

Monitoring Immune System Function and Reactivation of Latent Viruses in the Artificial Gravity Pilot Study

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Abstract

Numerous studies have indicated that dysregulation of the immune system occurs during or after spaceflight. Using 21 day -6° head-down tilt bed rest as a spaceflight analog, this study describes the effects of artificial gravity (AG) as a daily countermeasure on immunity, stress and reactivation of clinically important latent herpes viruses. The specific aims were to evaluate psychological and physiological stress, to determine the status of the immune system, and to quantify reactivation of latent herpes viruses. Blood, saliva, and urine samples were collected from each participating subject at different times throughout the study. An immune assessment was performed on all treatment and control subjects that consisted of a comprehensive peripheral immunophenotype analysis, intracellular cytokine profiles and a measurement of T cell function. The treatment group displayed no differences throughout the course of the study with regards to peripheral leukocyte distribution, cytokine production or T cell function. Shedding of Epstein barr virus (EBV), Cytomegalovirus (CMV), and Varicella zoster virus (VZV) was quantified by real time PCR in saliva and urine samples, respectively. There was no significant difference in CMV DNA in the treatment group as compared to the control group. EBV and VZV on the other hand showed a mild reactivation during the study. There were no significant differences in cortisol between the control and treatment groups. In addition, no significant differences between antiviral antibody titers (EBV-VCA, -EA, -EBNA, CMV) or tetramer-positive (EBV, CMV) were found between the two groups. EBV DNA copies in blood were typically undetectable but never exceeded 1,500 copies per 10⁶ PBMCs. Overall, these data indicate that the artificial gravity countermeasure and the 21 day head-down tilt bed rest regimen had no observable adverse effect on immune function.

Introduction

Space flight is a unique experience and results in adverse effects on human physiology. Changes have been reported in the musculoskeletal, neurovestibular,

cardiovascular, endocrine, and immune systems as well as others (12). As NASA goes forward, preparing for longer exploration missions to the Moon, Mars, and eventually beyond, effective countermeasures to these adverse effects on humans must be developed, verified, and implemented to ensure mission success. Many of the significant adverse effects of space flight on human physiology are caused by various stressors. These include isolation, confinement, anxiety, sleep deprivation, psychosocial interactions, and physical exertion. The physical environment further subjects the crew to noise, chemical and microbiological contaminants, increased radiation, and variable gravity forces (hyper- and hypo-gravity). Many of these stress factors are intermittent, but some are relatively constant (such as microgravity on long missions).

Research on astronaut physiology in space is complicated by exposure to these and other uncontrolled factors. Research is further slowed by the limited access to space and availability of microgravity-compatible technology for on-board analyses. For these reasons, high-fidelity ground-based analogs of space flight are essential tools for studying the effects of space flight on human physiology, developing countermeasures as needed, and testing the efficacy of countermeasures.

Various ground-based analogs have been developed and used to model specific aspects of space flight. Bed rest has been successfully used to model the effects of microgravity on musculoskeletal and some other physiological systems. The immune system is affected by space flight, and some analogs have been used to study various aspects of immunity at the cellular level. Some success in studying the effects of space flight on the immune system has also been achieved in specific animal models (8, 13). Stress models such as Antarctic science stations and others have been useful in study of the effect of stress on human immunity (14). Generally, human bed rest has not been demonstrated to be a highly useful model for human immunity studies. However, the use of artificial gravity (AG) as a countermeasure for some other physiological systems (e.g., musculoskeletal) may impact some elements of immunity. The immune system interacts with most if not all other physiological systems. From this perspective, measuring selected indicators of immune status makes a valuable contribution to the overall study of efficacy of artificial gravity as a countermeasure for other physiological systems. In the current study, we tested the combined effects of hypergravity and stress and other factors associated with bed rest on changes in immunity and reactivation of latent herpes viruses.

Methods

Fifteen human subjects completed the study. All subjects spent 41 days residing in the NIH-supported General Clinical Research Center (GCRC) located at the University of Texas Medical Branch (UTMB) in Galveston, TX and were deconditioned by 6° head-down tilt bed rest for 21 of those days. Subjects in the

Treatment group (n=8) received one hour of AG via centrifugation daily. Subjects in the Control group (n=7) did not receive any countermeasure. All human study protocols were approved by the Committee for the Protection of Human Subjects of the Johnson Space Center, Houston, TX, and informed consent were obtained from all subjects.

Blood, saliva, and urine samples were collected from each participating subject in this study according to the schedule shown in Figure 1. Simultaneously, psychological stress was measured in each subject at the time of saliva collection using a questionnaire (figure 1). Blood samples were used to measure concentrations of stress hormones and titers of antibodies to viruses and to conduct tests of immune system status, including measurement of virus-specific T-cell number and function. Shedding of viral DNA was quantified in saliva and urine samples, and urine samples were used to measure excretion of stress hormones.

Psychological Stress

Psychological stress was measured by asking each participating subject to complete two questionnaires, the Perceived Stress Scale (PSS) and the Positive Affect and Negative Affect Scale (PANAS). These questionnaires were completed first thing in the morning, on days before, during, and after the subjects' saliva was collected. The questionnaires were scored and data were analyzed statistically.

The 10-item PSS (1) was used to measure the extent to which participants perceived their recent life circumstances as stressful (that is, unpredictable, uncontrollable, and overloading). Participants rate each of the 10 statements on a 5-point scale (0 = never to 4 = very often), so that scores range from 0 to 40. The scale is internally consistent and has demonstrated moderate correlations with other measures of appraised stress (15).

Physiological Stress

Plasma and salivary cortisol measurements were done by methods previously described (9).

Immune System Status

Immunophenotype analysis

A moderate four-color flow cytometry antibody matrix was created that assessed all the major leukocyte/lymphocyte subsets, as well as activated and memory/naïve T cell subsets. Cell surface markers were stained first combining 100 ul of EDTA whole blood and 10 µg of each appropriate labeled monoclonal antibodies. Staining was performed by incubated at room temperature for 20 minutes. Red blood cells were lysed using Beckman-Coulter optilyse as described by the manufacturer.

Stained leukocytes were then fixed in 1.0% paraformaldehyde in PBS for 10 minutes and analyzed on a Beckman-Coulter Epics XL flow cytometer. The cytometry panel is outlined in **table 1 (Brian, please change the Table number from 2 to 1)**. The white blood cell count (WBC) and differential were performed at the indicated timepoints by the Johnson Space Center clinical laboratory using a Beckman-Coulter Hematology Analyzer.

Intracellular cytokine analysis

Lymphocyte and monocyte cytokine production were assessed for specific cell subsets at the single-cell level utilizing intracellular flow cytometry. The unique advantage of intracellular flow cytometry is the ability to assess the production of multiple cytokines simultaneously in positively identified cell sub-populations using multi-color flow cytometry. Whole blood cultures were set up by adding 100 μ l heparin whole blood to 1.0 culture media containing 10 ng/ml PMA, 1.0 μ g/ml ionomycin and 3 μ M monensin. Cultures were incubated for 4.0 hours at 37 degrees C. Following incubation, the supernatants were removed, the RBCs lysed as noted above, and the remaining WBCs were fixed in 4.0% paraformaldehyde for 10 minutes. To detect intracellular production of IFN γ or IL-2 (following surface marker staining), the fixed PBMC's were resuspended in 200 μ l of permeabilization buffer, consisting of 5.0% non-fat dry milk and 0.5% saponin in PBS to which 0.5 μ g of labeled mouse antibody to either IFN γ or IL-2 (or both) was added. The cells were incubated at room temperature for 25 minutes and then washed in PBS containing saponin. The cells were then resuspended in 1.0% paraformaldehyde for analysis.

T cell function

Immune suppression may consist of a reduced capacity for lymphocytes to respond to stimulus, even though the relative distribution of immune cells is unchanged. For this study the functional response of T cells was measured by activating whole blood cultures in the presence of T cell mitogenic stimuli, followed by measurement of surface activation marker expression on T cell subsets. Culture in the presence of anti-CD3 and anti-CD28 antibodies was used for T cell stimulation. These antibodies activate T cells by triggering T cell surface molecules, requiring the full complement of intracellular signaling to be utilized (as opposed to phorbol ester or ionomycin). T cell progression through a full activation cycle may be monitored by culturing cells for 24 hours and determining the expression of cell surface CD69 (early activation), CD25 (mid-activation, receptor for IL-2 that requires new gene synthesis).

Tetramer assay

HLA-A*0201-restricted tetramers (EBV BMLF; CMV pp65) were obtained from Beckman Coulter (San Diego, CA). PBMCs were incubated with PE- or APC-labeled MHC-tetramer complexes, along with CD8-PerCP (BD Biosciences), in dPBS with

2% FCS for 30 minutes at room temperature. Cells were then washed and fixed in 1% PFA and analyzed on a FACSCalibur flow cytometer using CellQuest software for data collection and analysis.

Peptide stimulation assay

Intracellular cytokine staining assays were performed as described elsewhere (2, 4). PBMCs were isolated from heparinized whole blood by density-gradient centrifugation and washed three times in Dulbecco's phosphate-buffered saline (dPBS) prior to use in functional studies. PBMCs were used immediately and stimulated with HLA-A*0201-restricted epitopic peptides (10 ug/ml/peptide), controls (PBS) or staphylococcus enterotoxin B (SEB) (10 ug/mL). Peptides used in this study were CMV pp65 (residues 495-503; NLVPMVATV) and EBV BMLF (residues 280-288; GLCTLVAML). Costimulatory monoclonal antibodies (mAbs) – CD28 and CD49d mAbs (1 ug/ml each) (BD Immunocytometry Systems) – were added to each tube. The tubes were vortexed and incubated for 6 h at 37°C with 5% CO₂, with the addition of Brefeldin A (10 ug/ml; Sigma) for the last 5 h. Following stimulation, cells were washed in PBS, incubated for 5 min at 37°C in 0.02% EDTA and washed in dPBS. Cells were then sequentially incubated for 10 minutes in FACSlyse and FACSPerm solutions (both from Becton-Dickinson Biosciences), washed, and stained with anti-IFN- γ (PE), anti-TNF-a (FITC), anti-CD8 (PerCP), and anti-CD69 (APC) antibodies. Samples were then fixed in 1% paraformaldehyde and analyzed by four-color flow cytometry using a FACSCalibur cytometer and CellQuest software (both BD Biosciences).

Viral Reactivation Assessment

Viral DNA by polymerase chain reaction

PCR was performed on either urine, saliva, or 1×10^6 peripheral blood mononuclear cells. 3.0 ml of each urine sample or 2.0 ml of saliva was concentrated to ~200 uL by centrifugation using a Microsep concentrator 100K (Pall Filtron Corp., Northborough, MA). Extraction of genomic/viral DNA from concentrated urine was performed using the QIAamp Viral RNA Kit (Qiagen Inc., Santa Clarita, CA) according to the manufacturer's instructions. EBV and VZV DNA were quantitated by real-time PCR using an ABI 7700 sequence detector (3, 5, 7). CMV DNA was quantitated using the same methodology but with primers that targeted the immediate early gene (11).

Plasma viral antibody levels

Anti-viral antibody titers were determined by indirect immunofluorescence as previously described (7, 9, 10). Commercially prepared substrate slides and control sera (Bion Enterprises, Park Ridge, IL) were used for determining IgG antibody titers to EBV-viral capsid antigen (VCA) and early antigen (EA), EBV-nuclear antigen

(EBNA), VZV and CMV. Two-fold dilutions of plasma or serum from each subject were prepared, and the endpoint titer was determined as the highest dilution of serum still able to demonstrate immunofluorescence-positive cells. All specimens were batch analyzed and read blind-coded.

Results

Psychological and Physiological stress

PSS and PANAS results

A total of 318 behavioral measurements for Perceived Stress Scale (PSS) and Positive Affect and Negative Affect Scale (PANAS) scores were collected from all the 15 subjects (8 treatment and 7 control subjects) during the 41 day period of the study. No significant differences were found in the PANAS, and PSS in these subjects during 21-day study as compared to the before bed rest values or between the control and treatment group (data not shown).

Cortisol levels

Salivary cortisol levels were measured in 7 control and 8 treatment subjects. No significant changes were observed between the treatment or control subjects or between the time intervals before, during or after the 21 day bed rest. Likewise with plasma cortisol levels, no significant differences were found either between or within the treatment or control groups.

Immune assessment

The peripheral immunophenotype was assessed for all control and treatment subjects and consisted of a leukocyte differential, lymphocyte subsets, and the following T cell subsets: CD4/CD8 ratio, memory/naïve (CD45RA+/-), early activated (CD69+) and late activated (HLA-DR+). The control subjects displayed an elevated percentage of granulocytes that persisted over the duration of the head-down phase and resolved by R+9 (figure 2B), that was not observed in the treatment subjects (figure 2A) ($p=0.6$). This change was not statistically significant. In general, no other consistent differences were observed in the peripheral leukocyte distribution for any of the subsets measured for either the treatment or control subjects (figures 2A and 2B respectively). Intracellular IFN γ and IL-2 were assessed for both the CD4+ and CD8+ T cell subsets following 5 hours stimulation with PMA+ionomycin. The treatment subjects displayed a significant decrease in the CD4+/IFN γ + subset and a non-significant trend towards reduced CD8+/IFN γ + (figure 2C) at BR+7 as compared to baseline values. This change was not evident in the control subjects. In fact, the control subjects demonstrated an increase in CD4+/IFN γ percentage that was not

significant (figure 2D). There were no obvious mission associated changes observed for the CD4+/IL-2 and CD8+/IL-2 subsets. T cell function was assessed by stimulating T cells with soluble CD3/CD28 antibodies for 24 hours and measuring the percentage of responding CD69+/CD25+ T cell subsets. There appeared to be no observable alterations in T cell function for either the treatment (figure 2E) or the control (figure 2F) subjects.

Four of the treatment subjects were HLA-A2 positive, while only 1 of the control subjects was positive. This limited data analysis for the MHC tetramers and peptide-stimulation assays. However, no differences were found in the levels of either CMV or EBV-specific CD8+ T-cells as revealed by MHC tetramer staining nor functional differences as determined by intracellular cytokine synthesis (IFN- γ and TNF- α) following viral-peptide stimulation (all data not shown).

Viral Reactivation

Antibody titers to EBV-VCA, -EA, EBNA, and CMV were measured in the control and treatment groups over the course of the study. No significant changes were found in any of the antiviral IgG antibodies between or within the 2 groups (data not shown).

Salivary EBV was detected frequently in both control group and treatment group. However, its frequency and copy number did not change significantly between these two groups or before, during and after the 21 day bed rest period. The frequency of VZV DNA was found in fewer of the saliva samples collected from treatment subjects than the samples from control. Similarly, CMV was also found less frequently in samples collected from treatment subjects as compared to the control subjects (Table 2). EBV DNA levels in peripheral blood PBMCs were also measured. The levels of EBV DNA were generally undetectable and never exceeded 1,500 copies per 10^6 PBMCs; no differences were found between the 2 groups.

Discussion

As peripheral phenotypic changes are generally associated with subject pathology, it was not unexpected that there were essentially no peripheral phenotype alterations in both the treatment and control subjects. This is consistent with healthy individuals who are essentially 'isolated' from society for a period of time. We did not expect them to become 'ill'. However, data from spaceflight and other ground-based spaceflight analogs has indicated that immune functional changes can be expected from healthy individuals experiencing unexpected, potentially stressful conditions like spaceflight. The reduced percentage of IFN γ producing T cell subsets observed

at BR+7 in the treatment group, but not the control group (figure 2C), may be an initial stress response to daily centrifugation. The fact that this alteration corrects itself by BR+14 lends support to this idea. There were no observed changes in the T cell function assessments for either treatment or control subjects (figures 2E and 2F). These data indicate that there was no observable immune-related pathology during the bed rest phase for either subject group. In addition, there was no other adverse effect on generalized immune function. Thus, neither the 21 day bed rest nor the daily AG treatment appeared to adversely affect immunity.

We have previously reported increase viral (EBV, VZV and CMV) reactivation during space flight as well as ground-based space analog studies (6). In this study, though there was an increasing trend in the EBV reactivation during the 21day bed rest period in the subjects in both groups (EBV frequency 21%), these increases were not significantly different. It is interesting to note that VZV and CMV which are not shed in normal healthy subjects, showed a slight decrease in the subjects who had undergone AG treatment as compared to the control group suggesting that AG may help in lowering the CMV and VZV shedding in these subjects.

In summary, our data suggest that stress associated with 21days of bed rest along with artificial gravity countermeasure did not have a major affect on the behavioral assessment scores, salivary cortisol and reactivation of EBV, CMV and VZV.

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