Immune Status, Latent Viral Reactivation, and Stress During Long-Duration Head-Down Bed Rest

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Introduction: As logistical access for space research becomes more limited and NASA prepares for exploration-class missions, ground-based spaceflight analogs will increase in importance for biomedical countermeasures development. A monitoring of immune parameters was performed during the NASA Flight Analogs Project bed rest study (without countermeasure); to establish 'control' data against which future studies (with countermeasure) will be evaluated. Some of the countermeasures planned to be evaluated in future studies may impact immune function. ***Methods:*** The immune assessment consisted of: leukocyte subset distribution, early T cell activation, intracellular cytokine profiles, latent viral reactivation, virus specific T cell levels and function, stress hormone levels, and a behavioral assessment using stress questionnaires. ***Results:*** In general, subjects did not display altered peripheral leukocyte subsets, constitutive immune activation, altered T cell function, or significant latent viral reactivation (EBV, VZV). Levels of constitutively activated T cells (CD8+/CD69+) and virus-specific T cells (CMV and EBV) decreased during the study. Cortisol levels (plasma and saliva) did not vary significantly during 90-d bed rest. ***Conclusions:*** These data demonstrate the absence of significant immune system alteration and physiological stress during 90-d bed rest, and establish control data against which future studies (including countermeasures) may be compared.

Keywords: immune function, cytokines, viral reactivation, microgravity.

NUMEROUS STUDIES have shown that there appears to be a dysregulation of the immune system following spaceflight of varying duration. Specific findings include altered leukocyte distribution and cytokine production postflight (6,26) and latent viral reactivation during spaceflight (16,20,24,25). There have recently been excellent reviews of what is currently known regarding the status of the immune system during spaceflight (1,23). Although the cause of this phenomenon is unknown, a synergy of spaceflight effects such as radiation, physiological stress, or disrupted circadian rhythms are likely causal factors.

The goal of NASA and the space life science community is to determine the clinical risks associated with all spaceflight effects on human physiology, so that countermeasures may be developed prior to the initiation of space missions beyond Earth orbit. This need has been heightened by the impending lunar/Mars program, soon to be initiated by NASA. Due to the state of the Space Shuttle and ISS programs, it is currently difficult to perform in-flight human physiology research. As mission availability, up-mass/volume, and crewmember on-orbit science time decreases, the use of ground-based spaceflight analogs will increase.

The choice of analog largely depends on the physiological system of interest. Closed chamber studies are excellent for psychological research, whereas prolonged bed rest is regarded best for bone and muscle loss. Arctic/Antarctic missions also simulate many aspects of long-duration spaceflight, but are a poor analog for bone loss. NASA has recently determined that the bed rest analog represents the most practical model for broad, multi-system spaceflight physiology research. This is in spite of the fact that the bed rest analog is not the best analog for some spaceflight effects on human physiology, such as immune dysfunction. Although some immune changes have been previously reported using the bed rest analog (altered leukocyte subsets, diminished cellular immunity, altered cytokine profiles), the data vary widely (2,9,21,22,29). These variations could be due to differences in study setting, duration, etc. Regarding some physiological changes, the bed rest model frequently differs from spaceflight (8,17,18).

The panel of immune tests presented here has been included in the NASA Flight Analogs Project (FAP) bed rest studies in order to monitor the effect that countermeasures (to be tested in future bed rest studies) may have on immunity. This article is one in a series of reports on the FAP, which is designed to lay the groundwork for a standard bed rest protocol. The immune data are considered 'control' for the future studies where countermeasures such as exercise or medications will be used. The assays selected for the FAP immune assess-

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ment activity determine: 1) peripheral leukocyte distribution; 2) early T cell activation and intracellular cytokine profiles; 3) viral specific immunity and viral reactivation; and 4) physiological and psychological stress levels. The specific immune assessment panel is presented as follows:

- General Immune Assessment
  - Comprehensive immunophenotype
  - T cell function
  - T cell intracellular cytokine profiles
- Viral Specific Immunity
  - Viral specific T cell number
  - Viral specific T cell function
  - Plasma viral antibody levels
- Viral Reactivation
  - Plasma EBV DNA level
  - Saliva EBV/VZV DNA level
- Neuroendocrine Stress-Response
  - Plasma, saliva cortisol levels
- Psychological Stress
  - PSS score
  - PANAS score

METHODS

Subjects/Samples

Study methods are as described by Meck et al. (13). Bed rest and test protocols were reviewed and approved by the Johnson Space Center Committee for the Protection of Human Subjects, the UTMB Institutional Review Board, and the UTMB General Clinical Research Center Science Advisory Committee. Subjects received verbal and written explanations of the bed rest and test protocols prior to providing written informed consent. Data from 10 subjects (5 men and 5 women) are presented in this article (subjects 4-13). The immune assessment described here was not conducted on subjects 1-3. Unfortunately, whereas subjects 4–7, 12, and 13 completed the full 90-d bed rest duration, the bed rest duration of subjects 8–11 was truncated after ~50 d due to hurricane Rita impacting the Houston/Galveston area. For this reason all data are represented as follows: $N = 10$ for all data points through BR50; whereas $N = 6$ for all data presented beyond BR50. For the immune assessment activity, whole blood, saliva, and 24-h urine samples, as well as a psychological questionnaire were collected from all subjects. For this study the sample consisted of 5.0 ml heparin and 10.0 ml ethylenediaminetetraacetic acid (EDTA)-treated (anticoagulated) whole blood. Blood samples were collected by standard phlebotomy techniques. The complete schedule for all blood, saliva, and urine sample collection is shown in **Fig. 1**. 'BR−' indicates the number of days prior to bed rest start; 'BR' indicates the number of days during bed rest; and 'BR+' indicates the number of days after completion of bed rest that samples were collected.

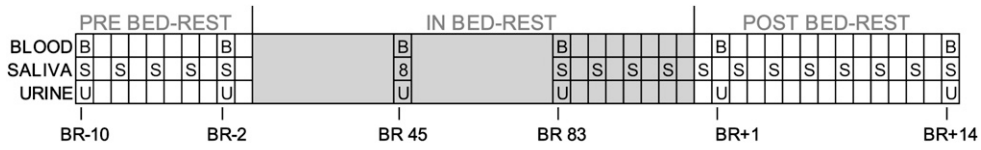
Immunophenotype Analysis

A 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. The peripheral immunophenotype was assessed for all subjects and consisted of: leukocyte differential, lymphocyte subsets, CD4/CD8 ratio, and memory/naïve T cell subsets. The surface markers used for immunophenotype analysis are outlined in **Table I**. 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 by combining 100 μl of EDTA whole blood and 10 μg of each appropriate labeled monoclonal antibodies. Staining was performed by incubating at room temperature for 20 min. Red blood cells were lysed using Beckman-Coulter (San Diego, CA) Optilyse as described by the manufacturer. Stained leukocytes were then fixed in 1.0% paraformaldehyde in PBS for 10 min and analyzed on a Beckman-Coulter Epics XL flow cytometer.

Early T Cell Activation

T cells were activated during whole blood culture for 24 h in the presence of antibodies which trigger the TCR (anti-CD3) and provide co-stimulation (anti-CD28). Culture was performed by combining 100 μl of heparinized blood, 1.0 ml RPMI media, and 10 μl each of anti-CD3 and anti-CD28. Cultures were incubated for 24 h at 37°C. Following culture, T cell progression through a full activation cycle was monitored by determining the expression of CD69 (early activation) and CD25 (mid-activation, receptor for IL-2 that requires new gene synthesis). Following incubation, 800 μl of supernatant was removed and discarded from the cell pellet. The cell pellet was then resuspended in the remaining media. A four-color staining of cell surface markers (CD25/FITC, CD69/PE, CD8/ECD, and CD3/PC5) was performed as described above. Flow cytometry was performed on a Beckman-Coulter XL flow cytometer. The gating strategy consisted of T cell resolution and separation into CD4 and CD8 subsets, followed by enumeration of both total CD69+ and CD69+/CD25+ dual positive events.

Intracellular cytokine analysis: T cell cytokine production was assessed for specific cell subsets at the single cell level using intracellular flow cytometry. Whole blood cultures were set up by adding 100 μl heparin whole blood to 1.0 ml culture media containing 10 ng · ml^{-1} PMA, 1.0 μg · ml^{-1} ionomycin, and 3 μM monensin. Cultures were incubated for 4.0 h at 37°C. Following incubation, the supernatants were removed, the red blood cells lysed as noted above, and the remaining WBC were fixed in 4.0% paraformaldehyde for 10 min. To detect intracellular production of IFNγ or IL-2 (following surface marker staining), the fixed peripheral blood mononuclear cells (PBMC) were resuspended in 200 μl of permeabilization buffer (5.0% non-fat dry milk and 0.5% saponin in PBS) to which 0.5 μg of labeled mouse antibody to human IL-2 (FITC), IFNγ (PE), CD8 (ECD), and CD3 (PC5) was added. The cells were incubated at room temperature for 25 min and then washed in PBS containing saponin. The cells were then resuspended in 1.0% paraformaldehyde and analyzed on a Beckman Coulter



KEY: B Single blood collection
S Single saliva collection
8 Eight saliva collections, every other day for 16 days surrounding blood collection
U 24 hour urine collection

Fig. 1. Immune assessment sampling matrix for 90-day bed rest. Whole blood, saliva, and 24-h urine samples were collected according to this schedule.

XL flow cytometer. The gating strategy consisted of T cell identification, resolution of CD4 and CD8 subsets, and plotting/enumeration of IFNγ and IL-2 expressing cells.

Plasma Viral Antibody Levels

Anti-viral antibody titers were determined by indirect immunofluorescence as previously described (20,24,25). Commercially prepared substrate slides and control sera (Bion Enterprises, Park Ridge, IL) were used for determining IgG antibody titers to Epstein-Barr virus (EBV) viral capsid antigen (VCA) and early antigen, EBV-nuclear antigen, Varicella-Zoster virus (VZV), and cytomegalovirus (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.

Tetramer Assay

HLA-A*0201-restricted tetramers (EBV BMLF; CMV pp65) were obtained from Beckman Coulter (San Diego, CA). PBMC were incubated with PE- or APC-labeled MHC-tetramer complexes, along with CD8-PerCP (BD Biosciences, San Jose, CA) in dPBS with 2% FCS for 30 min 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 (7,11). PBMC 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. PBMC were used immediately and stimulated with HLA-A*0201-restricted epitopic peptides (10 ug $\cdot$ ml^{-1} per peptide), controls (PBS), or staphylococcus enterotoxin B (SEB) (10 μg $\cdot$ ml^{-1}). Peptides used in this study were CMV pp65 (residues 495-503; NLVPMVATV) and EBV BMLF (residues 280-288; GLCTLVAML). Co-stimulatory monoclonal antibodies—CD28 and CD49d monoclonal antibodies (1 μg $\cdot$ ml^{-1} each) (BD Immunocytometry Systems)—were added to each tube. The tubes were vortexed and incubated for 6 h at 37°C with 5% CO_2, with the addition of Brefeldin A (10 μg $\cdot$ ml^{-1}; 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 min in FACSLyse and FACSPerm solutions (both from Becton-Dickinson Biosciences), washed, and stained with anti-IFN-g (PE), anti-TNF-a (FITC), anti-CD4 or 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). Flow cytometry data was analyzed and presented using Flow Jo Software (Tree Star, San Carlos, CA); 50,000 events were analyzed for each sample.

Viral DNA by Polymerase Chain Reaction

PCR was performed on either urine, saliva, or 1.0 x 10^6 PBMC. Using a Microsep concentrator 100K (Pall Filtron Corp., Northborough, MA) 3.0 ml of each urine sample or 2.0 ml of saliva was concentrated to ~200 μl by centrifugation. Extraction of genomic/viral DNA from concentrated urine was performed using the QIAamp

TABLE I. FLOW CYTOMETRY PANEL AND LEUKOCYTE SUBSET DATA FOR THE FLIGHT ANALOGS PROJECT. FOR EACH CELL SUBSET, DATA ARE PRESENTED AS MEAN ± SEM.

Cell Subset	Markers	BR−10	BR−2	BR45	BR83	BR+1	BR+14
Granulocytes	CD14, CD45	60 ± 5	54 ± 3	54 ± 4	57 ± 2	56 ± 2	56 ± 3
Lymphocytes	CD14, CD45	34 ± 5	40 ± 3	40 ± 4	37 ± 2	33 ± 3	39 ± 3
Monocytes	CD14, CD45	4 ± 0	4 ± 0	5 ± 0	4 ± 0	4 ± 0	4 ± 0
T cells	CD3	67 ± 4	72 ± 3	70 ± 3	70 ± 3	70 ± 3	69 ± 3
B cells	CD19	14 ± 3	11 ± 2	14 ± 2	14 ± 2	14 ± 2	12 ± 2
NK cells	CD16+CD56	13 ± 3	11 ± 3	11 ± 2	10 ± 2	9 ± 2	12 ± 2
T cells/CD4+	CD3, CD4	57 ± 4	60 ± 3	62 ± 3	60 ± 4	58 ± 4	59 ± 4
T cells/CD8+	CD3, CD8	37 ± 4	35 ± 3	33 ± 3	35 ± 4	36 ± 4	36 ± 4
Memory CD4	CD4, CD45RO	59 ± 7	56 ± 6	57 ± 6	64 ± 3	62 ± 2	64 ± 2
Memory CD8	CD4, CD45RO	39 ± 5	38 ± 5	37 ± 4	41 ± 3	40 ± 3	45 ± 3

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 (10,15,20). CMV DNA was quantitated using the same methodology but with primers that targeted the immediate early gene (28).

Stress Hormone Assessments

The measurement of hormones has been previously described in detail (24,26). Cortisol was measured by EIA. Plasma cortisol was assessed at each timed blood collection and saliva cortisol was assessed for each saliva sample collected (Fig. 1).

Psychological Questionnaire

Psychological evaluation of all subjects was performed using the Perceived Stress Scale (PSS) and the Positive Affect and Negative Affect (PANAS). Pre-bed rest evaluations were performed every other day from BR−10 to the initiation of bed rest. Evaluations during the bed rest period were performed at the following timepoints: days BR37, BR39, BR41, BR43, BR45, BR47, BR49, BR51, BR83, BR85, BR87, and BR89. Post-bed rest evaluations were performed starting at the end of the bed rest period (BR+0) and continued every other day through BR+12.

Statistical Analysis

Due to the low *N* for this study, some measurements (immune function, viral specific immunity) are not statistically analyzed. For these analyses, single subject data or mean data are presented. Where statistical analysis was performed (phenotype, cortisol, psychological data, viral DNA), one-way analysis of variance was used to study significant differences across different times during the study. The method of generalized estimating equations with a logit link was used to find significant difference between the phases (pre-, during, and post-bed rest). The differences between sampling periods were considered significant if $P < 0.05$.

RESULTS

No consistent changes were observed for either the total WBC or the relative percentage of neutrophils, basophils, or eosinophils (data not shown). The mean leukocyte subset data are presented in Table I. No significant changes were observed in any of the subsets measured for the duration of the study. Levels of early (CD4/CD69, CD8/CD69) and late (CD4/HLA-DR, CD8/HLA-DR) activated T cell subsets were also assessed. No changes were observed in the levels of CD4+/CD69+ T cells, with levels remaining low throughout the study (data not shown). Constitutive levels of CD8+/CD69+ T cells may range from 2–10% in healthy normal subjects. These cells likely represent normal immune activity to everyday environmental challenges. During the bed rest studies, those subjects with a detectable baseline value displayed reductions in the level of CD8+/CD69+ T cells (**Fig. 2A**). Among those subjects who had detectable levels (> 2.0%) of either CD4+ or CD8+/HLA-DR+ late activated T cells (4 and 8 subjects, respectively), the levels decreased during bed rest in all cases (data not shown).

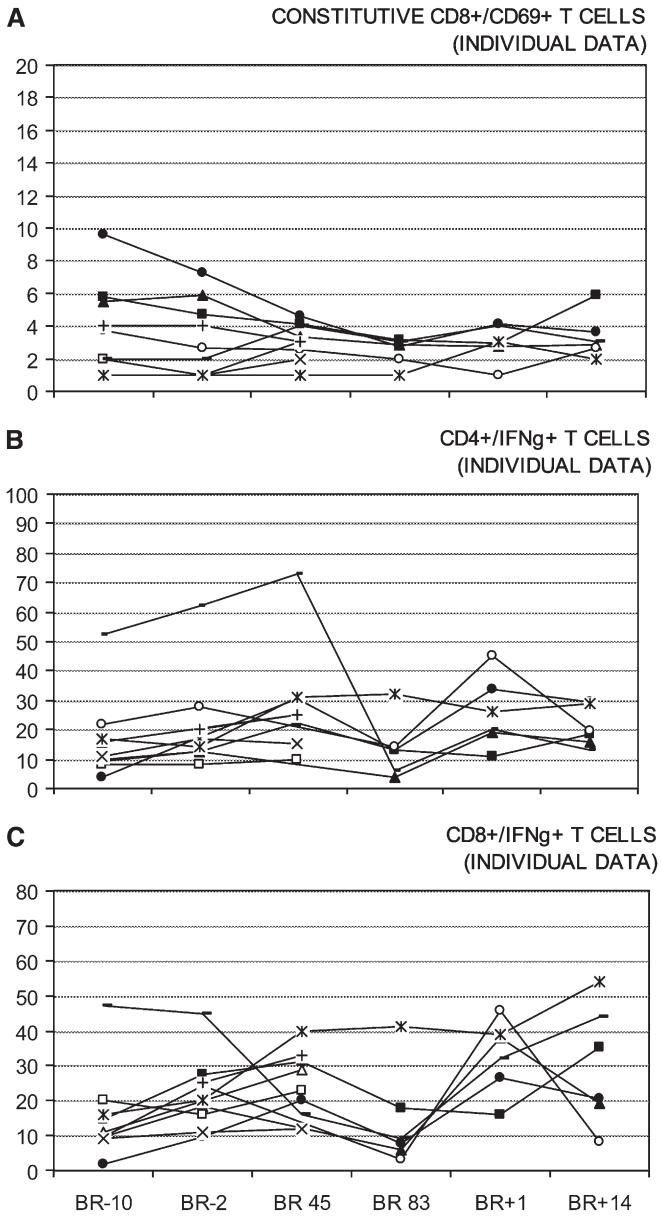


Fig. 2. Selected general immune data for subjects participating in 90-d bed rest. A) Single subject data regarding constitutive levels of peripheral blood activated CD8+ T cells; B and C) Single subject data representing levels of CD4+ and CD8+ T cells (respectively) capable of being stimulated to produce interferon-gamma following whole-blood activation in the presence of PMA+ionomycin.

T cell function was assessed both by intracellular IL-2 and IFNg detection in T cell subsets following PMA/ionomycin stimulation for 4 h, and CD69+/CD25+ expression following anti-CD3/CD28 antibody stimulation for 24 h in whole blood culture. The percentage of T cells capable of being stimulated to produce IL-2 tended to vary among the subjects with no changes evident for either the CD4 or CD8 subset (data not shown). There were no significant changes in the percentage of CD4+ T cells capable of being stimulated to produce IFNg,

although some subjects displayed reductions by BR83 (**Fig. 2B**).

Among the six subjects who completed the full 90-d study duration, five subjects demonstrated reductions in the percentage of CD8+ T cells capable of being stimulated to produce IFNg by BR83 that rebounded sharply after reambulation at BR+1 (**Fig. 2C**). T cell functional assessments of CD69/CD25 expression following mitogenic stimulation did not show any consistent changes (data not shown).

Viral immunity and EBV/CMV reactivation were assessed by determining viral antibody titers (EBV VCA, EBV early antigen, EBV nuclear antigen, and CMV), EBV DNA levels, and both the number (via tetramer) and function (via intracellular IFNg following peptide stimulation) of CMV and EBV viral specific T cells. No significant changes in EBV or CMV antibody titers were observed during the study (**Fig. 3**); no changes in VZV antibody titers were observed either (data not shown). The mean levels of both EBV and CMV specific T cells dropped dramatically from a high point at BR−10 to their lowest point at BR83 (Fig. 3B). The levels for both subsets then trended to rise at BR+1 through BR+14. The functional capability of the viral specific T cells was determined via viral peptide pulsing followed by intracellular IFNg levels. Although the levels were too small to draw conclusion or significance from, mean levels appeared slightly elevated at the BR83 point for both subsets (Fig. 3B). EBV/IFNg T cells were also elevated at BR+14.

Peripheral leukocyte EBV DNA (assumed to be present in B cells) was assessed to determine active reactivation of latent virus. Of the 10 bed rest subjects who participated in this study, 9 did not display EBV reactivation via this assay (defined as $>$ 200 copies per ml). The lone exception was a single 90-d subject who displayed obvious viral reactivation on BR+1 that was not present at BR83 and had resolved by BR+14 (**Fig. 4A**). EBV copies were also measured in the saliva by real time PCR. There was an increase in the salivary EBV copy number during bed rest when compared with pre- and post-bed rest (**Fig. 4B**). Two subjects showed peaks of EBV copies in the middle of the bed rest phase. Their mean + SE values for EBV copies for pre-bed rest were 293 $\pm$ 117.9, during bed rest were 803 $\pm$ 303.6, and post-bed rest were 134 $\pm$ 39.6 copies $\cdot$ ml^{-1}; however, these differences were not found to be statistically significant. Reactivation of latent VZV was measured by determining VZV copies in the DNA extracted from the saliva collected from all 10 subjects pre-, during, and post-bed rest. Evidence of VZV DNA was detected only in 2 of the 10 subjects (data not shown). Urine samples collected from these subjects were analyzed for CMV. CMV DNA was detected in only three subjects.

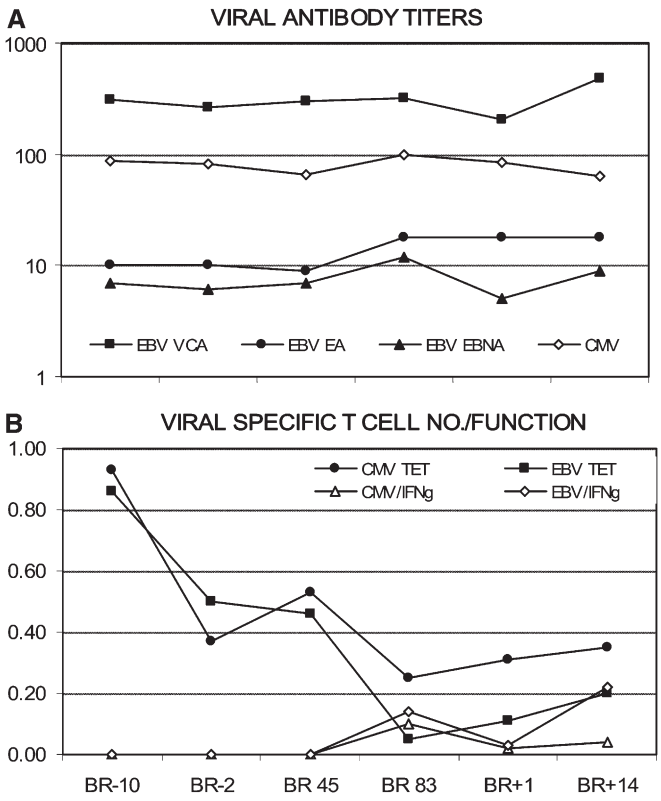


Fig. 3. Mean subject data representing A) the peripheral blood plasma levels of EBV and CMV antibodies; and B) peripheral blood levels of CMV and EBV specific T cells (CMV TET and EBV TET, respectively) as well as levels of CMV and EBV specific T cells capable of being stimulated to produce interferon gamma (CMV/IFNg and EBV/IFNg, respectively).

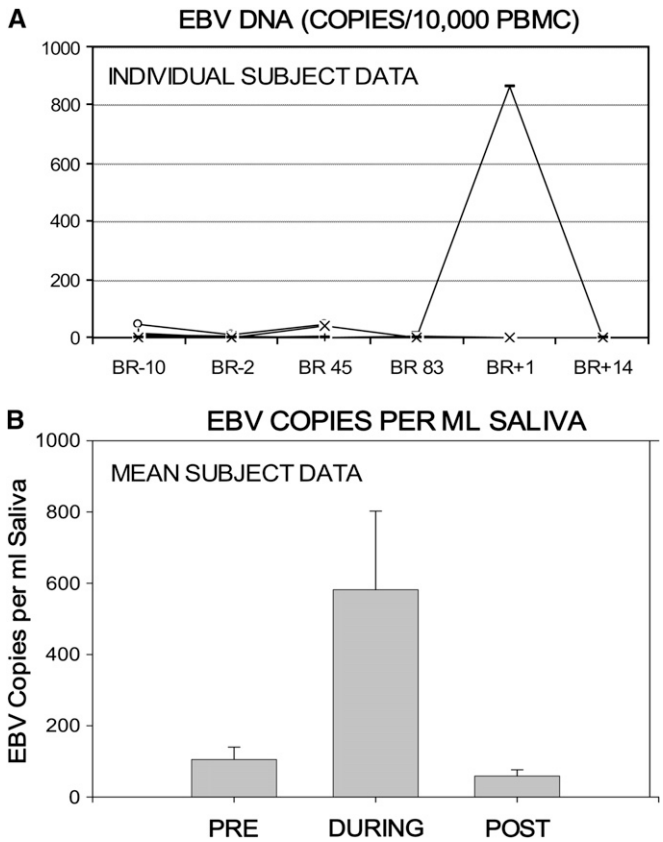


Fig. 4. A) EBV copies per 1x10^6 peripheral blood mononuclear cells; and B) salivary EBV copies per 1.0 ml saliva during 90-d bed rest.

Neuroendocrine stress response was assessed by determining plasma and salivary cortisol levels. For salivary cortisol, 5 pre-bed rest samples, 12 mid-bed rest samples, and 8 post-bed rest samples were collected from each subject (Fig. 1). Plasma cortisol was assessed for each blood collection throughout the study (6 total per subject, Fig. 1). For analysis, all pre-, mid-, and post-bed rest values were averaged. Plasma cortisol levels tended to vary among the subjects, and no study-specific changes were detected in any of the subjects (**Table II**). Though there were some sporadic increases in the salivary cortisol levels found during the study (data not shown), the mean values and standard error in both studies did not show any significant changes (Table II).

A total of 25 behavioral measurements for PSS (3) and PANAS (31) scores were scheduled to be administered for each subject during the study. No significant difference was found in the Positive Assay score (**Fig. 5A**), the negative scores (**Fig. 5B**), or Perceived Stress Scores (**Fig. 5C**) in the subjects during bed rest as compared to the pre-bed rest values.

DISCUSSION

Although the cause of spaceflight-associated immune dysfunction is unknown, it is likely that a synergy of factors such as physiological stress, microgravity, radiation, isolation, and an altered microbial environment all contribute to the phenomenon. The various ground-based spaceflight analogs may recreate some of these spaceflight factors, but no analog recreates all of them. Bed rest is an excellent analog for the microgravity effects on bone and muscle loss; however, the true validity of bed rest for immune dysfunction is debatable. Previous bed rest studies that have assessed immunity have indeed reported that some immune changes are associated with the bed rest model, but the data tended to vary between studies (2,9,21,22,29). The reasons for this variation are likely related to differences in bed rest duration (10 d to 120 d), assay and mitogen choices, culture techniques, etc.

FAP was initiated to use a ground-based model (head-down bed rest) to evaluate, compare, and refine candidate countermeasures to spaceflight deconditioning. The purpose of the immune assessment standard measure is two-fold. First, to establish 'baseline' immune data using a standardized bed rest protocol that may be used for comparison to data from future bed rest studies that will include countermeasures. Some countermeasures (such as exercise, nutritional supplementation, vibration, and medication) planned for use in bed rest have the potential to influence immunity. Second, the bed rest immune assessment is included to generate data using this analog against which data from other ground-based spaceflight analogs may be compared. There are a multitude of ground-based spaceflight analogs that are likely to be more appropriate with regards to spaceflight-associated immune dysfunction. These include undersea-based NASA Extreme Environment Mission Operations (NEEMO missions) and Canadian Arctic simulated planetary exploration at Haughton Crater on Devon Island (Haughton-Mars Project on Devon Island).

Some of the assays included in this study measure immune changes typically associated with illness or pathology (peripheral leukocyte subset changes), whereas other assays measure the subjects' capability to respond when challenged (T cell function, cytokine production

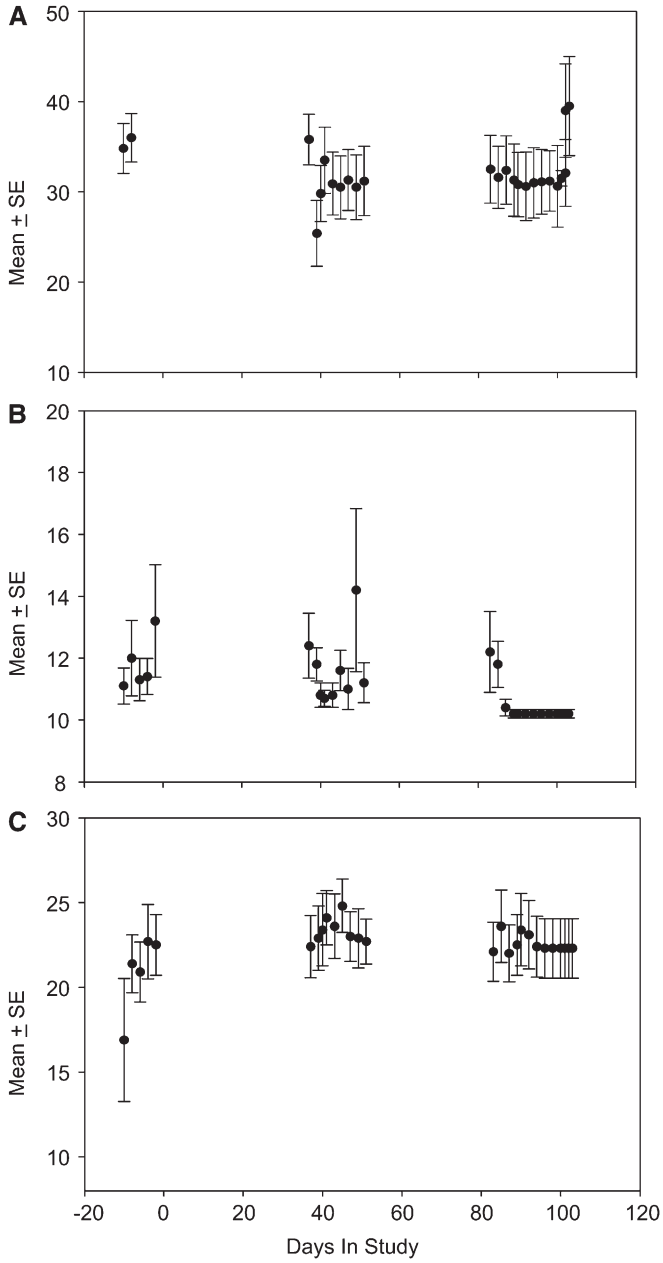


Fig. 5. A) Mean Positive Assay; B) Negative Assay; and C) Perceived Stress scores pre-, during, and post-bed rest.

TABLE II. CORTISOL LEVELS (pg · ml^{-1}) DURING FLIGHT ANALOGS PROJECT BED REST STUDIES (MEAN ± SE).

	Pre	During	Post
Plasma cortisol	25 ± 2	26 ± 2	25 ± 2
Saliva cortisol	0.98 ± 0.07	0.70 ± 0.05	0.36 ± 0.02

patterns). Immune function may be dramatically altered in the absence of pathology or illness, as is expected when otherwise healthy normal subjects experience extreme stress. This phenomenon has been observed in both Arctic and undersea ground-based spaceflight analogs (4,5), marathon runners (12,27), and medical students taking exams (30), and may likely occur with astronauts during long-duration spaceflight. For this reason, assays selected for the FAP determine: the current immune status; immune functional potential; latent viral reactivation (as an adverse outcome that can be measured); and both physiological and psychological stress levels. Using the major advantage of a ground-based spaceflight analog, assessments were performed during the bed rest studies at BR45 and BR83. The typical BR+0 assessment used for most post-spaceflight crewmember assessments was moved for FAP to BR+1. This change was made after consideration that there is no 'landing' event, such as the high-level re-entry G forces, that occurs analogous to that following a space mission. The BR+1 assessment was designed to observe the readaptation to unit gravity that occurs following a bed rest study. Pre-bed rest baseline data (BR−10, BR−2) and post-bed rest 'recovery' data (BR+14) were also assessed for all subjects.

Among the 10 bed rest subjects, no changes were seen in the peripheral leukocyte distribution during bed rest (Table I). This is fairly expected, as the subjects were not ill during the study. A rise or fall in the level of the various immune cell subsets is usually associated with disease, related to proliferation or sequestration at a site of inflammation. In healthy subjects, the level of constitutively activated (expressing CD69) CD8+ T cells may range from 2–10%. This population likely represents normal subclinical immune activity to everyday environmental challenges. In all six subjects who completed a full 90-d study, there was an obvious decline in the level of CD8+/CD69+ activated T cells (Fig. 2A). This was unexpected, and is probably the result of being relatively isolated from pathogens and everyday challenges to the immune system for the duration of the 90 d. The decline in peripheral levels of both EBV and CMV viral specific T cells evident throughout the study also lends support to the concept that the subjects were 'isolated' from an immunologic perspective (Fig. 3B). It should be noted, however, that this assay is restricted to HLA-A2 positive individuals for technical reasons, and unfortunately could only be performed on two subjects. The decline in constitutively activated and viral specific T cells could indicate the subjects were actually 'healthier' during bed rest, at least from the perspective of an absence of illness. It is notable, however, that the goal of the analog is to re-create spaceflight-induced alterations in multiple physiological systems, not to induce pathology. Although no obvious changes were observed in T cell functional responses to mitogens and intracellular IL-2 production, there was a definite rise in intracellular IFNg production at BR+1 for both T cell subsets (Fig. 2B and C). This observation is probably a stress response to the readaptation to the '1-G' environment. Subjects typically are extremely sore for several days following bed rest as their bodies re-adapt to bearing weight, which may induce this stress response. There was essentially no quantifiable EBV reactivation as measured by peripheral blood cell EBV DNA (Fig. 4A); however, the trend towards an increase in EBV copies in the saliva of these subjects during this study (Fig. 4B) was consistent with previously reported bed rest studies (19), in-flight space studies (25), and an undersea ground-based spaceflight analog study (14). Reactivation and shedding of VZV in saliva is usually not seen in healthy human subjects (15). The fact that two subjects shed VZV in their saliva indicates the reactivation of VZV. The clinical significance of this asymptomatic shedding remains to be tested in future spaceflight and spaceflight analog studies.

The plasma and salivary cortisol data presented here indicate that no significant physiological stress occurred in the subjects during these bed rest studies. This is in contrast to results from flight studies which have demonstrated a significant increase in cortisol during and after spaceflight which also correlated with immune alterations and latent virus reactivation (24–26). Changes in the psychological measures (significant decreases in positive affect, a trend toward higher perceived stress scores) observed in some subjects may suggest an impact on the subjects' mood and to a lesser extent on their level of perceived stress. It should be noted that as a whole these results were quite variable and showed no consistent trend in mood or perceived stress over the duration of the study. Instances of changes in mood captured by the questionnaire were usually coincident with personal stress-related events unrelated to the study itself. Bed rest creates an artificial isolation environment where it was anticipated that many of life's daily frustrations that may impact overall levels of stress would be eliminated. It was observed, however, that this was not the case during this study.

To summarize, a set of coordinated assays has been developed that is designed to assess: 1) immune status and function; 2) latent viral reactivation; and 3) physiological/psychological stress. This battery of testing is appropriate to measure spaceflight-associated immune dysfunction in astronaut crewmembers. The testing has been applied to the Flight Analogs Project in an attempt to coordinate multiple-system physiological alterations in a logistically appropriate spaceflight analog. Although alterations were observed, they probably do not reflect the magnitude of spaceflight-associated immune alterations observed in crewmembers. The data collected during these studies will, however, be used as a 'control' against which future bed rest studies will be compared. Several of the future bed rest studies will include active countermeasures that may influence immunity. This data will also be extremely useful in comparing the bed rest analog to other ground-based spaceflight analogs.

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