Preclinical Therapy

Viral Response to Chemotherapy in Endemic Burkitt Lymphoma

Weihua Tang1, Paula Harmon2, Margaret L. Gulley1,4, Charles Mwansambo5, Peter N. Kazembe6, Francis Martinson3, Clifford Wokocha5, Shannon C. Kenney6, Irving Hoffman3, Carlie Sigel1, Susan Maygarden1, Mariah Hoffman7, and Carol Shores2,4

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); OF1–10. ©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. 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.
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 analogues 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 specimen 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 years 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.) and 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 a 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 BamHIW 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 solution using the RNeasy Mini Kit (Qiagen) and total nucleated cells in three 40× microscopic fields.

<table>
<thead>
<tr>
<th>Table 1. Primer and probe sequences used in qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target gene</strong></td>
</tr>
<tr>
<td>ABL1</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>TaqMan probe</td>
</tr>
<tr>
<td>BZLF1</td>
</tr>
<tr>
<td>Reverse primer</td>
</tr>
<tr>
<td>TaqMan probe spanning spliced mRNA</td>
</tr>
</tbody>
</table>

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

Table 1. Primer and probe sequences used in qRT-PCR.
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 χ² 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 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 were further studied.

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 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 cytomorphic 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 normalization to the number of nucleated cells after 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 normalization to the number of nucleated cells after 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 normalization to the number of nucleated cells after 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 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 normalization to the number of nucleated cells after 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 normalization to the number of nucleated cells after treatment.
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).

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

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Day collected posttherapy</th>
<th>EBER-expressing cells as a proportion of total nucleated cells</th>
<th>Fold change in total cell no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before therapy</td>
<td>After therapy*</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.23</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.36</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.66</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.27</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.73</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>0.70</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>0.18</td>
<td>0.71</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.21</td>
<td>0.54</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>0.69</td>
<td>0</td>
</tr>
<tr>
<td>Mean</td>
<td>4</td>
<td>0.40</td>
<td>0.11</td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>0.44</td>
<td>0.50</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>0.23</td>
<td>0.13</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>0.39</td>
<td>0.00</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0.61</td>
<td>0.22</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0.39</td>
<td>0.24</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>0.27</td>
<td>0.50</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>0.21</td>
<td>0.33</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>0.15</td>
<td>0.67</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>0.42</td>
<td>0.23</td>
</tr>
<tr>
<td>Mean</td>
<td>1</td>
<td>0.33</td>
<td>0.31</td>
</tr>
<tr>
<td>Median</td>
<td>1</td>
<td>0.33</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*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).
expression, EBV viral load increased above pretreatment levels in 50% of day 1 aspirates, implying that viral repli-
cation had already occurred within the first 24 hours of
treatment. Viral load then decreased but remained detect-
able at days 3 to 5. It is uncertain how much of the re-
main ing viral DNA is intracellular versus packaged in
virions or existing as naked viral genomes from dying tu-
mor cells.

The time course of events implies that replicative in-
fec tion 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 re-
ceptors, 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 infec-
tion. 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 addi-
tional patients for evaluation just 1 day after the initia-
tion 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 impli-
cating 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 pro-
teins 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 character-
istic 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 con-
cert with the disappearance of malignant-appearing cells.
In future studies, one could explore whether the initial re-
response to chemotherapy, in terms of serially measured
viral loads or infected cell counts, predicts long-term out-
come. 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 re-
sult from the elimination of infected tumor cells or from
EBER downregulation in tumor cells. Lytic infection is as-
associated with diminished EBER expression, at least in
some lesions and cell models (36, 37). Although tran-
scriptional regulation of EBER genes is poorly understood,
recent evidence shows that MYC could bind to and acti-
vate the EBER1 gene promoter (38–40). Promoter meth-
ylation is a proposed mechanism for silencing EBER1 and
EBER2 transcription (36, 41). Altered availability or en-
hanced degradation of EBERs must also be considered
as potential cause(s) of diminished EBER expression (42).
Relief from the tumorigenic and antiapoptotic ef-
ects 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 begin-
ning 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
Burkitt lymphoma and for other EBV-related malignancies that, in aggregate, are estimated to affect \( \sim 1\% \) 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.

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

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Day collected posttherapy</th>
<th>Fold change in cell no. (APOB gene)</th>
<th>EBV copies/100,000 cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before therapy</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.89</td>
<td>684,877</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0.01</td>
<td>209,501</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0.42</td>
<td>990,627</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.08</td>
<td>232,444</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.22</td>
<td>538,058</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1.40</td>
<td>24,399,000</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0.07</td>
<td>3,488,468</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>1.04</td>
<td>7,169,565</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>0.17</td>
<td>2,201,256</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>1.10</td>
<td>330,844</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>0.02</td>
<td>1,968,976</td>
</tr>
<tr>
<td>Mean</td>
<td>4</td>
<td>0.49</td>
<td>3,837,602</td>
</tr>
<tr>
<td>Median</td>
<td>4</td>
<td>0.22</td>
<td>990,627</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>2.65</td>
<td>10,556,522</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>0.13</td>
<td>4,119,544</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>0.02</td>
<td>470,307</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>0.01</td>
<td>133,907</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0.03</td>
<td>170,152</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0.77</td>
<td>487,755</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>0.89</td>
<td>2,334,907</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>0.26</td>
<td>15,911,481</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>0.02</td>
<td>2,072,262</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>0.87</td>
<td>152,632</td>
</tr>
<tr>
<td>Mean</td>
<td>1</td>
<td>0.56</td>
<td>3,640,947</td>
</tr>
<tr>
<td>Median</td>
<td>1</td>
<td>0.20</td>
<td>1,280,008</td>
</tr>
</tbody>
</table>

*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 \((P < 0.001)\).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Gregory Sturtz, Mervi Eeva, Nana Feinberg, Bruce Israel, and Rosalie Dominik of the University of North Carolina at Chapel Hill for their administrative and technical expertise. We also thank Debbie Kamwendo MS and the staff of the UNC Project in Lilongwe. Finally, we thank the study subjects and their parents for brave and altruistic participation in this investigation.

**Grant Support**

American Academy of Otolaryngology-Head and Neck Surgery CORE Resident Research Grant, the UNC Medical Endowment fund, and NIH supplement grant R01-CA66519 (S.C. Kenney).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 09/09/2009; revised 01/15/2010; accepted 02/06/2010; published OnlineFirst 03/16/2010.
References


Viral Response to Chemotherapy in Endemic Burkitt Lymphoma

Weihua Tang, Paula Harmon, Margaret L. Gulley, et al.

Clin Cancer Res  Published OnlineFirst March 16, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-09-2424

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.