

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 a daily artificial gravity (AG) countermeasure treatment on immunity, stress, and reactivation of clinically important latent herpes viruses. Blood, saliva, and urine samples were collected from each of the 15 male test subjects (8 treatment, 7 control) periodically throughout the study. The immune assessment consisted of a comprehensive peripheral immunophenotype analysis, intracellular cytokine profiles, and measurement of T cell function. With the exception of mild reactivation of Epstein-Barr (EBV) and Varicella zoster (VZV) viruses, no significant changes in immune function were observed, suggesting that the AG countermeasure and the 21 day head-down tilt bed rest regimen had no adverse effect on immune function.

1. 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 [1]. 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 [2,3]. Stress models such as Antarctic science stations and others have been useful in study of the effect of stress on human immunity [4]. 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 AG 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.

2. 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 at the Johnson Space Center, Houston, TX, and the Institutional Review Board at UTMB; informed consent was obtained from all subjects.

Blood, saliva, and urine samples were collected from each participating subject in this study according to the schedule shown in Fig. 1. Simultaneously, psychological stress was measured in each subject at the time of saliva collection using a questionnaire (Fig. 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.

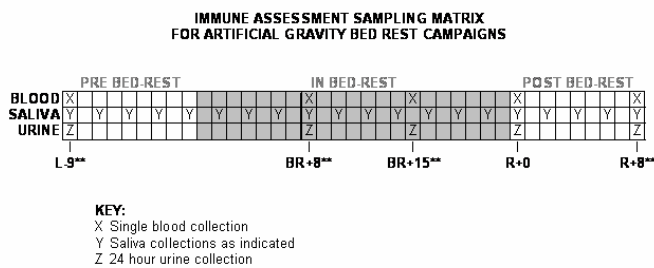


Fig. 1. Schedule for blood, saliva, and urine collection.

2.1 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 [5] 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 [6].

2.2 Physiological Stress

Plasma and salivary cortisol measurements were done by methods previously described [7].

2.3 Immune System Status

2.3.1 Immunophenotype analysis

A moderate four-color flow cytometry antibody matrix was created to assess all the major leukocyte/lymphocyte subsets, as well as activated and memory/naïve T cell subsets. Cell surface markers were stained first combining

100 µl 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. The white blood cell count (WBC) and differential were performed at the indicated time points using a Beckman-Coulter Hematology Analyzer.

Table 1. Flow cytometry panel for Bed Rest Campaigns.

FL	FL	FL	FL	CELL TYPES
CD1	CD1	CD4		WBC
CD	CD1	CD1	CD4	T cells, NK
CD	CD	CD		T cell
CD45R	CD45R	CD	CD	Memory/naïve T cell
HLA-	CD6	CD	CD	Early-, late-activated T cell

2.3.2 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 µl heparin whole blood to 1.0 culture media containing 10 ng/ml PMA, 1.0 µg/ml ionomycin and 3 µM monensin. Cultures were incubated for 4.0 hours at 37° 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 IFNγ or IL-2 (following surface marker staining), the fixed PBMC's were resuspended in 200 µl of permeabilization buffer, consisting of 5.0% non-fat dry milk and 0.5% saponin in PBS to which 0.5 µg of labeled mouse antibody to either IFNγ 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.

2.3.3 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 compliment 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).

2.3.4 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.

2.3.5 Peptide stimulation assay

Intracellular cytokine staining assays were performed as described elsewhere [8,9]. 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- α (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).

2.4 Viral Reactivation Assessment

2.4.1 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 [10,11,12]. CMV DNA was quantitated using the same methodology but with primers that targeted the immediate early gene [13].

2.4.2 Plasma viral antibody levels

Anti-viral antibody titers were determined by indirect immunofluorescence as previously described [7,12,14]. 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.

3. RESULTS

3.1 Psychological and Physiological stress

3.1.1 PSS and PANAS results

A total of 318 behavioral measurements for PSS and PANAS scores were collected from all 15 subjects during the 41 day study. No significant differences were found in PANAS or PSS during 21-day bed rest period (compared to the pre-bed rest values) nor were any differences found between the control and treatment groups.

3.1.2 Cortisol levels

Salivary cortisol levels were measured in all 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.

3.2 Immune assessment

The peripheral immunophenotype was assessed for all 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, that was not observed in the treatment subjects. 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. 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 + at BR+7 as compared to baseline values (Fig. 2). 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. No changes were 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 or the control subjects.

Four of the treatment subjects were HLA-A2 positive, while only one 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.

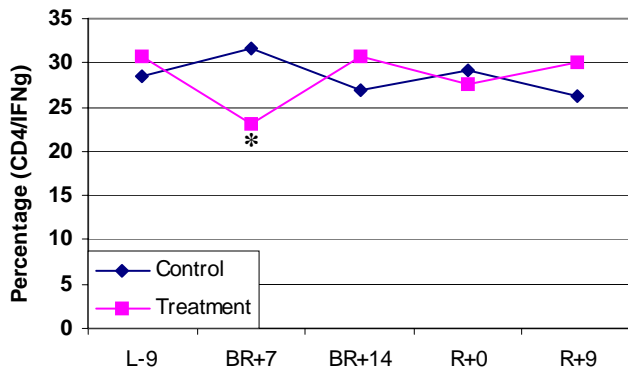


Fig. 2. CD4/IFN γ Profiles.

3.3 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 groups.

Salivary EBV was detected frequently in both control group and treatment group. However, its frequency and copy number did not change significantly between the groups or before, during, and after bed rest. 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 10⁶ PBMCs; no differences were found between the groups.

Table 2. Viral shedding in 15 subjects before, during and after a 21 day AG study.

a) EBV (frequency of EBV shedding in 14 healthy adults was 3.4%)

	EBV copies				Frequency	
	Treatment		Control		Treatment	Control
	Mean	SD	Mean	SD	%	%
Before	17.2	32.1	29.6	80.6	21.4	20.0
During	43.8	88.4	79.7	145.7	22.8	32.1
After	40.1	45.8	58.7	38.8	18.0	24.2

b) VZV (VZV copies as measured by real time PCR were between 23 and 43/ml saliva) and CMV (frequency of CMV shedding in 14 healthy adults was less than 1.6%)

	*Number of samples positive for VZV		*Number of samples positive for CMV	
	Treatment	Control	Treatment	Control
Before	1/44	3/35	1/18	1/16
During	1/92	4/67	1/30	3/24
After	1/40	1/40	0/18	1/13

4. DISCUSSION

As peripheral phenotypic changes are generally associated with subject pathology, it was not unexpected that there were essentially no peripheral phenotype alterations in any of the 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 (Fig. 2), 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. These data indicate that there was there 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 [15]. In this study, though there was an increasing trend in the EBV reactivation during the 21 day 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 21 days 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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