed reduction in HIV-1 RNA load of ≥ 1 $\log_{10}$ copies/mL by week 16. DM, both CCR5- and CXCR4-using viruses detected; R5, CCR5-using virus only detected; rHL, recurrent HL.

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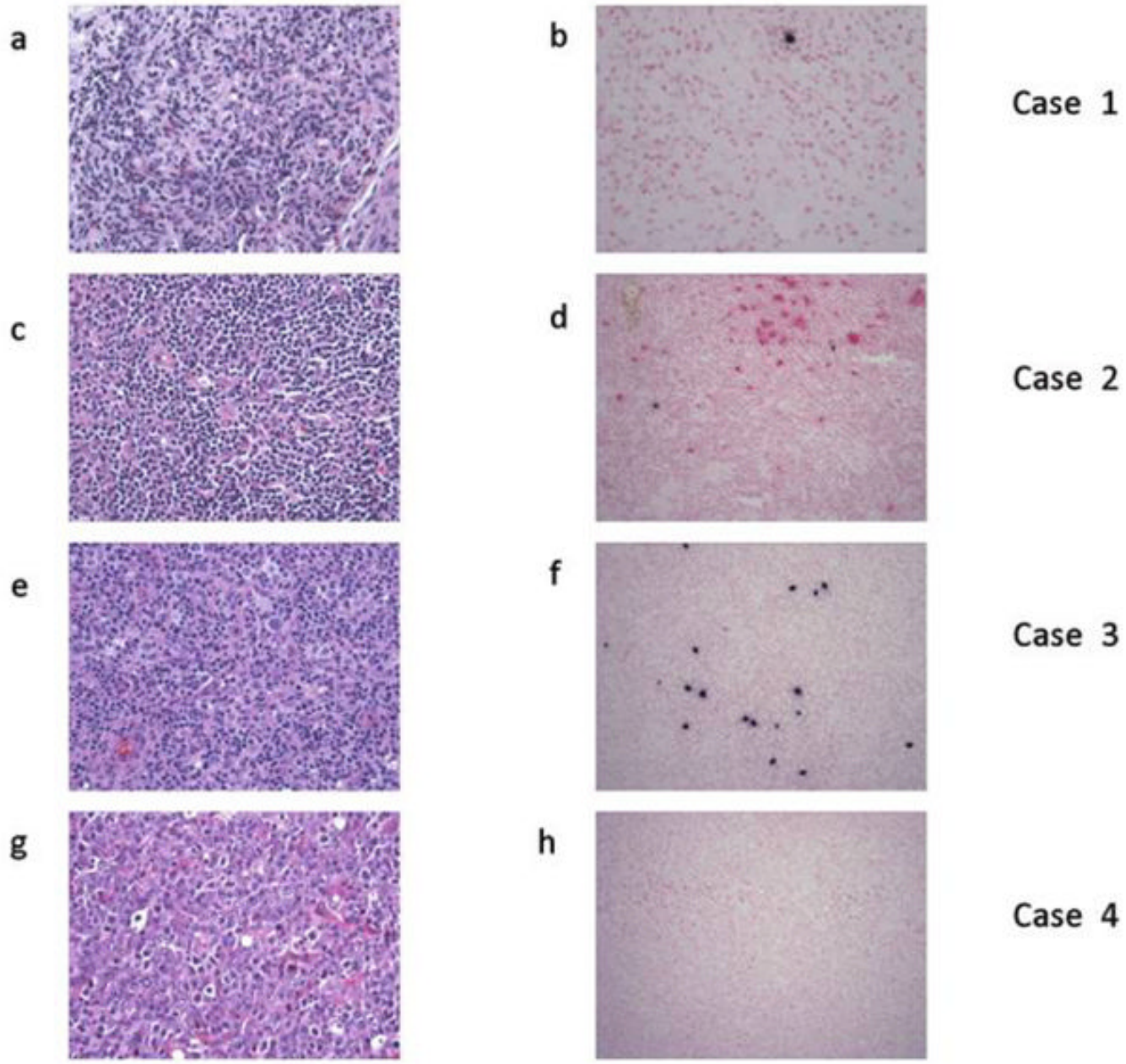

Figure 2.

Lymphoma histology and corresponding Epstein-Barr virus (EBV) immunohistochemistry of the 4 cases of lymphoma diagnosed in AIDS Clinical Trials Group A5211. *A*, Case 1 (Hodgkin lymphoma [HL]). *B*, Negative EBV stain findings in case 1, with the exception of Reed-Sternberg cells. *C*, Case 2 (diffuse large B cell non-Hodgkin lymphoma). *D*, Negative EBV stain findings in case 2, with the exception of Reed-Sternberg cells. *E*, Case 3 (HL). *F*, Strongly EBV-positive lymph node biopsy specimen from patient 3 (case 3). *G*, Case 4 (diffuse large B cell non-Hodgkin lymphoma. *H*, Negative EBV stain findings in case 4.

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Table 1

Plasma Epstein-Barr virus (EBV) DNA levels in patients from the AIDS Clinical Trials Group A5211 who had detectable plasma EBV DNA at baseline, by vicriviroc treatment arm.

	Plasma EBV DNA level, copies/mL, by treatment arm and patient					
	5 mg	10 mg		15 mg		
Week	Patient 13	Patient 17	Patient 87	Patient 10	Patient 85	Patient 88
0	632	3135	1430	3317	2941	5688
2	<600	<600	1364	<600	24,027	622
16	<600	NA	<600	<600	<600	<600
48	<600	NA	<600	1010	<600	<600

NOTE. All patients continued to receive vicriviroc treatment at each time point. NA, not available.

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Table 2

Plasma Epstein-Barr virus (EBV) DNA levels in patients who developed lymphoma while enrolled in AIDS Clinical Trials Group 5211.

Patient	Diagnosis	Week of onset	Week of study completion	Initial treatment arm, mg	Plasma EBV load, copies/mL			
					Week 0	Week 2	Week 16	Week 48
1	HL		23	10	3135	<600	NA	NA
2	DLBCL	26	48	15	<600	<600	<600	NA
3	HL	54	48	5	<600	2443	1995	NA
4	DLBCL	25	48	5	<600	NA	<600	NA

NOTE. DLBCL, diffuse large B cell lymphoma; HL, Hodgkin lymphoma; NA, not available.

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