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Characterization of Epstein–Barr Virus Reactivation in a Modeled Spaceflight System

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ABSTRACT

Epstein–Barr virus (EBV) is the causative agent of mononucleosis and is also associated with several malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma, among others. EBV reactivates during spaceflight, with EBV shedding in saliva increasing to levels ten times those observed pre-and post-flight. Although stress has been shown to increase reactivation of EBV, other factors such as radiation and microgravity have been hypothesized to contribute to reactivation in space. We used a modeled spaceflight environment to evaluate the influence of radiation and microgravity on EBV reactivation. BJAB (EBV-negative) and Raji (EBV-positive) cell lines were assessed for viability/apoptosis, viral antigen and reactive oxygen species expression, and DNA damage and repair. EBV-infected cells did not experience decreased viability and increased apoptosis due to modeled spaceflight, whereas an EBV-negative cell line did, suggesting that EBV infection provided protection against apoptosis and cell death. Radiation was the major contributor to EBV ZEBRA upregulation. Combining modeled microgravity and radiation increased DNA damage and reactive oxygen species while modeled microgravity alone decreased DNA repair in Raji cells. Additionally, EBV-infected cells had increased DNA damage compared to EBV-negative cells. Since EBV-infected cells do not undergo apoptosis as readily as uninfected cells, it is possible that virus-infected cells in EBV seropositive individuals may have an increased risk to accumulate DNA damage during spaceflight. More studies are warranted to investigate this possibility. J. Cell. Biochem. 114: 616–624, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: EPSTEIN–BARR VIRUS; EBV; RADIATION; BIOREACTOR; MICROGRAVITY; REACTIVATION

Epstein–Barr virus (EBV) is a human herpesvirus known to infect at least 90% of adults. EBV can cause infectious mononucleosis during primary infection and is also associated with several types of malignancy [Cohen, 2000]. After primary infection, EBV persists within host B-lymphocytes in a latent state and can reactivate periodically to produce infectious virus, which is often shed in saliva. The majority of individuals who are infected with EBV have occasional asymptomatic reactivation. The fact that this reactivation is asymptomatic can be attributed to a properly functioning immune system along with various genetic and behavioral factors, which generally minimize the risk for symptomatic EBV disorders [Rickinson and Kieff, 2006]. However, disruption of the immune system can lead to increased viral loads and EBV-associated clinical conditions [Thorley-Lawson, 2005]. Evidence has been published that spaceflight disrupts immune function enough to allow EBV reactivation, detected by increased viral load and lytic viral transcripts such as EBNA1, BZLF1, and BHRF1 [Pierson et al., 2005; Stowe et al., 2011]. Additionally, astronauts undergo immunological changes that may disrupt the balance between viral activation and immune control [Crucian et al., 2008; Gueguinou et al., 2009]. The potential consequences of this spaceflight EBV reactivation with combined immune dysregulation are unknown.

Not only have previous spaceflight studies found increased EBV activation [Pierson et al., 2005; Stowe et al., 2011] and increased DNA damage due to actual spaceflight [Fry et al., 1994], but also

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decreased DNA repair due to modeled microgravity exposure [Kumari et al., 2009; Mognato et al., 2009]. Furthermore, expression of EBNA1 has been shown to increase DNA damage in B-lymphocytes [Gruhne et al., 2009a], and BHRF1 increases the potential for EBV-infected cells to survive inclement conditions [Henderson et al., 1993; Marshall et al., 1999]. Consequently, long duration astronaut crews may be more susceptible to EBV-associated disorders due to the combination of EBV infection and reactivation, immunological changes, and DNA damage during spaceflight.

Therefore, in this study we sought to address how a modeled spaceflight environment, including both radiation and modeled microgravity, affects EBV-infected cells. Cells were evaluated for viability, apoptosis, cell death, reactive oxygen species (ROS) expression, DNA damage, and DNA repair. Additionally, cells were evaluated for the expression of two viral antigens expressed during lytic activity. ZEBRA (also known as BZLF1, Zta, or Z) is an EBV immediate-early transcription factor that broadly upregulates EBV lytic gene expression and increased expression of ZEBRA indicates increased EBV lytic activity. BHRF1 is an EBV early lytic antigen that can also be expressed during latency. It has antiapoptotic effects and enhances cell survival [Henderson et al., 1993; Marshall et al., 1999; Kieff and Rickinson, 2006; Watanabe et al., 2010]. EBV may confer protective effects on the host cell, preventing cellular apoptosis through the expression of this protein [Blaise et al., 2002].

MATERIALS AND METHODS

CELL LINES

Both EBV-negative (BJAB) and EBV-positive (Raji) cell lines were used for this study. BJAB is an EBV negative cell line isolated from a Burkitt's lymphoma patient in 1965 and does not express any EBV antigens [Epstein and Barr, 1965]. Raji is derived from Burkitt's lymphoma and has an abortive EBV replicative system that does not produce virus capable of transforming other cell lines [Seigneurin et al., 1977]. BJAB cells were generously donated by Jeffery Cohen while Raji cells were provided by Raymond Stowe. All cells were grown at 37°C in a 95% air, 5% CO_2 atmosphere in complete culture medium (RPMI 1640 with 10% fetal bovine serum [FBS], 25 U/ml penicillin, 25 μg/ml streptomycin, and 2 mM L-glutamine; Gibco, Invitrogen, Grand Island, NY).

EXPERIMENTAL CONDITIONS

The experimental models used involved incubation of BJAB and Raji cell lines in either T-25 flasks or in modeled microgravity conditions. The microgravity environment was modeled using a 10 ml rotating wall vessel bioreactor (Synthecon, Inc., Houston, TX) held constant at 10 revolutions per minute. The bioreactor and upright T-25 control flasks (Corning, Tewksbury, MA) were seeded with 1×10^6 cells/ml in 11 ml medium. For irradiation, cells suspended in culture medium were exposed to 3 Gy ^{137}Cs gamma radiation using the biological irradiator facility at Johnson Space Center. Cells were irradiated under constant rotation providing even doses across the circumference of the tube and returned to the incubator immediately after irradiation. Cells were harvested at varying times after being placed in the bioreactor or flask. Experiments were conducted in duplicate or triplicate and repeated 2–3 times. Four experimental conditions (environments) were evaluated: the control flask, the irradiated flask, the bioreactor (modeled microgravity), and the irradiated bioreactor (spaceflight model).

CHEMICAL INDUCTION OF VIRAL LYTIC CYCLE

Epstein–Barr virus infected cells were induced to activate the viral lytic cycle with 3 mM sodium butyrate (SB; Sigma–Aldrich, St. Louis, MO) and 100 nM 12-*O*-tetradecanoyl phorbol 13-acetate (TPA; Sigma–Aldrich). Control cells received fresh media without SB and TPA.

VIABILITY AND APOPTOSIS ASSAYS

Viability, apoptosis, and cell death were evaluated using Guava ViaCount and Guava Nexin assays (EMD Millipore, Billerica, MA) according to the manufacturer's instructions [Harris et al., 2005].

FLOW CYTOMETRY FOR ZEBRA

Flow cytometry procedures for ZEBRA were adapted from the protocols published by Chang et al. [2010] and Guo et al. [2010]. Approximately 1×10^6 cells were fixed with 4% paraformaldehyde for 10 min then washed with PBS (Gibco). Next, cells were permeabilized with PBS/0.2% Triton X-100 (Sigma–Aldrich) for 3 min, and subsequently washed twice with PBS. After permeabilization, cells were incubated with 1:50 PBS-diluted mouse monoclonal anti-BZLF1 (ZEBRA) IgG_1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Cells were washed with PBS then incubated with 1:200 diluted AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen) for detection of BZLF1. For all flow cytometry assays, at least 10,000 events per sample were analyzed using a Beckman Coulter XL flow cytometer. Analysis was performed by first configuring cytometer settings to resolve target cells from debris on a forward scatter versus side scatter plot. Target cells were then "gated" and intracellular analyte mean fluorescence intensity for gated target cells was obtained by plotting emission for the AlexaFluor488 (or other fluorophore) labeled antibody versus side scatter. Both cytometry controls (staining with the labeled secondary antibody only) and experimental controls consisting of uninfected or unstimulated target cells were used to determine the threshold for positive. Fluorescence channel voltages were set so that negative events were located in the first axial decade, and the number of events beyond the threshold for positive was quantified.

FLOW CYTOMETRY FOR BHRF1

Approximately 1×10^6 cells were fixed with 4% paraformaldehyde for 10 min then washed with PBS. Next, cells were permeabilized with buffer (0.5% saponin/5% non-fat milk/PBS) and incubated with 1:50 diluted mouse monoclonal anti-BHRF1 (vBcl-2) IgG_1 antibody (Santa Cruz Biotechnology, Inc.). Cells were washed with PBS then incubated in the permeabilizing buffer with fluorochrome phycoerythrin (PE)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.) for detection of BHRF1 and analyzed as above.

CYTOKINESIS BLOCK MICRONUCLEUS (CBMN) ASSAY

Formation of micronuclei (MN), nucleoplasmic bridges (NPB), and nuclear buds (NB) was measured by the method published by Fenech [Fenech, 2007]. Briefly, cells were treated as described in "experimental conditions" for 24 h in the flask or bioreactor environments, irradiated, and then cytokinesis was blocked by the addition of 4.5 μg/ml cytochalasin-B (Sigma–Aldrich). Cells were incubated for at least 28 h in their respective treatment environment with cytochalasin-B followed by spotting onto slides for analysis. Slides were fixed with 3:1 methanol/acetic acid (Sigma–Aldrich) for 15 min, washed with PBS and deionized water, and the nuclei stained with Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). *Scoring Criteria for CBMN assay:* MN, NPB, and NB were counted only in binucleated cells with clear nuclear margins. Frequencies of MN, NPB, and NB were counted in 1,000 cells per slide. The nuclear division index was recorded as a measure of cellular proliferation and the number of MN per binucleated cell was also catalogued as a measure of DNA damage intensity.

DNA DAMAGE RESPONSE BY FLOW CYTOMETRY

For detection of ataxia-telangiectasia, mutated (ATM), 1×10^6 cells were removed from the experimental vessel and washed once with PBS. The cells were fixed with 4% paraformaldehyde for at least 10 min and washed twice with PBS. Permeabilizing buffer containing 5% non-fat milk and 0.5% saponin in PBS were added with the polyclonal rabbit anti-ATM antibody (Novus Biologicals, Littleton, CO) and incubated overnight at 4°C. Cells were washed twice and incubated with goat anti-rabbit IgG AlexaFluor 488 labeled (Invitrogen) secondary antibody for 2 h at room temperature. Finally, the samples were washed twice with PBS and analyzed by flow cytometry as above.

DETECTION OF REACTIVE OXYGEN SPECIES

To detect ROS, cells were stained with 2,7-dichlorofluorescin diacetate (DCFDA; Invitrogen) [Gruhne et al., 2009a], a membrane-permeable indicator which fluoresces after its acetate group is cleaved by intracellular oxidases. Approximately 1×10^6 cells were stained with 10 μM DCFDA for 30 min at 37°C, washed twice in PBS, then analyzed by flow cytometry.

STATISTICAL ANALYSES

For comparison of two groups, the Student's *t*-test was used. If data did not have a normal distribution, the data were transformed to use the parametric *t*-test. For data sets with more than two comparison groups, normal data with equal variances were analyzed by ANOVA. To assess the relationship between radiation and modeled microgravity, a two-way ANOVA was used with a Bonferroni test for post-hoc comparisons and a one-way ANOVA was also used to assess inter-group differences. Data were tested for the normality assumption using the Shapiro–Wilk test. Data that did not have a normal distribution were normalized, as necessary, using various transforms and analyzed by ANOVA for parametric data. Simple transformations (e.g., log, ln, etc.) were first evaluated and if no simple transformation was successful, complex transformations were assessed (e.g., logit). When data with an abnormal distribution were not able to be transformed, a non-parametric test was employed, or data were accepted for use in a parametric test based on the uncertainty of normality in datasets with low "n". Analyses were conducted using Stata and SigmaStat12 software packages. A *P*-value less than or equal to 0.05 was considered significant. In the results, main effects are indicated in the figure caption and data are displayed as mean ± standard deviation (SD). Asterisks (*) in figures indicate a statistical difference in means of $P \leq 0.05$ and the (&) symbol on figures indicates a significant interaction between the modeled microgravity and radiation factors.

RESULTS

CELL VIABILITY, APOPTOSIS AND DEATH IN A MODELED SPACEFLIGHT ENVIRONMENT

Raji and BJAB cells were evaluated for any changes in cell viability, apoptosis, and death due to the modeled spaceflight environment by Guava ViaCount (Fig. 1) and Guava Nexin (data not shown) methods. BJAB cells showed significant decreases in mean viability values when comparing the combination of modeled microgravity and radiation (Viacount: 82.5 ± 4.9% and Nexin: 81.9 ± 3.5%) to control values (Viacount: 89.6 ± 1.8% and Nexin: 89.6 ± 1.7%) after 72 h incubation (Fig. 1A, top). Additionally, BJAB cells exhibited increased apoptosis (Fig. 1B) and cell death (Fig. 1A, bottom) according to the Guava ViaCount assay; thus, most of the change in viability was related to cell death. Similar results were attained for BJAB cells using the Guava Nexin assay for viability and early apoptosis (data not shown). For viability and apoptosis, statistical main effects (i.e., an effect due to one or more of the experimental conditions [environments] evaluated: radiation alone, modeled microgravity alone, or an interaction between the two) were detected for both modeled microgravity alone (viability $P < 0.003$; apoptosis, $P = 0.001$) and radiation alone (viability $P < 0.001$; apoptosis, $P < 0.001$) by the ViaCount assay as well as for both modeled microgravity (viability $P = 0.01$; apoptosis, $P < 0.001$) and radiation alone (viability $P = 0.02$; apoptosis, $P = 0.01$) by the Nexin assay; however, a significant interaction between modeled microgravity and radiation was not detected by either assay. Therefore, it is possible to attribute the changes in viability, apoptosis, and cell death specifically to radiation and modeled microgravity; however, the combination of factors does not statistically enhance the effect of either factor alone, and differences were small (<10%).

In contrast to BJAB cells, Raji cell viability (Fig. 1C, top), apoptosis (Fig. 1C, bottom), and cell death (Fig. 1D) were not affected by the modeled spaceflight environment to the same extent. There were no significant differences in mean values for Raji cells with either the Guava ViaCount or Guava Nexin methods; however, the ViaCount assay did detect a significant main effect due to radiation alone for viability ($P = 0.045$) and apoptosis ($P = 0.031$) but not death ($P = 0.176$). Therefore, in Raji cells, any change in viability was attributable to apoptosis, rather than cell death. There were no main effects due to either modeled microgravity (viability: $P = 0.396$; apoptosis: $P = 0.08$; death: $P = 0.615$) or the combination

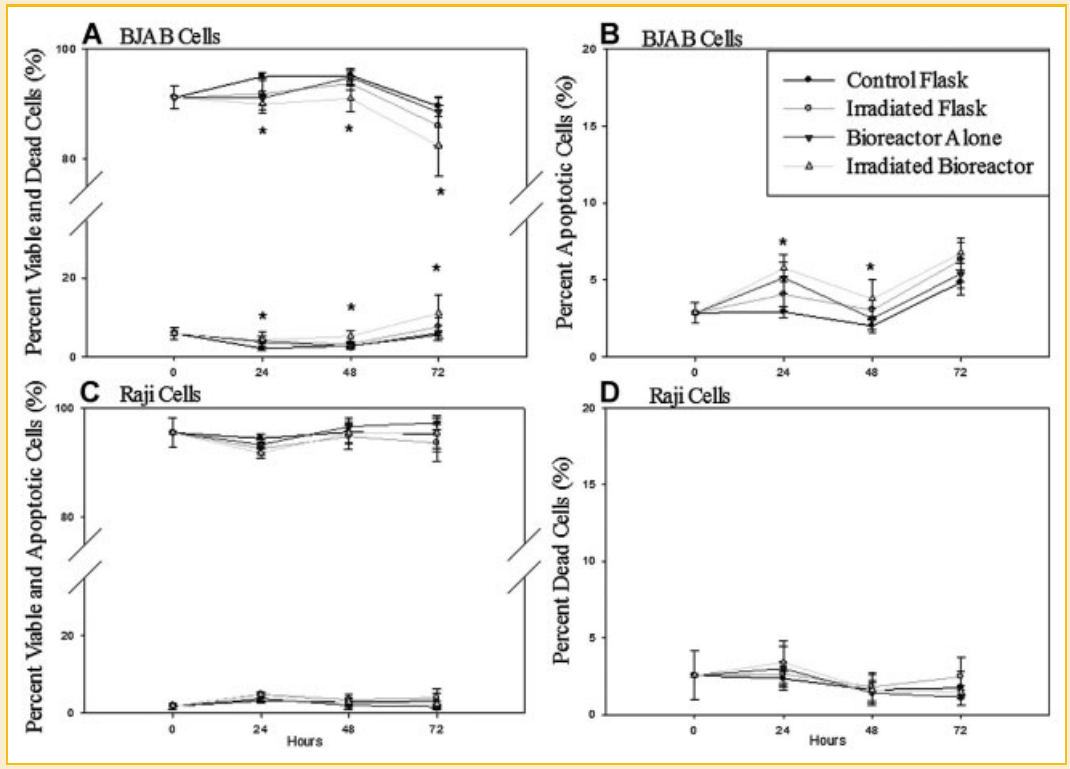

Fig. 1. Viability, apoptosis, and cell death in BJAB and Raji cells measured by Guava ViaCount assay. A: Percent of viable (top) and dead (bottom) BJAB cells (B) percent apoptotic BJAB cells (C) percent of viable (top) and apoptotic (bottom) Raji cells, and (D) percent of dead Raji cells after exposure to control, irradiation alone, modeled microgravity alone, and modeled microgravity with irradiation for 72 h. Figures show mean and SD of three experiments with two replicates each. Asterisk (*) indicates a statistical difference of at least $P = 0.05$ between the irradiated bioreactor environment (combination) and the control flask. A: Viable Cells: Main effect for the bioreactor alone ($P < 0.003$) and radiation alone ($P < 0.001$). Dead Cells: Main effect for the bioreactor alone ($P = 0.015$) and radiation alone $P < 0.001$). B: Apoptotic Cells: Main effect for the bioreactor ($P = 0.001$) and radiation alone ($P < 0.001$). C: Viable Cells: Main effect for radiation alone ($P = 0.045$). Apoptotic Cells: Main effect for radiation alone ($P = 0.031$). D: No main effects detected.

of radiation and modeled microgravity (viability: $P = 0.954$; apoptosis: $P = 0.930$; death: $P = 0.884$). Furthermore, there were no main effects for Raji cells detected by the Nexin assay for radiation alone (viability: $P = 0.317$; apoptosis: $P = 0.484$; death: $P = 0.149$), modeled microgravity alone (viability: $P = 0.542$; apoptosis: $P = 0.413$; death: $P = 0.481$), or the combination (viability: $P = 0.950$; apoptosis: $P = 0.669$; death: $P = 0.783$). Therefore, there were no main effects due to the Nexin assay indicating that modeled microgravity alone, radiation alone, and the combination of radiation and modeled microgravity do not affect the outcomes of viability, apoptosis, or death for Raji cells.

EXPRESSION OF EBV BHRF1 (VBCL-2) AND ZEBRA IN RAJI CELLS

Because EBV-infected Raji cells demonstrated very little change in viability and apoptosis due to the modeled spaceflight environment (Fig. 1C and D), evaluation of EBV BHRF1 (vBcl-2) was undertaken (Fig. 2). BHRF1 was expressed in an increasing proportion of the cell population due to radiation alone ($28.3 \pm 1.6\%$), modeled microgravity alone ($52.0 \pm 13.1\%$), and the combination of modeled microgravity and radiation ($61.5 \pm 9.1\%$) as compared to the control

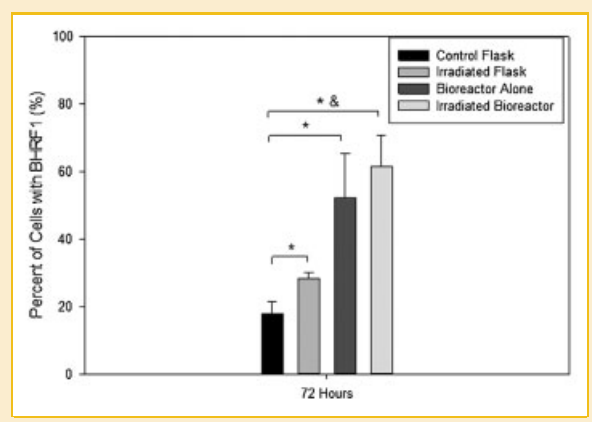

Fig. 2. Percent of Raji cells expressing vBcl-2 (BHRF1) after 72 h in the control flask, irradiated flask, bioreactor alone, or the bioreactor with irradiation measured by flow cytometry. Mean and SD of three experiments. Asterisk (*) indicates a statistical difference of at least $P = 0.05$ between the experimental conditions and the control flask. Main effect detected for the interaction between the bioreactor and radiation ($P < 0.001$), radiation alone ($P < 0.001$), and the bioreactor alone ($P < 0.001$).

flask (17.9 ± 3.5%). All mean values were significantly increased from the control flask (all $P < 0.003$) and a statistically significant, additive interaction between modeled microgravity and radiation was detected ($P < 0.001$) indicating that the combination upregulates the expression of BHRF1 more than either factor alone. BHRF1 was also evaluated by confocal microscopy in order to determine whether or not protein localization was affected by the modeled spaceflight environment. No change in localization was detected (data not shown).

Expression of EBV ZEBRA was assessed in order to identify whether or not the modeled spaceflight environment could affect the reactivation of EBV (Fig. 3). Positive controls (i.e., chemical induction with 3 mM SB/100 nM TPA) showed increased expression of ZEBRA in approximately 30% of cells at 72 h after treatment (Fig. 3A–D). In the modeled spaceflight environments, the percentage of cells expressing ZEBRA was increased due to radiation ($P < 0.001$), modeled microgravity alone ($P < 0.001$), and the combination ($P < 0.001$) compared to the control flask (Fig. 3E). There was also a significant interaction between modeled microgravity and radiation ($P = 0.001$). ZEBRA was evaluated by fluorescence microscopy in order to ensure that the localization of ZEBRA in experimental environments matched positive control localization (an additional measure of viral activation). Small vacuole-like structures, reminiscent of viral replication compartments, were observed in positive controls as has been reported in the literature [Takagi et al., 1991; Takahashi and Ohnishi, 2004]. This matched the fluorescence patterns in the irradiated bioreactor (data not shown).

DNA DAMAGE AND REPAIR IN THE MODELED SPACEFLIGHT ENVIRONMENT

DNA damage was assessed by the cytokinesis block micronucleus assay (CBMN) in the modeled spaceflight environment to determine the effects of EBV and spaceflight environmental conditions on chromosomal instability. DNA damage was measured by the percentage of cells with micronuclei (MN), nucleoplasmic bridges (NPB), and nuclear buds (NB; Table I). When MN were analyzed, both BJAB and Raji cell lines showed a similar pattern in DNA damage after exposure to either radiation or modeled microgravity alone, or in combination. A significant interaction was detected between modeled microgravity and radiation ($P < 0.003$) for both cell lines, indicating that both radiation and modeled microgravity are contributing to DNA damage in the spaceflight environment. Control BJAB cells had significantly fewer cells with MN (3.9 ± 0.2%) than control Raji cells (6.7 ± 1.1%; $P = 0.002$). After radiation, Raji cells (29.3 ± 4.4%) had a higher percentage of cells with MN than BJAB cells (20.2 ± 1.1%; $P < 0.001$). After modeled microgravity alone, both cell lines had indistinguishable levels of MN (Raji, 16.3 ± 0.5%; BJAB, 16.0 ± 0.8%; $P = 0.393$). Finally, the percentage of cells with MN after exposure to combined modeled microgravity and radiation was increased from control levels in both cell lines; although this was slightly higher in Raji (37.7 ± 1.5%) than in BJAB cells (35.0 ± 1.4%; $P = 0.009$).

NPB were evaluated as a measure of chromosome rearrangement [Fenech, 2007; Thomas et al., 2003] (Table I). BJAB cells had a low constitutive level of NPB that did not change much after exposure to radiation or modeled microgravity, but doubled after exposure to

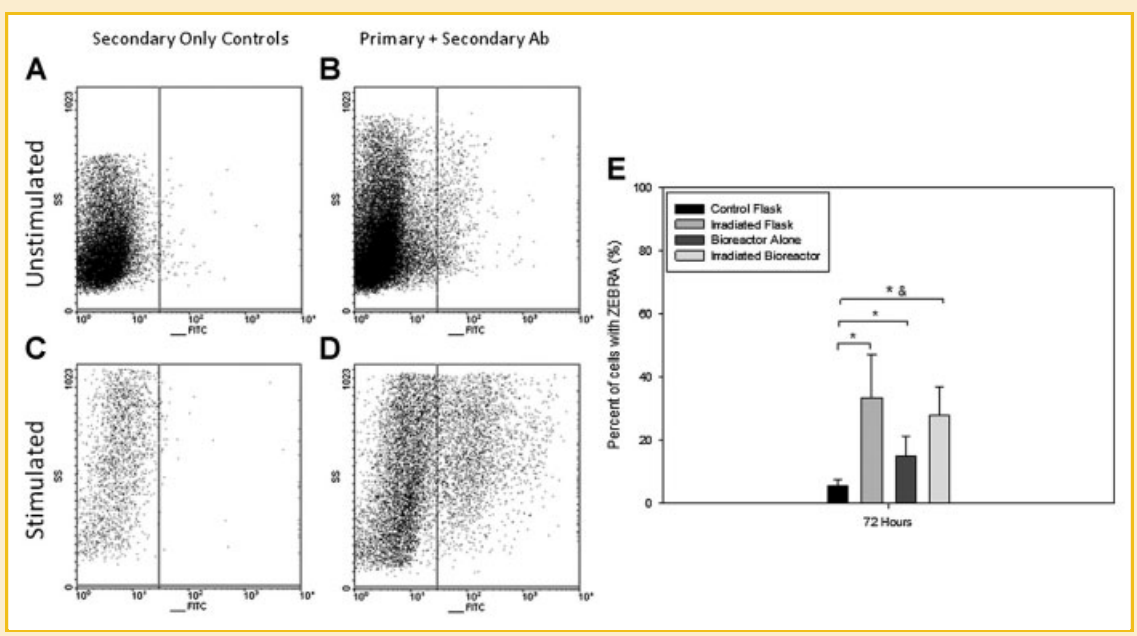

Fig. 3. Expression of EBV immediate-early ZEBRA. A–D: Percent of Raji cells expressing ZEBRA after induction with 3 mM sodium butyrate and 100 nM TPA for 4 days. A: Unstimulated cells with secondary antibody only, that is, cytometry control (B) Unstimulated cells with both primary and secondary antibodies, that is, the negative experimental control (C) Stimulated cells with secondary antibody alone (D) Stimulated cells with primary and secondary antibodies (i.e., positive experimental control). E: Percent of Raji cells expressing ZEBRA (BZLF1) after 72 h in the control flask, irradiated flask, bioreactor alone, or the bioreactor with irradiation measured by flow cytometry. Mean ± SD of three experiments. Main effect detected for the interaction between the bioreactor and radiation ($P = 0.001$), radiation alone ($P < 0.001$), and the bioreactor alone ($P = 0.005$).

TABLE I. DNA Damage in BJAB and Raji Cells Detected by the CBMN Assay

	BJAB			Raji		
Vessel type	MN	NB	NPB	MN	NB	NPB
Control flask	3.96 ± 0.2	0.18 ± 0.07	0.06 ± 0.05	6.02 ± 0.5	0.65 ± 0.3	0.30 ± 0.2
Irradiated flask	20.2 ± 1.1*	0.45 ± 0.09*	0.04 ± 0.05	32.0 ± 1.4*	1.18 ± 0.1*	0.72 ± 0.2*
Bioreactor alone	15.9 ± 0.8*	0.52 ± 0.05*	0.05 ± 0.05	16.33 ± 0.7*	1.00 ± 0.2*	0.92 ± 0.1*
Irradiated bioreactor	35.0 ± 1.4*	1.71 ± 0.3*	0.15 ± 0.06	38.27 ± 1.7*	1.27 ± 0.3*	1.15 ± 0.2*

Data expressed as mean percentages ± SD. Significant differences ($P \leq 0.05$) in means from the control flask are indicated by an asterisk.

the combination of radiation and microgravity. Raji cells had a higher control level of NPB and up to 1.2 ± 0.2% NPB ($P < 0.001$) in the combined radiation and modeled microgravity environment; however, levels of NPB remained in the 1% range after exposure to all environments. NBs provide a measure of gene amplification and the formation of MN (Table I) [Fenech, 2007]. Raji cells had a higher basal level of NBs than BJAB cells, which provides some insight into the higher number of MN in Raji cells. The combination of modeled microgravity and radiation increased the percentage of BJAB cells with NBs to a level on par with Raji cells but the overall percentage of nuclear buds was small for both cell lines.

Reactive oxygen species (ROS) can be generated by a wide variety of normal and pathological processes. In this study, ROS were assessed by determining the percentage of cells with ROS over threshold levels (Fig. 4). BJAB cells had a smaller percentage of cells expressing ROS as compared to Raji cells (17.0 ± 1.5% vs. 41.8 ± 2.8%, respectively) in control conditions as well as after exposure to radiation alone (29.8 ± 2.8% vs. 57.9 ± 3.6%, respectively), modeled microgravity alone (13.9 ± 5.6% vs. 51.1 ± 2.9%, respectively), and the combination (22.8 ± 4.9% vs. 60.7 ± 1.3%, respectively). While radiation alone ($P < 0.001$) and modeled microgravity alone ($P = 0.006$) had significant effects for the percent of BJAB cells expressing ROS, no significant interaction was detected ($P = 0.253$). Alternatively, there were significant effects for Raji cells due to both radiation ($P < 0.001$) and modeled microgravity alone ($P < 0.001$), and a significant interaction between modeled microgravity and radiation was also detected ($P = 0.04$).

Ataxia-telangiectasia, mutated (ATM) is a protein kinase that is involved in the DNA damage response to double strand breaks. ATM phosphorylates approximately 30 proteins, including p53 and H2AX, to activate cell cycle checkpoints, apoptosis, and DNA repair [Yang et al., 2003; Lavin et al., 2006; Tomita, 2010] and is known to be activated by ionizing radiation [Canman et al., 1998]. There was no change in the percentage of BJAB cells expressing ATM (Fig. 5A); however, in Raji cells (Fig. 5B), both of the modeled microgravity environments (modeled microgravity alone: 80.8 ± 3.1%; combined modeled microgravity and radiation: 80.1 ± 4.1%) had decreased percentages of cells expressing ATM when compared to those grown in a normal flask (control: 85.2 ± 2.9%; radiation alone: 85.1 ± 6.5%). Only modeled microgravity had a significant effect ($P = 0.017$) indicating exposure to modeled microgravity reduced the percentage of cells expressing ATM in Raji cells. Furthermore, increased cytoplasmic localization of ATM was found in EBV-positive Raji cells in control conditions and after exposure to modeled microgravity in both cell lines (data not shown).

DISCUSSION

In order to assess the effect of spaceflight factors, such as radiation and microgravity on latent EBV infection, a modeled spaceflight

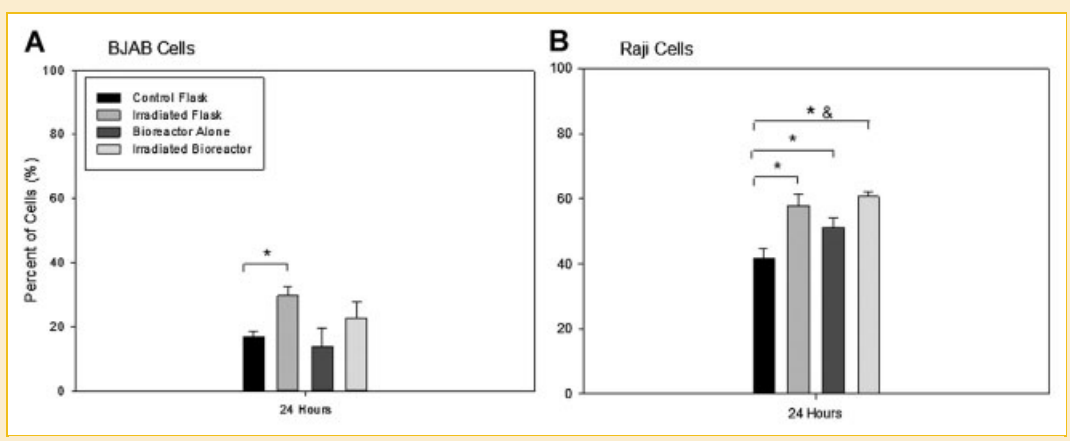

Fig. 4. Percent of BJAB (A) and Raji (B) cells with reactive oxygen species. For BJAB, radiation ($P < 0.001$) and modeled microgravity ($P = 0.006$) had significant effects; however, there was not a significant interaction ($P = 0.253$). The mean of the irradiated flask was increased from the control flask ($P < 0.001$). For Raji, radiation and modeled microgravity had significant effects ($P < 0.001$) and a significant interaction was detected ($P = 0.04$). The means of radiation alone ($P < 0.001$), modeled microgravity alone ($P < 0.001$), and the combination ($P = 0.04$) were increased from the mean of the control flask.

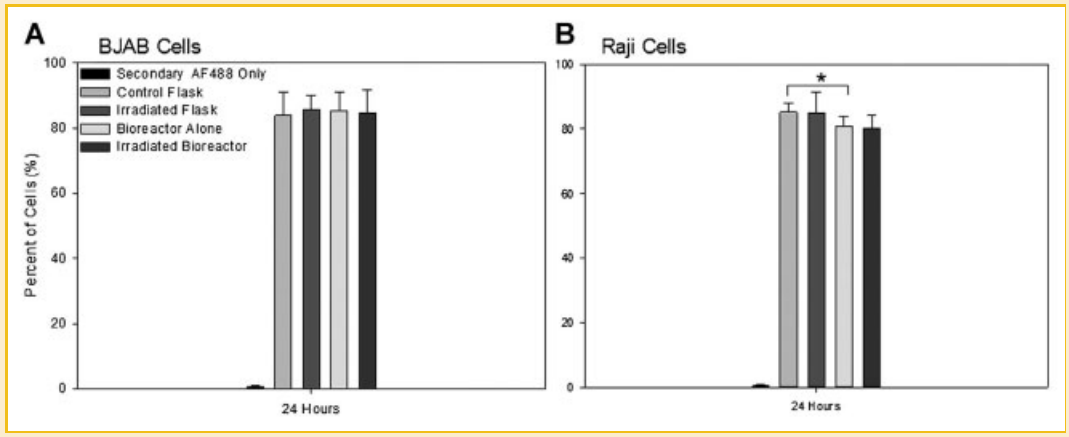

Fig. 5. Percent of BJAB (A) and Raji (B) cells expressing ATM. For BJAB, no significant differences were found. For Raji, modeled microgravity had a significant effect ($P = 0.017$). The mean of modeled microgravity alone was decreased from the mean of the control flask.

environment with one or both factors was investigated. An EBV positive cell line (Raji) was compared to an EBV negative cell line (BJAB) to determine the role of EBV and the modeled spaceflight environment.

We found that there was significantly increased apoptosis and cell death, and decreased viability in the EBV-negative cell line after exposure to modeled spaceflight whereas these differences were not evident in the EBV-positive cell line. In order to further investigate the effects of EBV on apoptosis, a known EBV anti-apoptotic protein, BHRF1 (or vBcl-2), was evaluated [Marshall et al., 1999]. The combined modeled microgravity/radiation environment induced increased levels of BHRF1 more than either radiation or modeled microgravity alone suggesting that the combined environment allows EBV to amplify viral anti-apoptotic proteins to prevent apoptosis. Therefore, the production of BHRF1 in the EBV-positive Raji cells may contribute to the ability of Raji cells to survive the modeled spaceflight environment better than BJAB cells; however, other viral or cellular factors are likely involved [Zuo et al., 2011].

Although stress plays a role in herpesvirus reactivation [Glaser and Kiecolt-Glaser, 1998], spaceflight environmental factors may also contribute to increased reactivation in space. ZEBRA is an EBV lytic antigen that is one of the first EBV genes activated upon the initiation of the lytic cycle. Radiation alone, modeled microgravity alone, and the combination all significantly increased activation of ZEBRA over control values. A statistically significant interaction between modeled microgravity and radiation was detected for ZEBRA. However, this interaction may be antagonistic because the combined environment was not increased from radiation or modeled microgravity alone. These data are consistent with studies by Ferrieu et al. [2003] which suggested increased EBV lytic activity due to 2–4 Gy radiation, and studies by Long and Hughes [2001] and Long et al. [1999], which suggested no change or decreased activation of ZEBRA due to modeled microgravity alone in most cases. However, one experiment in a Long and Hughes [2001] study indicated increased EBV activation in P3HR-1 cells after being cultured for 4–7 days in a bioreactor followed by exposure to TPA for 2–3 h. The experiments from our study suggest that radiation is likely the major spaceflight environmental factor contributing to the reactivation of EBV in our model.

Both EBV and radiation can contribute to genomic instability and DNA damage [Kamranvar et al., 2007; Gruhne et al., 2009ab]. We investigated the risk for DNA damage associated with the presence of EBV in B-lymphocytes after exposure to gamma radiation and modeled microgravity. DNA damage was assessed by three parameters: percentage of cells with MN, percentage of cells with NPB, and percentage of cells with NB, which were all measured by the CBMN assay. Increased DNA damage was detected in both EBV negative (BJAB) and EBV positive (Raji) cell lines after exposure to the modeled spaceflight environment. The combination of modeled microgravity and radiation increased DNA damage markers in both cell lines, suggesting that both modeled microgravity and radiation contribute to the DNA damage observed during spaceflight. However, DNA damage was also increased in EBV-positive Raji cells as compared to EBV-negative BJAB cells, which would indicate that EBV also contributed to increased DNA damage.

Previous research has found that EBV latency protein, EBNA1 can increase DNA damage by upregulating ROS. In agreement with this study, a greater percentage of Raji cells were expressing ROS than in BJAB cells. Furthermore, all of the model environments increased ROS in Raji cells while radiation was the only environment that increased the percentage of BJAB cells expressing ROS. This may indicate that either the higher basal level of ROS expression may make ROS production in Raji cells more sensitive to environmental changes, or activation of EBV could increase ROS since higher EBV ZEBRA was associated with increased ROS (Figs. 3 and 4).

ATM expression analyses indicated that modeled microgravity may inhibit DNA repair. Therefore, decreased capacity to repair DNA damage could explain why increased DNA damage was detected after exposure to modeled microgravity in both cell lines. While it is uncertain whether DNA damage is induced by modeled microgravity itself, or dysregulation of DNA repair, either could account for the accumulation of DNA damage in the modeled microgravity environment [Canova et al., 2005; Kumari et al., 2009]. EBV appears to induce some DNA damage in Raji cells according to the CBMN

assay and previous studies, perhaps by upregulating ROS production pathways [Gruhne et al., 2009ab]. Moreover, exposure to the combined radiation/modeled microgravity environment increased DNA damage. The amalgamation of these different factors likely resulted in the observed DNA damage in BJAB and Raji cells. Increased DNA damage in spaceflight due to radiation could be amplified by EBV infection. It appeared DNA damage was higher in EBV-infected Raji cells, which also had higher ROS and reduced ATM expression as compared to their non-EBV infected counterparts. Additionally, EBV and modeled microgravity appeared to affect the nuclear localization of ATM, likely further reducing its efficacy (data not shown). While we did not see a large change in the percentage of cells expressing ATM, we did not investigate changes in ATM kinase activity which may also be modulated by modeled microgravity. Whether ATM-dependent repair is significantly affected by EBV infection and modeled microgravity still remains relatively unknown.

There are several conclusions from this study. First, EBV-infected cells survive the model spaceflight environment better than non-EBV infected cells, which could be related to the increased expression of BHRF1, amongst other factors. Second, the EBV lytic antigen ZEBRA, was expressed in a greater percentage of cells after exposure to gamma radiation than after growth in control conditions, or conditions with modeled microgravity. This suggests that space radiation is a major environmental factor that contributes to increased lytic activity observed in our in vitro model of spaceflight, and may have played a role in previous studies of astronauts [Pierson et al., 2005; Stowe et al., 2011]. Thus, the cumulative data suggest that the modeled spaceflight environment may enhance virus-cell interactions to make the cell more favorable to viral DNA replication and EBV protein production. Finally, increased DNA damage, reduced DNA repair, and increased activation of viral anti-apoptotic proteins (i.e., BHRF1) are all consistent such that damaged cells may be able to survive and proliferate longer. These results are in agreement with the studies undertaken by Canova et al. [2005] and Kumari et al. [2009].

There are several limitations associated with this study. The cell and spaceflight models are by no means exactly replicative of actual space conditions. For example, B-lymphocytes were evaluated alone, without the rest of the immune system, including the cells with which B-cells interact with frequently, namely T-lymphocytes. It is possible that healthy, functioning T-lymphocytes would limit some of the EBV activity observed during the course of this study. There was also no adrenocortical stress response present in this simplified model of B-lymphocyte function. Additionally, the spaceflight environment was modeled and, thus, does not provide an entirely accurate depiction of conditions encountered in actual spaceflight. Gamma radiation is only a small subset of the radiation which is present in space, and the bioreactor only models certain aspects of the microgravity environment. While the bioreactor does provide a low-fluid shear environment which essentially removes the major forces of gravity and fluid shear from a cell-based system, gravity is still present. Furthermore, fluid shear in the bioreactor closely models fluid shear expected in germinal centers but is much less than expected in arterial blood vessels. During spaceflight, B-lymphocytes would still be exposed to fluid shear in the circulatory system; however, would encounter low fluid shear, similar to the bioreactor, in germinal centers [Resto et al., 2008], thus the bioreactor only models certain aspects of the microgravity environment.

There is no doubt that cells in spaceflight are exposed to greater levels of radiation than on Earth [Fry et al., 1994] and would be expected to have greater levels of DNA damage [Kawata et al., 2004; Matsumoto et al., 2004]. Although it appears there is an increased risk for DNA damage during spaceflight due to the combination of radiation, modeled microgravity [Ferrieu et al., 2003; Canova et al., 2005; Kumari et al., 2009], and EBV infection [Gruhne et al., 2009a], it is possible that reduced cellular immune function in response to spaceflight may exacerbate the effect of these factors [Crucian et al., 2008]. Therefore, increased DNA damage, with decreased apoptosis and DNA repair, could potentially lead to an increased risk for EBV-associated disorders during long duration spaceflight; however, this will require further investigation.

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