Cancer Therapy: Preclinical

Clinical Cancer Research

Viral Response to Chemotherapy in Endemic Burkitt Lymphoma

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Abstract

Purpose: Some EBV-directed therapies are predicted to be effective only when lytic viral replication occurs. We studied whether cyclophosphamide chemotherapy induces EBV to switch from latent to lytic phases of infection in a series of EBV-associated Burkitt lymphomas.

Experimental Design: Children with first presentation of an expanding, solid maxillary or mandibular mass consistent with Burkitt lymphoma underwent fine-needle aspiration just prior to the initiation of cyclophosphamide therapy and again 1 to 5 days later. Aspirated cells were examined for latent and lytic EBV infection using *in situ* hybridization to EBV-encoded RNA (*EBER*), immunohistochemical analysis of the lytic EBV proteins BZLF1 and BMRF1, reverse transcription PCR targeting BZLF1 transcripts, and EBV viral load measurement by quantitative PCR.

Results: Among 21 lymphomas expressing *EBER* prior to chemotherapy, 9 of 10 still expressed *EBER* on day 1 after therapy whereas only 2 of 11 (18%) specimens still expressed *EBER* at days 3 to 5, implying that chemotherapy was fairly effective at eliminating latently infected cells. Neither of the lytic products, BZLF1 or BMRF1, were significantly upregulated at the posttherapy time points examined. However, EBV genomic copy number increased in 5 of 10 samples 1 day after treatment began, suggesting that viral replication occurs within the first 24 hours.

Conclusion: Cyclophosphamide may induce the lytic phase of EBV infection and is fairly effective in diminishing *EBER*-expressing tumor cells within 5 days. These findings provide the rationale for a trial testing synergistic tumor cell killing using cyclophosphamide with a drug like ganciclovir targeting lytically infected cells. *Clin Cancer Res; 16(7); 2055–64. ©2010 AACR.*

EBV is a human gammaherpesvirus that establishes a persistent infection in more than 90% of the world's population. EBV DNA has been detected in numerous tumors including Hodgkin and non-Hodgkin lymphomas, undifferentiated nasopharyngeal carcinoma, gastric carcinoma, and primary brain lymphomas in AIDS patients. EBV is closely associated with endemic Burkitt lymphoma in sub-Saharan Africa.

Like other herpesviruses, EBV exists inside cells in two alternative modes: latent infection and lytic replication (1). In the latent form of infection, EBV persists largely within memory B lymphocytes in which only a small fraction of viral genes are expressed including EBV-encoded RNA (*EBER*). Upon cell division, replication of the viral genome is mediated by host cell DNA polymerase (1). The lytic form of infection is required for packaging of virions permitting transmission from host to host. In the lytic phase, most viral genes are expressed in a carefully orchestrated sequential fashion mediating viral genomic replication, encapsidation, and release of infectious virions accompanied by lysis and death of the host cell (1).

Induction of EBV replication can be achieved using radiation therapy, chemotherapy, or other manipulations inducing the lytic phase (2–5). Lytic phase infection promotes the destruction of EBV-positive tumor cells, at least in cell culture models and in transgenic mouse models (6–11). It is timely and important to translate successful laboratory models into clinical practice. Latently infected malignancies such as Burkitt lymphoma may respond to lytic induction with associated host cell death. In fact, lytic cell death may be even more effective *in vivo* than *in vitro*, when immune recognition of foreign viral antigens contributes to cell destruction.

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Translational Relevance

The presence of the EBV genome within malignant cells of endemic Burkitt lymphoma makes EBV an appealing target for therapy. Mechanistic data in cell lines and mouse models suggests that ganciclovir enhances tumor cell killing by chemotherapy *only* if the chemotherapy induces lytic viral replication. Our prospective study of naturally infected African Burkitt lymphoma patients found evidence of viral replication within the first 24 hours after starting cyclophosphamide. This result supports the design of a phase two trial of cyclophosphamide and ganciclovir in endemic Burkitt lymphoma.

A clinical trial by Chan and colleagues explored the feasibility of lytic induction by azacytidine in 10 patients with various EBV-related malignancies (12). Azacytidine is a DNA methyltransferase inhibitor that reactivates the transcription of genes that were silenced by CpG island methylation. When administered to patients, there was significant demethylation of CpG islands in promoters of latent and lytic EBV genes encoding immunogenic proteins. By immunohistochemistry, only one patient had detectable lytic protein expression, suggesting that the effect was minimal, transient, or promptly progressed to cell death (12).

Histone deacetylase inhibitors such as sodium butyrate are well known inducers of lytic replication in Burkitt lymphoma cell lines (5, 13, 14). A clinical trial of the histone deacetylase inhibitor, romidepsin, in patients with T-cell lymphoma resulted in secondary EBV-related lymphoproliferations in 2 of 120 treated patients (15). Although these lymphoproliferations are likely to be multifactorial, it is feasible that histone deacetylase inhibitors and other chemotherapeutic agents initiating lytic replication (like methotrexate; ref. 16) could function to enhance killing of EBV-related neoplasia at the cost of increasing risk of secondary EBV-related lymphoproliferations. Interestingly, withdrawal of methotrexate therapy usually leads to regression of the associated EBV-driven lymphoproliferation, presumably by relieving the drug's immunosuppressive effect (17).

If lytic cycle inducers could be rendered even more potent, perhaps they might be capable of eliminating every malignant cell of an infected tumor. Intriguing studies show that some lytic phase proteins (e.g., EBV thymidine kinase and BGLF4) could phosphorylate nucleoside analogue drugs such as ganciclovir to a toxic form, enhancing killing of the host cell and also of surrounding cells (e.g., latently infected tumor cells; refs. 2, 9, 18, 19). Therefore, the addition of nucleoside analogue drugs might achieve outcomes beyond what could be achieved with cytotoxic therapy alone. In a clinical trial of arginine butyrate plus ganciclovir in 15 patients with EBV-related lymphoma, 4 patients had complete response and 6 others had partial response (20). Most responses occurred within the first week of therapy, and tumor lysis was unexpectedly rapid in several patients (20).

Whether there is synergy between traditional chemotherapy and nucleoside analogue therapy in patients with Burkitt lymphoma remains to be tested. Burkitt lymphoma is the most common lethal malignancy of children between the ages of 3 to 15 years in the tropical regions of Africa, and this cancer is nearly always EBV-associated (21–23). In nearly all cases, the EBV gene expression pattern is very restricted. LMP1 and LMP2 are *not* expressed and, due to Qp promoter usage for EBNA1 transcription, only EBNA1 but not the other EBNAs are expressed (24, 25). In resource-poor settings in Africa, Burkitt lymphoma is often treated using single-agent cyclophosphamide with survival rates of 64% among children with maxillary or mandibular tumors compared with only 33% for those having more disseminated disease (24, 26, 27).

Despite the potential for application to human disease, it has yet to be proven that chemotherapy induces lytic EBV gene expression *in vivo* in patients with Burkitt lymphoma. Demonstrating lytic EBV expression in tumor tissue following treatment with chemotherapy may justify a new combined therapeutic approach to treating EBV-related malignancies. In the current study, we examined the extent to which lytic replication is induced by single-agent cyclophosphamide in African children with Burkitt lymphoma. Fine-needle aspirates (FNA) of tumor were collected before and again 1 to 5 days after the initiation of cyclophosphamide therapy, and the specimens were examined for evidence of latent and lytic EBV infection using histochemical and molecular methods. Our findings lay the groundwork for a possible clinical trial testing the addition of ganciclovir to initial chemotherapy as a way to enhance cancer-specific cytotoxicity.

Materials and Methods

Subjects. This research was approved by the University of North Carolina Medical Institutional Review Board and the Malawi National Health Sciences Research Committee. Consent was obtained from each patient's guardian. Children were admitted to the pediatric Burkitt Lymphoma ward of Kamuzu Central Hospital in Lilongwe, Malawi from August 2007 to June 2008. Patients ages 3 to 15 y old were recruited if they had a rapidly expanding mass involving the maxilla or mandible that was superficial and amenable to safe biopsy. All patients were studied at initial presentation of disease, and none had a previous history of cancer or cancer treatment. All patients were scheduled to receive cyclophosphamide therapy. Patients were excluded if they had hemoglobin levels of <8 g/dL, platelet counts of <50,000/μL, or known intolerance or allergy to lidocaine. One study-specific pediatrician (C. Wokocha) performed all FNA procedures. Treatment plans did not change as a result of enrollment. Patients

were treated with cyclophosphamide (40 mg/kg i.v.), with a plan to repeat every 2 wk for at least six cycles.

Prior to the first round of cyclophosphamide, the skin overlying the mandibular or maxillary mass was anesthetized with topical 2.5% lidocaine and 2.5% prilocaine (EMLA Cream, AstraZeneca) and an FNA was obtained using a 23-gauge needle. The aspirated material was promptly transferred into a vial containing 20 mL of PreservCyt solution (Hologic, Inc.), pumping the syringe several times to rinse. In the first half of the study, the aspiration procedure was repeated 3 to 5 d after initiation of the first round of cyclophosphamide therapy. After interim analysis of laboratory data, subsequent patients had their second aspiration on day 1 rather than on days 3 to 5 to test whether viral replication occurred earlier in the time course following initiation of treatment. Specimens were refrigerated for up to 6 wk before shipment in batches to University of North Carolina at Chapel Hill, where 10 mL of the aspirate was used to make a paraffin-embedded cell block and the remaining 10 mL was used for nucleic acid extraction.

In situ *hybridization.* To localize latently infected cells, *in situ* hybridization was done using an *EBER* probe, with an oligo-dT probe run in parallel as an RNA preservation control, on a BenchMark XT system (Ventana Medical System) according to the instructions of the manufacturer. Results were interpreted by two pathologists (M.L. Gulley and W. Tang) who characterized each specimen as having abundant, few, or no detectable *EBER*-expressing cells. Nuclear *EBER* signal was considered legitimate evidence of EBV infection. The proportion of *EBER*-expressing cells was calculated based on counts of *EBER*-positive cells and total nucleated cells in three 40× microscopic fields.

Real-time PCR to measure EBV viral load. To measure EBV genomic copy number, quantitative PCR targeting the *Bam*HIW segment was done on DNA extracted from scrolls of each paraffin-embedded cell block using the QIAamp DNA Mini Kit (Qiagen, Inc.). In four subjects, there was insufficient DNA in the paraffin-embedded sample so total nucleic acid was extracted from PreservCyt-suspended cells using the the QIAamp DNA Mini Kit. Viral load was measured as previously described (28) on an ABI 7500 real-time PCR instrument using primers and a TaqMan probe targeting the EBV *Bam*HIW segment in duplex with an assay targeting the human *APOB* gene that serves as a normalizer for the number of cells represented in the reaction. DNA from the Namalwa Burkitt lymphoma cell line was used as a standard by which to quantitate both EBV and *APOB* (28). EBV genomic copy number was reported as EBV copies per 100,000 cells.

Quantitative reverse transcription PCR to detect EBV lytic gene expression. EBV lytic gene expression was detected by quantitative reverse transcription PCR (qRT-PCR) targeting *BZLF1* mRNA. First, RNA was extracted from 10 mL of FNA preservative using the RNeasy Mini Kit (Qiagen) and DNA was removed by on-column DNase digestion for 15 min at room temperature using the RNase-Free DNase Set (Qiagen). Total RNA was eluted into 30 μL of RNase-free water and cDNA was synthesized immediately using random hexamers and the High Capacity cDNA Archive Kit (Applied Biosystems). RNA preservation and cDNA quality was confirmed using qRT-PCR targeting human *ABL1* exons 10 and 11. Primers and TaqMan probes listed in Table 1 were chosen to span introns so that any residual genomic DNA would be unlikely to amplify (29). Positive controls included RNA from the B95.8 and Namalwa cell lines. No template controls verified the absence of amplicon contamination.

Immunohistochemistry to detect EBV lytic gene expression. To detect EBV lytic protein expression in cell blocks, immunohistochemistry was done using antibodies targeting the immediate-early lytic protein BZLF1 and the early lytic protein BMRF1. Briefly, unstained sections were deparaffinized, rehydrated, and then incubated overnight at 4°C in monoclonal antibody targeting either BZLF1 (ZEBRA, M7005; DAKO Corporation) or BMRF1 (clone no. G3-E31) using the blocking and detection protocols in the Super-Sensitive Non-Biotin HRP Detection Kit (BioGenex). Oral hairy leukoplakia was used as a positive control for both lytic proteins. A control stain of the B cell marker CD20 (Chemicon International) was run in parallel to verify protein detectability

Table 1. Primer and probe sequences used in qRT-PCR

Target gene	Sequence	Position*
ABL1		
Forward primer	5-agagctgcagagcacagagaca-3	
Reverse primer	5-gctcttttcgagggagcaatg-3	
TaqMan probe	5-FAM-atggtccagaggatcgctctctccct-TAMRA-3	
BZLF1		
Forward primer	5′-acgcacaccgaaaccacaa-3′	90,397-90,379
Reverse primer	5′-cttaaacttggcccggcatt-3′	90,162-90,181
TaqMan probe spanning spliced mRNA	FAM-aatcgcattcctccagcgattctgg-TAMRA	90,227-90,242; 90,367-90,375

*Position in the EBV genome relates to National Center for Biotechnology Information accession no. AJ507799.

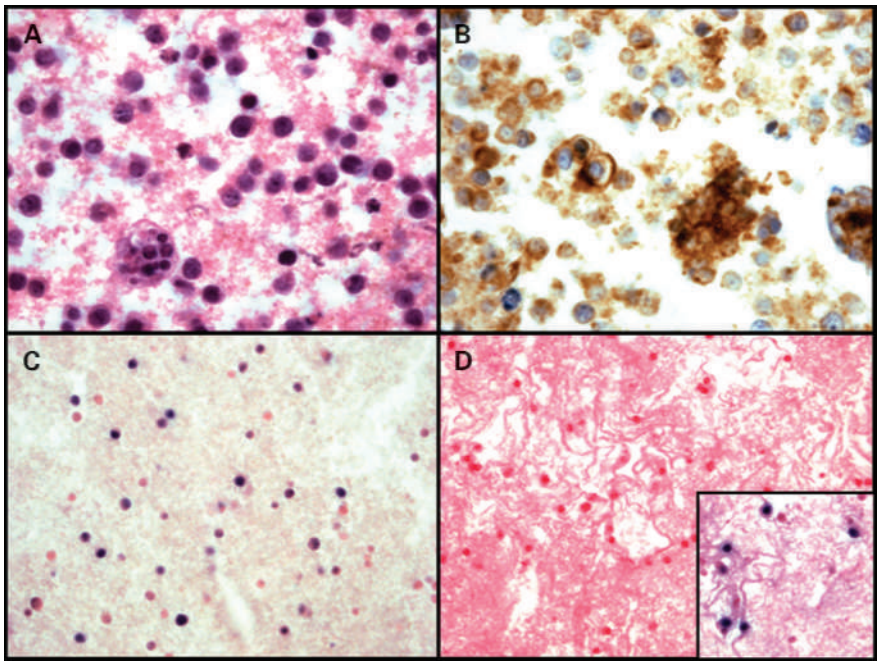

Fig. 1. Cytologic and histochemical features show loss of *EBER*-expressing Burkitt lymphoma cells after cyclophosphamide therapy. A, in a newly diagnosed patient, H&E staining reveals the features of Burkitt lymphoma including atypical lymphoid cells with fine, blast-like chromatin, and a large cell-laden macrophage (bottom left). B, immunohistochemistry reveals CD20 localized to cytoplasm and membrane of atypical cells. C, *EBER* is expressed by *in situ* hybridization in many nuclei, whereas smaller lymphoid cells stain only with the eosin counterstain; D, in an aspirate from the same patient 4 d after the start of chemotherapy, *EBER* is not expressed whereas a control hybridization targeting oligo-dT (inset) shows that RNA is preserved in most cells.

in each specimen. Results were interpreted by two pathologists (M.L. Gulley and W. Tang) who characterized each specimen as having abundant, few, or no detectable BZLF1 or BMRF1-expressing cells.

Statistical analysis. Association of *EBER* positivity between 1-d and 3- to 5-d treatment groups was assessed using a χ^2 test. Exact Wilcoxon rank-sum tests were used to compare differences in median viral loads and median *EBER*-expressing cell proportions, respectively, between specimens collected on treatment day 1 versus specimens collected on days 3 to 5 (SAS 9.2; SAS Institute, Inc.).

Results

Clinical and cytologic features. Thirty-three patients ranging from 4 to 14 years old with 20 males and 13 females were enrolled. Only those patients confirmed to have EBV-related Burkitt lymphoma based on clinical and cytologic features were further studied.

The first 16 enrolled patients had a second FNA collected 3 to 5 days after starting the first round of cyclophosphamide. Five of the 16 patients were excluded because initial hemoglobin levels were <8 g/dL (n = 1), the subject left the hospital before the second FNA was done (n = 1), or FNA was *EBER*-negative at both time points (n = 3). Among the latter three subjects with *EBER*-negative aspirates, the first had cytologic features of an *EBER*-negative diffuse large cell lymphoma, the second had rare atypical large cells in a background of acute and chronic inflammation, and the third had reactive-appearing mixtures of inflammatory cells. Further evaluation would be required to distinguish reactive or infectious processes from Hodgkin lymphoma, non-Hodgkin lymphoma, or other tumor types.

The next 17 enrolled patients had a second FNA collected only 1 day after starting the first round of cyclophosphamide. Seven of the 17 were excluded because initial hemoglobin levels were <8 g/dL (n = 1), an FNA sample was lost (n = 1), or the initial FNA was *EBER*-negative (n = 5). Among the latter five patients with *EBER*-negative aspirates, the first had multinucleated giant cells and abundant eosinophils, the second had a few CD20-negative large cells and many macrophages, and the next three patients had peripheral blood although the posttreatment aspirate of one patient contained scattered *EBER*-positive large cells suggesting that the needle had missed an *EBER*-expressing tumor in the pretreatment sampling. Overall, a total of 21 patients had adequate FNAs before and after chemotherapy. There were no complications.

The 21 patients confirmed to have EBV-related Burkitt lymphoma based on clinical and cytologic features combined with *EBER*-positivity ranged from 5 to 14 years old, with 13 males and 8 females. Typical cytologic features included enlarged lymphoid cells, fine granular blast-like chromatin, and tingible body-laden macrophages, all of which are characteristic of Burkitt lymphoma. CD20 was expressed in larger atypical lymphoid cells in all cases (Fig. 1).

EBER expression is diminished or lost after therapy. Latent EBV infection was identified by *EBER in situ* hybridization in all 21 specimens collected before therapy was initiated. As shown in Table 2, *EBER* was still expressed in nearly all

specimens collected 1 day after treatment. By 3 to 5 days after treatment, *EBER* was undetectable in the majority of patients (Fig. 1). The difference is statistically significant ($P = 0.0019$).

Total nucleated cell number was visibly decreased in all posttherapy specimens compared with pretreatment levels. There was often a lower proportion of atypical lymphoid cells and, in some cases, no cytomorphologic evidence of malignancy in the posttherapy specimens. *EBER*-expressing cells were only rarely visible by microscopy in patients sampled 3 to 5 days after therapy. In contrast, *EBER*-expressing cells were easily identified in all but one patient (no. 15) sampled on day 1 after therapy (see Table 3). These findings suggest that cyclophosphamide has a rapid effect in diminishing latent infection to nearly undetectable levels in the majority of patients within 5 days after the initiation of treatment.

EBV DNA levels implicate viral replication after chemotherapy. Human *APOB* gene copy number was measured by real-time PCR as a surrogate for the number of nucleated cells represented in each DNA amplification reaction. *APOB* results helped adjust for the fact that the number of cells aspirated differed from specimen to specimen. *APOB* levels typically went down after therapy (average 2-fold decrease), which correlates with the cytologic observation of fewer nucleated cells after treatment.

EBV viral load in tumor aspirates was measured by real-time PCR, and the results were rendered quantitative by virtue of normalization to the number of nucleated cells as represented by the human *APOB* gene results. EBV viral load was high in all 21 lymphoma specimens tested at the time of initial diagnosis before starting therapy. As shown in Fig. 2, EBV load decreased precipitously in the 11 samples that were aspirated 3 to 5 days after treatment (average 9-fold loss of EBV). However, in specimens collected just 1 day after therapy, EBV viral load often did not decrease substantially compared with diagnostic specimens, and in fact it *increased* in half of the specimens (5 of 10 cases; Table 4). The average increase in EBV load was 30-fold in the 10 tumors sampled at day 1 compared with pretreatment levels. Even after excluding the outlier subject (case no. 16) with an exceptionally high increase in EBV load, the average viral load still increased 8-fold among tumors sampled at day 1 compared with pretreatment levels. An increase in EBV load suggests that the EBV genome had replicated during the first day after initiation of chemotherapy. This increase in viral load, in the face of diminishing numbers of cells, is consistent with lytic viral replication.

EBV lytic gene BZLF1 expression is detected in few Burkitt lymphoma tissues. To test whether lytic infection was the explanation for increased EBV viral load immediately after treatment, viral lytic gene expression was examined by qRT-PCR targeting the EBV lytic transcript, *BZLF1*. BZLF1 is normally expressed very early in the lytic phase and is considered to be the master switch controlling exit from latency to lytic viral replication.

Extracted RNA was successfully amplified in all specimens using PCR with a TaqMan probe targeting an endogenous control transcript (human *ABL1* mRNA). BZLF1 transcripts were expressed in only four samples, three collected before the initiation of therapy (from patient nos. 4, 11, and 19) and only one collected after therapy. The latter, from patient no. 12, was aspirated on day 1 after therapy and expressed low-level BZLF1. Overall, these data suggest no significant upregulation of this lytic viral factor at the posttherapy time points examined. All three of the tumors having evidence of lytic infection prior to therapy had dramatic drops in EBV viral load after chemotherapy.

EBV lytic proteins were not expressed by immunohistochemistry. To further characterize any lytic infection, immunohistochemistry was done using monoclonal antibodies against BZLF1 and BMRF1 which represent markers of lytic EBV infection. Microscopic examination revealed no detectable expression of either protein in any of the pretherapy or posttherapy aspirates. Control staining showed successful detection of both proteins in oral hairy leukoplakia tissue. Furthermore, each aspirate in this study was successfully stained for a protein (CD20) that is ubiquitously expressed in normal and malignant B lymphocytes, diminishing the likelihood that technical failure was responsible for the negative viral lytic expression results in these aspirates. These data suggest that our immunohistochemical method is not sensitive enough to detect these lytic proteins, or that the timing of specimen collection was not optimal for detecting transient expression of these factors. It should be noted that BZLF1 is an immediate early lytic factor and BMRF1 is an early factor, meaning that both are expressed at an early

Table 2. Frequency of latent EBV detection in tumors before and after the start of cyclophosphamide therapy

	Before therapy	After therapy*	
		Day 1 ($n = 10$)	Days 3-5 ($n = 11$)
Positive *EBER* stain	21	9	2
Negative *EBER* stain	0	1	9

*Results are significantly different in day 1 versus days 3 to 5 collections ($P = 0.0019$).

Table 3. Proportion of *EBER*-expressing cells declines in most tumors by 4 to 5 d after start of cyclophosphamide therapy, as assessed by microscopy

Case no.	Day collected posttherapy	*EBER*-expressing cells as a proportion of total nucleated cells			Fold change in total cell no.
		Before therapy	After therapy*	Fold change†	
1	5	0.06	0	0	0.57
2	5	0.23	0	0	0.23
3	4	0.36	0	0	0.15
4	4	0.66	0	0	0.01
5	4	0.27	0	0	0.38
6	4	0.28	0	0	0.22
7	4	0.73	0	0	0.09
8	4	0.70	0	0	0.02
9	3	0.18	0.71	4.05	0.03
10	3	0.21	0.54	2.57	0.39
11	3	0.69	0	0	0.03
Mean	**4**	**0.40**	**0.11**	**0.60**	**0.19**
Median	**4**	**0.28**	**0**	**0**	**0.15**
12	1	0.15	0.23	1.59	0.61
13	1	0.44	0.50	1.12	0.26
14	1	0.23	0.13	0.57	0.16
15	1	0.39	0.00	0.00	0.32
16	1	0.61	0.22	0.36	0.00
17	1	0.39	0.24	0.62	0.68
18	1	0.27	0.50	1.82	0.53
19	1	0.21	0.33	1.60	0.05
20	1	0.15	0.67	4.58	0.37
21	1	0.42	0.23	0.55	0.60
Mean	**1**	**0.33**	**0.31**	**1.28**	**0.36**
Median	**1**	**0.33**	**0.24**	**0.87**	**0.35**

*Median *EBER*-positive cell proportion was lower in tumors sampled on days 3 to 5 compared with day 1 ($P = 0.017$).
†Median fold change in *EBER*-positive cell count was lower in tumors sampled on days 3 to 5 compared with day 1 ($P < 0.014$).

time point during the sequential process of viral replication and cell lysis.

Discussion

This study found EBV DNA levels, as measured by quantitative PCR, frequently increase within 1 day of the start of cyclophosphamide therapy for Burkitt lymphoma. By day 5, the viral load and the tumor burden are already greatly diminished. The initial increase in EBV levels in some day 1 tumors could reflect viral DNA replication. The mechanism of EBV DNA replication is hypothesized to be lytic infection, although evidence for expression of the early viral genes BZLF1 and BMRF1 was lacking at the time points that were examined.

The lytic phase of EBV infection is normally marked by sequential expression of a cascade of proteins that co-opt cellular functions leading to replication of the viral genome followed by packaging of viral DNA and export of infectious virions (1). The earliest marker of lytic viral infection is the expression of the immediate-early protein, BZLF1, followed by other early markers such as BMRF1 (10, 30, 31). These lytic genes are usually silent or are expressed only focally at low levels in Burkitt lymphoma tissues (3, 25).

The current study confirmed that BZLF1 and BMRF1 were not expressed in Burkitt lymphoma using immunohistochemistry, although the more sensitive rtPCR method revealed BZLF1 in 3 of 21 pretreatment tumors. Our study is the first to examine the expression of these lytic factors in the first few days after the initiation of chemotherapy. We showed low-level expression of BZLF1, detectable only by a sensitive rtPCR assay, in only one posttherapy aspirate. Despite a general lack of evidence for replicative gene

expression, EBV viral load increased above pretreatment levels in 50% of day 1 aspirates, implying that viral replication had already occurred within the first 24 hours of treatment. Viral load then decreased but remained detectable at days 3 to 5. It is uncertain how much of the remaining viral DNA is intracellular versus packaged in virions or existing as naked viral genomes from dying tumor cells.

The time course of events implies that replicative infection could be initiated within hours of exposure to cyclophosphamide. In cell line models, Countryman et al. showed that the kinetics of lytic induction depends on the host cell type and the dose and timing of the stimulus (32). In the Akata Burkitt lymphoma cell line stimulated by crosslinking surface immunoglobulin receptors, Wen et al. showed that BZLF1 mRNA was expressed as early as 1.5 hours and disappeared in 24 hours, whereas the encoded protein was expressed from 6 to 12 hours after induction (4). In humans, we found it difficult to track the kinetics of replicative infection. After enrolling our first cohort of Burkitt lymphoma patients and finding no replicative gene expression at time points of 3 to 5 days, we proceeded to enroll additional patients for evaluation just 1 day after the initiation of therapy and we still seem to have missed the putative window of replicative viral gene upregulation, although we were able to show viral load increases implicating a lytic phase of infection. Re-biopsy at multiple time points is difficult, so a detailed timeline of tumor-based profiles is probably not feasible in humans.

We hypothesize that transient expression of lytic proteins permits viral DNA synthesis whereas simultaneously triggering immune recognition of foreign immunogenic peptides leading to engulfment by macrophages, which contributes to the "starry sky" histology that is characteristic of Burkitt lymphoma. Lytic infection is notoriously difficult to document, even in oropharyngeal tissues in which replicative infection occurs routinely, as evidenced by periodic shedding of virions into the saliva (33).

In the current study, the proportion of *EBER*-expressing cells was similar to baseline at days 1 and 3, although by days 4 to 5, all specimens had undetectable *EBER* in concert with the disappearance of malignant-appearing cells. In future studies, one could explore whether the initial response to chemotherapy, in terms of serially measured viral loads or infected cell counts, predicts long-term outcome. Because blood sampling is less invasive than tumor aspiration, circulating markers of latent and replicative viral infection should be examined (34, 35).

The observed decline in *EBER*-expressing cells could result from the elimination of infected tumor cells or from *EBER* downregulation in tumor cells. Lytic infection is associated with diminished *EBER* expression, at least in some lesions and cell models (36, 37). Although transcriptional regulation of *EBER* genes is poorly understood, recent evidence shows that MYC could bind to and activate the *EBER1* gene promoter (38–40). Promoter methylation is a proposed mechanism for silencing *EBER1* and *EBER2* transcription (36, 41). Altered availability or enhanced degradation of *EBER*s must also be considered as potential cause(s) of diminished *EBER* expression (42). Relief from the tumorigenic and antiapoptotic effects of EBV might explain, at least in part, the efficacy of chemotherapy.

The antitumor effect of cyclophosphamide therapy is dramatic, and its introduction to clinical medicine beginning over 50 years ago represents one of the great historic breakthroughs in cancer management (43). Nevertheless, a significant fraction of treated patients eventually relapse and die. Clearly, more effective therapies are needed for

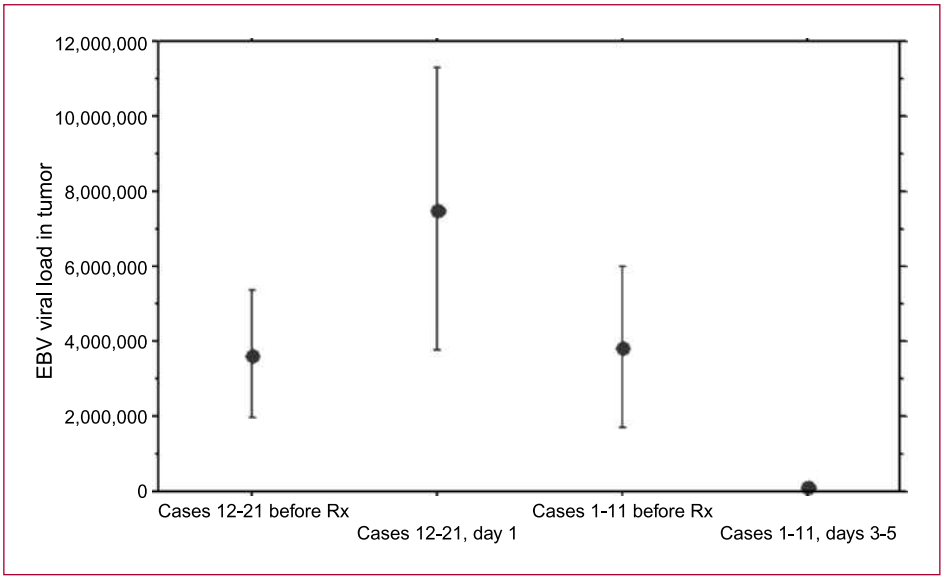

Fig. 2. EBV viral load may increase initially but then decreases consistently in posttherapy tumor specimens. EBV viral load, expressed in copies per 100,000 cells, is displayed as mean with SE bars in each clinical subgroup of Burkitt lymphomas. Both pretreatment groups have similar distributions of viral burden. Mean viral load is even higher among tumors sampled on day 1, but declines by days 3 to 5 after the start of chemotherapy.

Table 4. EBV viral load results as measured by real-time PCR

Case no.	Day collected posttherapy	Fold change in cell no. (*APOB* gene)	EBV copies/100,000 cells*		
			Before therapy	After therapy	Fold change
1	5	0.89	684,877	312,755	0.460
2	5	0.01	209,501	31,429	0.150
3	4	0.42	990,627	7,872	0.010
4	4	0.08	232,444	5,555	0.020
5	4	0.22	538,058	1,015	0.002
6	4	1.40	24,399,000	579,509	0.020
7	4	0.07	3,488,468	17,949	0.005
8	4	1.04	7,169,565	33,333	0.005
9	3	0.17	2,201,256	134,909	0.060
10	3	1.10	330,844	154,949	0.470
11	3	0.02	1,968,976	8,000	0.004
Mean	**4**	**0.49**	**3,837,602**	**117,025**	**0.110**
Median	**4**	**0.22**	**990,627**	**31,429**	**0.020**
12	1	2.65	10,556,522	3,824,590	0.360
13	1	0.13	4,119,544	**12,380,000**	**3.010**
14	1	0.02	470,307	758,015	**1.610**
15	1	0.01	133,907	23,529	0.180
16	1	0.03	170,152	**39,058,491**	**229.550**
17	1	0.77	487,755	364,000	0.750
18	1	0.89	2,334,907	**7,215,556**	**3.090**
19	1	0.26	15,911,481	242,857	0.020
20	1	0.02	2,072,262	1,736,190	0.840
21	1	0.87	152,632	**9,533,333**	**62.460**
Mean	**1**	**0.56**	**3,640,947**	**7,513,656**	**30.187**
Median	**1**	**0.20**	**1,280,008**	**2,780,390**	**1.225**

*Median viral load was higher in tumors sampled on day 1 compared with days 3 to 5 ($P < 0.001$). Median fold change in viral load was also higher in tumors sampled on day 1 compared with days 3 to 5 ($P < 0.001$).

Burkitt lymphoma and for other EBV-related malignancies that, in aggregate, are estimated to affect ~1% of the world's population. EBV is an appealing therapeutic target because it is likely to be involved in pathogenesis of the neoplasm and, when present, it is generally found within every malignant cell of a given infected malignancy (3, 9, 44–46). Considerable work has been done to explore the possibility of destroying EBV-infected cells by means of inducing lytic infection (6–9, 12, 45, 47–49). Infection terminating in cell lysis or immune destruction is an appealing end point for cancer therapy.

In conclusion, we found evidence supporting EBV lytic induction in Burkitt lymphoma patients almost immediately after the first dose of cyclophosphamide, a drug that is very commonly used in Africa as single-agent therapy. Evidence of lytic replication lays the groundwork for a clinical trial testing the efficacy of adding a nucleoside analogue to enhance cancer-specific cytotoxicity of cyclophosphamide.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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