INTEGRATED EVALUATION OF LATENT VIRAL REACTIVATION DURING SPACEFLIGHT

Introduction

This application proposes a continuation of our current effort, which has provided the first demonstration of viral reactivation during space flight. We have used the herpesvirus EBV as a model for latent viral reactivation and have shown that increased amounts of EBV DNA were shed by astronauts during space flight. Analysis of the Antarctic space flight analog indicated that the frequency of viral shedding may also increase (along with the increased numbers of virus) during long periods of isolation. However, a number of critical questions remain before the findings may be considered a significant health risk during extended space flight. These include:

- Are other latent viruses (e.g., other herpesviruses and polyomaviruses) in addition to EBV also reactivated and shed more frequently and/or in higher numbers during space flight?
- Is the viral reactivation observed in space flight and ground-based analogs mediated through the hypothalamus-pituitary-adrenal (HPA) axis resulting in a decreased cell-mediated immune response?
- How does detection of viral DNA by PCR analysis correlate with infectious virus?
- How does the amount of virus found during flight compare with viral levels observed in acute/chronic viral illnesses and in control individuals?

This expanded study will examine the phenomenon of viral reactivation from the initiating stress through the HPA axis with the accompanying suppression of the immune system resulting in viral reactivation. This information is essential to determine if latent viral reactivation among crewmembers represents a sufficient medical risk to space travel to require the development of suitable countermeasures.

Hypothesis

Increased viral reactivation and shedding during space flight results from physiological stress, is mediated through the hypothalamus-pituitary-adrenal (HPA) axis, and poses a potential health risk to long-duration space flight.

Specific Aims

The mechanism and significance of viral reactivation under space flight conditions will be addressed by the following specific aims:

Aim 1. To implement an integrated test regime to evaluate behavioral status, stress hormone levels, cell-mediated immune status, and viral reactivation in test subjects.

- This end-to-end analysis will be conducted on space flight crewmembers, stress model subjects, persons with compromised immunity, and control individuals.

- Viral levels in control and immunocompromised populations will assess the medical significance of viral findings in astronauts.

Aim 2. To broaden the scope of the current study to include other herpesviruses (CMV, HSV, HHV6) and polyomaviruses (JCV, BKV, SV40).

- The integrated test regime will be used to evaluate viral reactivation and shedding (frequency and quantitation) in appropriate body fluids (blood, urine, and saliva).
- Levels of reactivated infectious viruses shed in body fluids will be correlated with viral DNA detected by PCR.

Aim 3. To determine which component(s) of the HPA axis and immune system are involved in viral reactivation.

These studies will provide insights into the extent and mechanism of viral reactivation during space flight and will help establish the medical significance of the findings. This information is essential to determine if countermeasures are indicated to ensure the safety of long-duration space flights.

BACKGROUND AND SIGNIFICANCE

The launch of the Zarya began the era of international Space Station allowing for permanent human occupation and longer stays in space. Studies conducted on the ISS will inevitably lead to exploration missions to the Moon and eventually Mars. Living and working for extended periods in relatively crowded conditions in the closed, reduced gravity environment of spacecraft, and potentially altered immunity (89) poses difficult challenge to sustained human habitation. Since the inception of the U.S. space Program, prevention of infectious disease has been a high priority and the incidence of such illnesses in the Apollo, Skylab, Apollo-Soyuz Test Project, and the space station program has been described (91). The implement of the Health Stabilization Program has been highly successful in reducing infectious illnesses before or during spaceflight. Astronauts are free of many common pathogens found in the general population, such as, tuberculosis or hepatitis viruses. However, commensals may be an important threat especially if significant changes in the immune responses occur. The most common infections expected include superficial bacterial skin infections, urinary tract infections, and latent or persistent viral infections. These infectious disease risks will almost certainly increase with increasing mission duration. Infectious disease risks are also increased by: reduced gravity (absence of settling of infectious droplets), ease of general aerosol generation (large droplets of saliva and urine remain suspended), limited availability of diagnostics and environmental monitoring, limited isolation facilities, limited disinfection options in closed environment, and other unique features of living in space.

Eight identified types of herpesviruses commonly establish infections in humans. Herpes simplex type-1 (HSV-1), herpes simplex type-2 (HSV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), human herpesvirus-6

(HHV-6), human herpesvirus-7 (HHV-7), and human herpesvirus-8 (HHV-8) are all double-stranded DNA viruses that may establish a lifelong latent infection in the host (64, 93). These latent viruses are subject to intermittent reactivation; replication of the reactivated herpesvirus genome may give rise to both infectious and noninfectious (defective) viral particles. Reactivation of latent EBV infection can be triggered in spaceflight and in ground based space analogues like Antarctica by a variety of local or systemic stimuli, perhaps resulting from physical and psychological stresses. EBV in lymphoblastoid cells can be reactivated in the presence of hydrocortisone, dexamethasone, corticotropin-releasing factor, adrenocorticotropic hormone, or somatostatin (27). Since one or more of the herpesviruses infect nearly 100% of the adult population (64), they are prime candidates for studying the effects of spaceflight on latent viruses in astronauts. More than 90% of the adult population are believed to be infected by EBV (50,63). EBV infections are usually self-limiting and are characterized by fever, pharyngitis, and cervical lymphadenopathy. EBV is the causative agent of infectious mononucleosis, Burkitt's lymphoma, undifferentiated nasopharyngeal carcinoma, and diffuse polyclonal B-cell lymphoma (39,50). Most CMV infections in adults are asymptomatic; however, CMV infections can be severe in immunocompromised individuals (37). CMV infection may result in an infectious mononucleosis-like syndrome, central nervous system infections, and febrile illnesses (41). Salivary shedding of EBV and CMV by healthy individuals serves as a reservoir for reinfection (1,37,52,66). VZV infects the upper respiratory tract and causes chicken pox on primary infection and remains latent thereafter (25). Reactivated VZV causes episodes of zoster or "shingles"; reactivation may occur in the absence of clinical symptoms (25). In AIDS patients, reactivated VZV may cause severe systemic infections (97). HHV-6 is the etiological agent of exanthema subitum in children, an infectious mononucleosis-like disease later in life, and may cause a fatal acute pneumonia in organ-transplant patients (4,64). The persistence of HHV-6 infection has been attributed to the capacity of this virus to infect the parotid and salivary glands (64); saliva plays a central role in transmitting this infectious agent. While HSV or VZV establish latency in dorsal root ganglia, EBV establishes latency in B-lymphocytes. Unlike neurons, B-lymphocytes can replicate and move among tissues, and are almost always continuously subject to immune surveillance (58).

The polyomaviruses are small DNA viruses with oncogenic potential (11,18,24,79). Important members that infect humans are BK virus (BKV), JC virus (JCV), and SV40. All three primate polyomaviruses are cancer-causing in rodents, and SV40 and JCV DNAs have been associated with human tumors. Both BKV and JCV are ubiquitous; primary infections occur in childhood, with 70–80% of adults having antibody. Both viruses establish in the kidney in a persistent or latent form and may also be latent in B lymphocytes (2,61). Both BKV and JCV have also been found in human tonsillar cells. Immunosuppression causes reactivation, and virus is shed in the urine. Reactivation occurs after organ transplantation, immunosuppressive chemotherapy for malignancy, and during pregnancy, old age and AIDS (3,78). Immunocompetent individuals excrete low levels of virus in the urine, with JCV shed more often than BKV (2,55,88). Transmission of BKV and JCV occurs by unknown routes, but respiratory

secretions and urine are believed to be the means. Viremia during primary infection distributes virus to the kidney, and virus may enter the central nervous system (CNS) in lymphocytes. JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), a demyelinating, fatal disorder of the CNS that occurs on a background of immune deficiency (5). BKV infection has been linked to cases of cystitis and kidney disease in children, hemorrhagic cystitis (3,78), retinitis (9), acute respiratory disease (34), urethral stenosis (19), and encephalitis. JCV DNA has been detected in human brain tumors (48,75) and colorectal cancers (49).

SV40 is able to infect both monkeys and humans (12). SV40 establishes persistent infections in the kidneys, and virus shed in the urine is probably the means of transmission (79). Monkeys with simian AIDS develop SV40 disease (meningoencephalitis, nephritis, PML) (38,80), suggesting SV40 can cause disease in immunocompromised hosts. Millions of people worldwide were inadvertently exposed to SV40 from 1955 to 1963 when they were administered contaminated poliovaccines (12,79). Infectious SV40 had unknowingly contaminated batches of both inactivated and live attenuated forms of poliovaccine prepared in monkey kidney cells. SV40 was subsequently shown to be tumorigenic in hamsters and able to transform human cells in culture (13). SV40 DNA has been detected at relatively high frequency in several types of human tumors (mesotheliomas, brain tumors, osteosarcomas) (12,51). SV40 has been isolated from two patients with PML (99) and detected in immunocompromised children (14). The infection rate in humans by SV40 is unknown, but preliminary seroepidemiology studies suggest infection rates of approximately 2–10% (15,40).

Although less commonly disease-causing than herpesviruses, reactivated polyomaviruses also pose a risk to long-term space flight because of their tendency for CNS infections and their association with malignancies. Against the background of mutagenic radiation that host cells will be exposed to in deep space coupled with space flight-induced suppressive effects on the immune system, the potent tumor-inducing potentials of the polyomaviruses and Epstein-Barr virus (EBV) are of serious concern.

Psychological and other stressors can promote the release of pituitary and adrenal hormones that modulate numerous physiological processes including the immune system. The interaction of the immune system with the central nervous system is well documented (36,84). Psychological stress has been implicated in the down-regulation of the immune system and the onset of infectious, autoimmune, and neoplastic diseases (32,36,84,101). Suppression of the immune response is mediated, in part, through the action of stress hormones, and the results of this immunosuppression are remarkably similar to those listed above for astronauts after flight (36,57,101). Accordingly, it has been proposed that stress may be a major mediating factor in the blunted post spaceflight immune response of astronauts (57,58). The psychological and physical stressors associated with short and long duration spaceflight are expected to influence host-parasite relationships. In particular, unique problems associated with spaceflight (e.g. microgravity, crowded living conditions, and physical and psychological stress) can lead to down-regulation of the immune response (23,57,58,90,91,92). This is particularly

important since confinement in a closed environment may increase transmission of medically important microorganism (72,73,93).

After a person is infected with one of the human herpesviruses, the virus persists in a latent state for life. Under certain conditions these viruses can be reactivated. The mechanisms underlying the establishment of latent virus infection or viral reactivation are not completely understood. However, it is known that the cellular immune response plays an important role in the maintenance and replication of latent herpesviruses and reestablishment of control over virus replication after reactivation (25,26). Alterations in the cell-mediated immunity (CMI) may result in viral reactivation, which can be expressed as: (a) asymptomatic viral shedding, (b) a localized clinical infection (e.g., herpetic lip lesion) limited by the CMI to a cell-to-cell transfer of virus, or (c) a more severe disseminated infection. These four scenarios may result from different levels of overall CMI deterioration, or alternatively they may reflect changes in different CMI components or component functions. For example, antigen-specific T-cell responses may be defective for EBV but not for the other herpesviruses, or interferon production by T-cells may be blunted. Even in the absence of clinical disease, reactivation of latent virus may result in increased serum antibody directed against the reactivated herpesvirus (25,26).

Studies in the fields of endocrinology, neurosciences, immunology and psychology have shown interrelationships between CNS and the immune system (43). Early research demonstrate that the CNS communicates with immune system and that stress and depression can alter immune function (6). **Recently it has become clear that there is also a feed back loop to CNS from immune system to CNS** (7). There has been plenty of research relating to the effect of stressors on the immune system (42). Animal data have shown the positive effect of social stability in mediating immune changes caused by chronic stress (16). In humans, th epositive effect of social resources in mediating stress are also well recognized (53) even if the underlying physiological mechanism is not well understood. But there are hints. For example, Interleukin-1 released by macrophages after an infection can interact with hypothalamus modulating the Hypothalamic/ pituitary/ adrenal (HPA) axis to stimulate the production and secretion of cortisol through several intermediate steps (21,26,29). Glucocorticoid hormones are induced by tumor necrosis factor, interleukin-1 and interleukin-6, while the induction of these cytokines is, inturn, under negative conrol of glucocorticoids. (10). The activation of cytokines in the central nervous system can lead to profound changes in neural function ranging from mild behavioral disturbances to anorexia, drowsiness, increased slow-wave sleep, dementia, coma, destruction of neurons (74) B-cell lymphocytes contain corticotropin and can be stimulated by corticotropin-releasing hormone and inhibited by cortisol (74). Studies have shown changes in serum immunoglobulin due to healthy and unhealthy lifestyle. IgE concentrations increased in unhealthy lifestyle (83). Theorell et al (94) found a small but significant increase in IgG concentrations with increaseing job strains among those with low social support. Similar trends in immumoglobulin concentrations have been reported by other workers also (43,62,63).

The components of the nonspecific (e.g., macrophages, NK cells), cell-mediated (e.g., cytotoxic T-cells), and humoral (primary antibody response) immune systems act to contain and "cure" a primary herpesvirus infection, but often with the establishment of a latent viral infection (28,60). Both immune and non-immune host factors act to maintain the virus in the latent state. Herpesviruses also affect host immunocompetence by infecting cells of the immune system (22, 33). Such infections are known to alter the regulation and activation of components of the host immune response. For example, herpesvirus infection of monocytes/macrophages may result in suppressed synthesis of immunoregulatory cytokines such as IL-6, IL-1B, and tumor necrosis factor (TNF) alpha (33). NK cells are important in reducing viral load by attaching to and killing herpesvirus-infected cells; CMV-induced immunosuppression is known to decrease NK cell activity (22). A herpesvirus-induced immunosuppression could result in latent virus reactivation (e.g., herpesvirus or retrovirus) and dissemination, and may afford an opportunity for other pathogenic or opportunistic pathogenic microorganisms to establish a primary infection (22, 33).

Antibody response to vaccines has also been shown to be influenced by stress. Researchers have investigated correlation between stress and response to hepatitis B vaccine. One study showed a negative correlation (39), where as the other found a positive correlation with higher level of perceived stress (71). It is hypothesized that stress decreases our control over these viruses, and this can be shown with increased antibody titers. EBV antibody titers have been reported to be significantly higher during examination in medical students as compared to the baseline period before examination (32).

These viruses will be carried by the astronauts into space, and may represent a health threat especially during long duration missions. Reactivation of latent viruses and shedding may occur among people working and living in space and in other physically isolated extreme environments. Stress-induced changes in levels of specific hormones lead to alterations in the cell-mediated immune (CMI) response, resulting in increased reactivation and shedding of latent viruses in body fluids such as saliva, urine and blood. . Unique aspects of spaceflight, such as the ease of generation of aerosols from liquids such as saliva or urine, increase the risk of contact with infectious particles. Microgravity may allow exposure to larger droplets containing larger numbers of viruses. Astronauts experience psychological and physical stresses resulting in decreased immune function in during and after spaceflight (89). Altered cytokine production, altered distribution of leukocyte and lymphocyte subsets, and decreased delayed hypersensitivity have been observed in during space flight (89,90,91). These changes may result in increased reactivation of latent viruses, potentially increasing the risk of disease. Similarly, reactivation and shedding of these viruses may also occur in health and disease with variable daily stress. The upcoming launch of the first element of the International Space Station underscores the importance of maintaining crew health during increasingly long stays in space. Little is known as to how space flight affects human immunity to viruses and other infectious agents. However, the longer the mission, the greater the risk of latent viruses becoming reactivated; moreover, unique aspects of space flight, such as the generation of aerosols from liquids such as saliva or urine, increase the risk of contact with infectious particles. Although health risks associated with many infectious agents

can be reduced by an appropriate quarantine period before flight, latent viruses are not affected by this strategy.

Evidence provides additional support that stress hormones play an underlying role in space flight immune suppression. Stein and Schluter (85) measured inflight urinary interleukin-6 (IL-6) and cortisol excretion on SpaceLab Life Sciences (SLS)-1 and showed that cortisol fluctuated preflight, dramatically increased immediately after launch, and moderately increased again just prior to reentry. In addition, IL-6 was significantly elevated immediately after launch, and a larger spike was observed after landing. IL-6, an acute phase response protein, is a major inducer of B-cell terminal differentiation. Because the significance of the changes in the immune response associated with long-term space flight is not known, reactivation of latent viruses, such as EBV, is a potential problem for astronauts or cosmonauts on long missions (>90 days).

Experimental Design

Overview: The four Specific Aims will be achieved and the critical questions answered through the implementation of an integrated test regime to study four study groups. The **Integrated Test Regime** (outlined below) is designed to evaluate stress/behavior levels, stress hormone levels, cellular immunity status, and viral reactivation and shedding (Table 1).

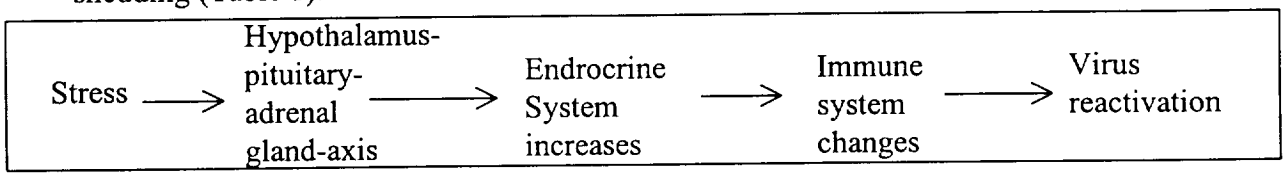

Table 1. Integrated Test Regime

Test Elements	Measurement parameters
Stress measurements	Questionnaire
Stress Hormones	In Plasma: cortisol, ACTH, HGH In Urine: cortisol, catecholamines (epinephrine, norepinephrine) In saliva: cortisol
Cellular Immunity	Delayed type hypersensitivity Cytokines (IL 4, IL 6, IL1, IL1, IL8, IL10, IFNγ, TNFα)
Viral Reactivation EBV CMV HSV1 and 2 HHV6 JCV, BK, SV40	Qualitative and quantitative PCR Viral culture Specific viral antibody titer

Subjects:

This **Integrated Test Regime** will be studied in four different study populations: 1) Space Shuttle/ISS crewmembers; 2) subjects from space-flight analogs; 3) patients with diminished immunity, and 4) healthy control subjects.

Study Group 1: Space Shuttle/ISS Crewmembers. This will address specific aim 1, 2 and 3, and will allow us to answer critical questions listed on page 1 of this proposal. This group will consist of 25 crewmembers. Saliva will be collected (three times a week: Monday, Wednesday, and Friday) for one month (at least 15 samples) at 4-6 months before the flight as a baseline from each participating crewmember. On Shuttle flights saliva will be collected everyday during flight and continued for two weeks (MWF) after flight; on ISS missions (>30 days) saliva will be collected early, mid-way, and late in the mission. Blood (10 ml) and 20 ml urine will be collected from each astronauts at their annual physical exam, 10 days before launch (L-10), and on the landing day (R+0), three days after (R+3) and seven days after (R+7) flight. Cell mediated immunity (CMI) will be measured by delayed type hypersensitivity (DTH) skin test at baseline (4-6 months before flight or at the time of annual physical exam, within 2-7 days after landing and at next annual physical exam. This sample schedule is summarized below in Table 2.

Table 2. Sample schedule for space crewmembers

Item	Preflight	Inflight	Postflight
Questionnaire	Annual Physical, L-10	Early, Mid, Late	R+3, R+7, R+14
Saliva	MWF; 15 samples	Everyday: Shuttle	MWF for 2 wks;

	4-6m before launch	Early/mid/late: ISS	
Blood (10ml EDTA)	Annual Physical, L-10		R+0, R+3, R+7
Urine (20 ml)	Annual physical, L-10	Every 3rd day: Space Shuttle Early/mid/late: ISS	R+0, R+3, R+7
CMI	Annual Physical or 4-6m before launch		Between R+2 and R+7; next annual physical exam.

Study Group 2: Ground-based Space Flight Analogs: Due to the limited access to the space flight, these studies will also be carried out in space analogs, such as Closed Chamber and Antarctica. This will address Specific aim 1, 2 and 3. This study group will include 25 expeditioners participating in the Australian Antarctic science station during the about 6 month Antarctic winter-over isolation. For Closed Chamber, 20 subject will be included to carry out these studies.The integrated test regime will be implemented, and sample collection is summarized in Table 3.

Table 3. Sample schedule for Antarctic expeditioners

Item	Pre isolation	Isolation	Post isolation
Questionnaire	Early, Mid and Late	Early, Mid and Late	Early, Mid and Late
Saliva	M W F; 15 samples 1-2 m before isolation	Every week	M W F; 15 samples
Blood (10ml EDTA)	1-2 month before isolation	Every month	1 month after
Urine (20ml)	1-2 month before isolation	Every month	1 month after
CMI	1-2 month before	Every 2-3 month	1-2 month after

Study Group 3: Diminished Immunity Patients

This will address the medical significance of viral reactivation in astronauts (third part of Specific aim 1) and will allow us to compare our data from astronauts with the immuno-compromised subjects. This group will include 25 patients with diminished cellular immunity. This group will include selected patient populations such as but not limited to: infectious mononucleosis, HIV-positive, and cancer chemotherapy. Saliva will be collected three times a week (Monday, Wednesday, and Friday) for one month (at least 15 samples). Blood (10ml EDTA) and urine (20ml) will be taken immediately before and after one month of saliva collection from these subjects: Cell mediated immunity of each participating individual will be measured by delayed-type skin hypersensitivity before and after one month of sample collection period (Table 4).

Table 4. Sample schedule for patient populations

Item	Sample schedule
Questionnaire	Early, Mid, Late
Saliva	M, W, F for 4 weeks; 15 samples
Blood 10ml EDTA	Before and after the 4 week study
Urine 20ml	Every week for 4 weeks
CMI	Before and after 4 weeks of study

Study Group 4: Healthy Control Subjects

The reactivation of viruses will be compared with apparently healthy normal individuals. This group will consist of 25 age/sex matched healthy individuals. The specimen schedule is the same as for the patient populations given above in Table 4.

Methods

Behavioral Measures of Stress

Stress is best measured with a convergence of mood, behavioral, physiological, and biochemical measures (4b). The proposed research will use this approach to stress assessment, measuring behavioral changes, mood, symptom reporting, and sleep disturbances, as well as physiological arousal indexed by changes in cortisol and catecholamines. In all cases they have proved to be useful and sensitive measures of stress, have tended to converge (suggesting that they are tapping different aspects of the same construct), and have proved to be stable, reliable, and valid. This is consistent with the notion that multi-level, simultaneous assessments of stress provides a more complete and potentially informative picture of stress responding.

All data collection for the questionnaires and behavioral tasks will be done on a laptop computer and all entries will be time-and-date stamped. In the space crew, a detailed assessment will be conducted at the ground-based assessments (annual physical, L-10, and R+3, R+7, and R+14). During Shuttle flight or ISS missions, a brief assessment will be conducted at the beginning, middle, and end of each flight/mission. For participants in the two control groups, the same detailed assessment as completed by the space crew will be collected at the beginning and at the end of the sampling period. The Balanced Inventory of Desirable Responding will be used to measure the extent to which people respond to questions in socially desirable ways (69b). It measures both impression management and self-deception and will be completed only at the first assessment.

Changes in anxiety will be assessed using the State Scale of the Spielberger State-Trait Anxiety Inventory (STAI) (84b). The State Scale of the STAI is a 20-item scale that provides information about a person's current level of anxiety. There is extensive normative data using this scale and it is widely used in research on clinical, medical, and student populations and has good internal consistency reliability (0.85 to 0.95) (84c). This questionnaire will be administered at all time points. The Trait portion of the STAI will be only completed at the first assessment point.

Poor sleep has been associated with subsequent decrements in mental health including symptom reporting, incident cases of mood and anxiety disorders, and immune function (15,34a, 34b). Therefore, sleep disturbances will be assessed using the Pittsburgh Sleep Quality Index (PSQI) (7). The PSQI is a self-rated questionnaire which assesses quality of sleep and sleep disturbances and has good internal (0.83) and test re-test reliability (0.65-0.85). Participants will also complete a brief sleep diary which specifically asks about sleep quality the night before. The sleep diary will be completed at all time points and the PSQI will be completed at all the ground-based assessments.

To examine specific aspects of job stress, participants will complete the Job Stress Survey (JSS) (94b). The JSS asks about the severity and frequency of 30 specific sources of occupational stress. Scales for job pressure and lack of organizational support were identified as the major dimensions of occupational stress. The scale has good internal and test-retest reliability. The JSS will be modified to specifically address job-related stressors experienced by astronauts both during ground training and flight. Some questions will specifically ask about the perception of crowding and issues of separation from family and friends. The JSS will be administered at all time points.

As stress has been found to influence attentional processes and reaction time (RT), participants will perform a series of brief RT tasks (77b). The computer will record both the speed and the accuracy with which the subjects complete the tasks. The RT tasks will be administered at all time points.

The Impact of Events Scale (IES) is a 15-item self-report scale that assesses the two most common categories of responses to stressful events: intrusion and avoidant thoughts. Intrusive thoughts or the tendency to ruminate on or to avoid thoughts about stressors appears to exacerbate the impact of stress (102). Reliability estimates of the scale in this population (internal consistency) ranged from 0.79 to 0.90 (37b). The IES will be completed at all the ground-based assessments.

Symptom reporting will be measured by the Brief Symptom Inventory (BSI) (19a). This widely used symptom inventory, a 53-item version of the Symptom Checklist 90-R (19b), yields an overall distress score (global severity index - GSI) as well as subscale scores reflecting somatization, anxiety, phobic anxiety, depression, hostility, obsessive-compulsive, interpersonal sensitivity, paranoid ideation, and psychoticism. Patients are asked to rate the level of distress associated with each symptom they have experienced during the past week. Reliability estimates for the various subscales range from 0.84 to 0.90, and the subscales correlate with the Minnesota Multiphasic Personality Inventory (MMPI) scales measuring similar constructs (19a,19c). The BSI will be completed at all the ground-based assessments, except not at R+7.

Table 5. Sample data collection for the behavioral measures

ANNUAL PHYSICAL	L-10, R+3, R+7, R+14	EARLY, MIDDLE, AND LATE IN MISSION/FLIGHT
• Balanced Inventory of Desirable Responding • Spielberger State-Trait Anxiety	• State Anxiety Inventory	• State Anxiety Inventory

Inventory • Pittsburgh Sleep Quality Index/Sleep Diary (PSQI/Sleep Diary) • Brief Symptom Inventory (BSI) • Impact of Events Scale (IES) • Job Stress Survey (modified for astronauts) • Attentional Tasks	• BSI (L-10, R+3, and R+14) • IES • PSQI/Sleep Diary • Job Stress Survey • Attentional Tasks	• Sleep Diary • Job Stress Survey • Attentional Tasks

Detection of Infectious Virus

The amplification of viral DNA by PCR from a clinical specimen does not indicate whether intact, infectious virus was present in the sample. Infectious virus is required to induce disease or to transmit an infection to susceptible contacts. Although it might be assumed that any sample containing viral DNA would contain some infectious virus, this needs to be proven using virus culture approaches.

Most viruses of priority in this study are difficult to culture and cannot be quantitated by plaque assays. Therefore, an alternative approach will be followed. Samples that are positive for viral DNA by PCR will be diluted and inoculated into cultures of susceptible cells. Seven to 10 days later, the cells will be lysed and assayed for viral DNA by PCR. Human diploid fibroblasts are the cells of choice to isolate CMV and VZV (49b) and also are susceptible to HSV (49b) and BKV (79b). COS-7 cells will be used for detection of JCV (34b) and CV1 cells for isolation of SV40 (51b). The PCR-based assays of infected cell lysates will not be quantitative but, if positive, will indicate that infectious virus was present in the original specimen. Serial dilutions of original inocula would provide rough estimations of relative levels of infectious virus present in samples. A different approach must be used for EBV as it infects only lymphocytes and fails to replicate in most cells; infectivity with that virus is measured using a lymphocyte transformation assay (96). This is a laborious assay, but will indicate if infectious EBV was present in a specimen.

DNA extraction

Saliva specimens will be concentrated with a Microsep 100K filtration unit (Filtron Technology Corporation, Northborough, MA) and extracted by a nonorganic extraction method (Qiagen Inc., Chatsworth, CA). Microcarrier gel (Molecular Research Company) will be added to facilitate DNA recovery at the proteinase K digestion step (Boehringer Mannheim, Indianapolis, IN). DNA will be resuspended in 50 µl of nuclease-free water (Amresco, Solon, ØH). EBV DNA for control studies was obtained from Sigma Chemical Co. (St. Louis, MO).

DNA amplification by PCR

PCR primers directed at the EBV polymerase accessory protein gene (BMRF1) are P1, 5',3'-GTCCAAGAGCCACCACACCTG (The Midland Certified Reagent Co., Midland, TX), and P2, 5',3'-biotin CCAGAAGTATACGTGGTGACGTAGA (Digene Diagnostics, Gaithersburg, MD) (17). Primers for HSV type 1 and 2, CMV, and HHV6, are as given in Appendix---.These primers will be used at a concentration of 200 µM with 10 µM deoxynucleic acid triphosphates (Perkin-Elmer, Branchburg, NJ). PCR will be optimized using buffer II (Perkin-Elmer) with 2.5 mM $MgCl_2$ (68). Dimethyl sulfoxide

(Sigma) will be added to a final concentration of 5% (69). AmpliTaq Gold (2.5 units) (Perkin-Elmer) will be added to the 100-µl reaction mixture; then 5 µl of the purified DNA was added to 20 µl of the reaction mixture. PCR cycle parameters for amplification of these viruses will be followed as described by the manufacturer (Digene Diagnostics, Gaithersburg, MD).

PCR fragments will be detected with the Sharp Signal System (Digene Diagnostics) after 24 hours. [67]. Positive and negative controls will be used in the amplification by PCR and detection of EBV by Digene's Sharp Signal Detection system. Our laboratory has verified that the sensitivity of the Digene detection system is up to 10 copies of DNA, as claimed by the manufacturer . Seronegative and seropositive samples were included in a control study done to compare results from the test subjects. No cross-reactivity was observed between the EBV primers used and DNA from other herpesviruses like HSV type 1, HSV type 2, CMV, and HHV6. PCR primers used for the detection of polyomaviruses are listed in Table (Appendix---). Reaction conditions will be as described (22).

Quantitative estimation of HSV

Competitive PCR will be used for determining viral load with Viral Quant kit (Biosource International). This is a quantitative adaptation of the PCR in which a known copy number of exogenous synthesized DNA, known as Internal calibration Standard (ICS), is mixed with sample DNA prior to extraction and amplification. The ICS has been constructed to contain PCR primer binding site identical to the viral DNA (to be quantified) and a unique capture binding site that allows the resulting ICS amplicon to be distinguished from the viral amplicon. In this procedure, viral DNA isolated from saliva or urine is amplified with Viral Quant format, primers, one of which is biotinylated. In case of EBV, these primers target a conserved sequence of EBER 1. The EBER1 gene is expressed during EBV latency as a small non-polyadenylated RNA transcribed by RNA pol III. Sequences recognized by the two EBV- specific amplification primers are identical between type 1 and type 2 strain of EBV (54). In case of CMV, these primers target a conserved sequence of gB (gpUL55), the protein product of which is critical for inducing neutralizing antibody response (56). During amplification, the biotin labeled primer is incorporated in to both ICS and viral (EBV or CMV) amplicons. Following PCR, the amplicons are denatured and hybridized to either ICS or EBV sequence-specific capture oligonucleotides. Capture oligonucleotides are prebound to microtiter wells. The bound amplicons are detected and quantified by addition of an enzyme-streptavidin conjugate followed by the substrate. The signal generated in the reaction is proportional to the amount of amplicon present. Since the ICS is amplified at an efficiency identical to the viral (EBV or CMV) DNA, it can serve as a standard for viral DNA quantitation. In addition, the detection of ICS serves as a sample-specific extraction control.

For viruses other than EBV and CMV, including EBV, CMV, HSV1 and 2 and HHV6 quantification will also be carried out with TaqMan 7700, DNA sequence detector from Perkin Elmar. Primers and probes specific for different herpes viruses to be used on this instrument will be designed after Kimura et al. (44).

Viral Antibody Titers:

Determine if antibody titers to EBV-specific antigens are increased after space

flight.

Preliminary data has shown an increase in anti-VCA IgG antibodies prior to flight, and 3-to-6-fold increases in anti-EA IgG antibodies after space flight. This specific aim will characterize in detail the humoral immune response to EBV lytic antigens. Blood samples will be spun at 3000 rpm for 15 min to collect plasma. Standard techniques (immunofluorescence assay) will be used on plasma specimens for determining IgG/IgM antibodies to EBV-VCA, -EA, and -EBNA (86,87). Measurement of an irrelevant antibody (i.e., tetanus) will be performed to confirm the specificity of EBV-antibody titer changes. This information will also be useful for documenting seroconversion by crewmembers. It is expected that increased antibody titers to EBV lytic antigens will be increased before launch (anti-VCA) and after landing (anti-VCA and -EA), thus demonstrating a significant humoral immune response to EBV replication.

Stress hormones

Measure stress hormones in plasma and urine.

The preliminary data indicates increased stress hormones both prior to and after launch. The measurement of hormones and immunoglobulins, which has been described previously in detail by Leach *et al.* (50), will be performed in collaboration with the Clinical Laboratory, NASA Johnson Space Center. Plasma adrenocorticotropic hormone, plasma and urinary cortisol, and angiotensin I will be measured by radioimmunoassay (RIA). Urinary catecholamines (epinephrine, norepinephrine) will be measured by high-performance liquid chromatography. IL-6, human IL-10, and viral IL-10 will be measured by ELISA. The amount of stress hormones are expected to be significantly increased after landing, and these changes will partially be responsible for the switch from latent to lytic EBV replication.

Phenotypic analysis of leukocyte sub populations will also be performed to correlate with the results from the WBC and differential hematology data (obtained from the JSC Clinical Laboratory) as well as specific aim 2. For antibody staining of leukocytes, a whole blood will be aliquoted into 12 x 75 mm tubes and stained with the following antibodies: anti- CD3, CD14, CD16, CD56, CD4, CD8, CD10, and CD11b. Red cells will be lysed (FACSLyse, Bectin-Dickinson), washed once, resuspended in 1% paraformaldhyde and analyzed by flow cytometry. It is expected that the number and function of EBV-specific T-cells will be decreased after space flight, and these results will directly correlate with the elevations in stress hormones (i.e., cortisol).

Delayed–type Hypersensitivity

The delayed–type Hypersensitivity (DTH) skin test is a reliable screening test for cell mediated immunity (CMI) (45,82). DTH skin testing will be done using a commercially available Multitest CMI applicator (Institut Mérieux, Lyon, France). The recall antigens applied with the multitest are 7 antigens and a glycerine control (46,62,63). The antigens in this test include tetanus, diphtheria, *Streptococcus*, tuberculin, *Candida albicans*, *Trichophyton*, and *Proteus*. Reactions is recorded after 48 h as the area of induration measured in millimeters with calipers; reactions were considered positive if the diameter of induration was $\geq$ 2 mm. Subject score is recorded as the total millimeters of induration from all 7 test antigens. The following criteria will be used to assess CMI

multitest results (62,100): subjects with one or less positive antigen reaction, with a subject score of less than 5 mm, will be designated anergic; subjects with subject scores of less than 10 mm will be hypoergic; and subjects with subject scores of more than 10 mm will be designated normal responders. All DTH testing will be conducted by trained nurses in the procedures.

Statistical analysis:

Sample Size

The number of subjects per group (*N*) and the number of observation days per subject (*n*) recommended for this study were determined by examining the power of the test (probability of finding a statistically significant difference) comparing the incidence rates (P) of positive EBV samples between two groups. The power calculation takes into consideration differences between subjects as well as daily variation for a given subject. Appendix 2 show estimates of power (test level $\alpha = .05$) for hypothetical comparisons between astronauts (P = 0.32) against (a) a normal healthy group (P = 0.10); (b) a normal moderately stressed group (P = 0.20) and (c) an immune-compromised group (P = 0.52). Based on these calculations, we chose to proceed with $N = 25$ subjects and $n = 12$ or more observation days per subject. This combination gives a power of 0.99, 0.54 and 0.83 for the comparisons in (a)-(c) respectively. The data collected from the present study will be statistically analyzed using repeat regression logistic and Analysis of Variance (ANOVA) wherever needed.

Study schedule will be as follows:

Collection of samples from control group	
Processing of samples from control group	
Analysis of data obtained from patient population	
Collection of samples from patient population	
Processing of samples from patient population	
Analysis of data obtained from space analogs	
Collection of samples from space analogs	
Processing of samples from space analogs	
Analysis of data obtained from space analogs	
Collection of samples from spaceflights	
Processing of samples from spaceflights	
Analysis of data obtained from spaceflights	
Preparation of final report	

Progress report on the previous grant

The first two years of the current effort produced several important findings and tangible products. For example,

- The first report of latent viral reactivation and shedding during space flight.

- The first report of quantitative increases in viral DNA in saliva during space flight.
- The first report of increased salivary EBV shedding correlating with diminished CMI response in Antarctic expeditioners.
- The development of a new flow cytometric method for the detection of EBV infected B cells.
- The development of highly sensitive CMV primers for PCR analysis.

This has resulted in 2 manuscripts published (Appendix 3,4), two manuscripts accepted (Appendix 5,6), 1 manuscripts submitted (Appendix 7), 2 manuscripts in preparation, 2 abstracts (Appendix 8,9), and 2 NASA Tech Briefs (Appendix), and 1 patent pending (CMV primers).

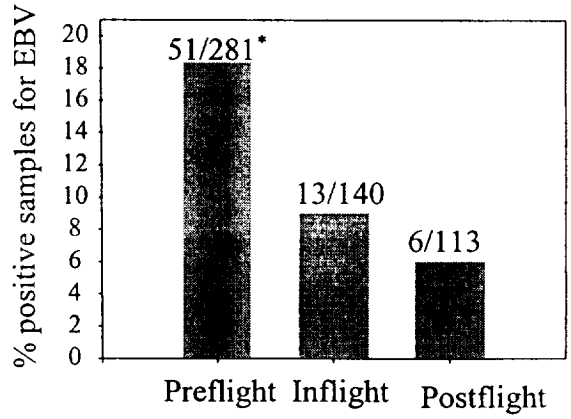

Figure 2. EBV DNA detected in saliva from astronauts before, during and after spaceflight. * denote number of positive/total saliva samples

We selected EBV as a model for our studies of latent viral reactivation in spacecraft and ground-based models of space flight. The high incidence (~90%) of EBV in the general population and the ease with which saliva specimens can be collected during space flight made EBV an ideal candidate for studying this phenomenon. Space flight constraints required the use of PCR assay for the viral DNA because freezers were not available to maintain virus viability. Storage of saliva at ambient temperatures required the development of a stabilization buffer to maintain sample integrity. **We were the first to report the shedding frequency of EBV in astronauts during space shuttle missions (67).** Eleven seropositive astronauts (8 men and 3 women) provided saliva specimens before, during, and after their space shuttle missions; the presence of EBV was determined by PCR. Preflight samples were collected upon rising every other day for two months, beginning about 6 months before launch. In-flight samples were collected daily. Postflight samples were collected on landing day and then every other day for two weeks. Ten astronauts shed EBV DNA during the preflight period; only two shed EBV DNA during flight. Chi-square test and analysis of variance confirmed that **viral shedding was higher before launch than either during ($p<0.001$) or after flight ($p<0.01$).** The frequency of appearance of EBV DNA was no different during vs. after flight (Figure 2). These data have been accepted for publication in Aviation, Space and Environmental Medicine (Appendix-5).

Reactivation of latent EBV infection may be triggered by a variety of factors, perhaps including the physical and psychological stresses associated with space flight. EBV in lymphoblastoid cells can be reactivated with two glucocorticoid hormones, hydrocortisone, dexamethasone, and corticotropin-releasing factor, adrenocorticotropic hormone (30). Although stress hormones were not measured on the missions included in this study, the effects of stress associated with space flight are known to vary considerably among astronauts. The viral shedding patterns in this study suggest that stress initiating viral reactivation is greatest before space flight.

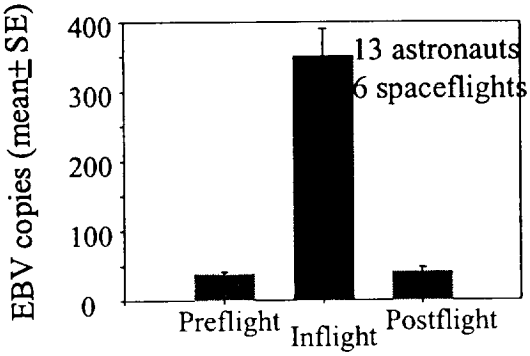

Figure 3. EBV copies in saliva from astronauts

Subsequently, we have also measured EBV DNA copies in 95 virus-positive saliva samples screened from 684 samples collected from 13 astronauts before, during, and after 6 space Shuttle flights (Figure 3). Quantification of EBV DNA was done with the BioSource Viral Quant EBV (PCR). EBV DNA was detected in 14% of the saliva specimens. The number of EBV DNA copies was found to be significantly higher ($p < 0.05$) in saliva collected during the flight than before and after flight. The number of EBV copies (mean $\pm$ SE) was 38 ± 3.1 before, 350 ± 39.7 during, and 40 ± 7.2 after flight. Of the 94 EBV-positive saliva samples, 66 were obtained before, 19 during, and 9 after flight. **The increased number of EBV DNA copies found in saliva during space flight indicates larger numbers of infectious EBV particles are produced during space flight, perhaps resulting in increased risk of disease.** These results were presented at the 99th Annual meeting of American Society for Microbiology, Chicago., IL. (Appendix 8).

We have compared the EBV DNA copies measured in saliva samples collected before, during and after space shuttle and Shuttle Mir. Inflight EBVcopy number were found to be about ten fold higher than that of pre or post flight samples. Though, these inflight EBV copies were much less than that of HIV patients, there was some overlap of upper limits of astronaut's values with the lower limits of HIV patients (Table 6).

Recently Kimura et al. (26) have reported EBV copies from patients with symptomatic EBV infection. They reported that 316 copies per microgram of PBMNC DNA were enough to diagnose symptomatic infection. Two more studies used the level of 500 copies of EBV DNA/10^5 cells as indicators of symptomatic EBV infection (51a, 51b)

Table 6. EBV DNA copies in different populations

	EBV DNA copies / ml	
	Average	Ranges
Pre-post/flight	40	6-109
Inflight Shuttle	350	130-738
Inflight Mir	231	16-1130
HIV patients	3700	<600-6200
Infectious mononucleosis (26)	158	NA
EBV related LPD (44)	5011	NA
Chronic active EBV infection (44)	12,590	NA
Symtomatic EBV infection (76,77b)	500 copies per 10^5 cells	NA

To complement our space flight studies, we have studied viral reactivation in the Antarctic and Lunar Mars Space Test Project-Closed Chamber ground-based space analogs. Sixteen expeditioners from two Australian Antarctic stations were tested for EBV DNA shedding patterns. Although the temporal shedding pattern varied, EBV DNA was detected more frequently in the samples collected during the isolation period than either pre-isolation or post-isolation periods. Cell-mediated immunity (CMI) was assessed periodically with cutaneous CMI Multitests on the forearm of each subject. More than 80% of the subjects had reduced CMI response at all 5 of the measurement times. In most subjects EBV shedding was found to occur more frequently during periods of anergy or hypoergy (Figure 4 &5). We concluded that extreme environmental conditions and the stress associated with total physical isolation caused a decline in CMI, resulting in an increase in EBV reactivation early during the winter-over period. **This is the first report of increased salivary EBV shedding with diminished CMI response in Antarctic expeditioners.** These data were presented at 98th General Meeting of American Society for Microbiology (Appendix 9) and a manuscript is in press in the *Journal of Medical Virology* (58b) (Appendix 6).

We also measured EBV DNA in saliva before, during, and after a 90-day closed chamber study (Lunar Mars Space Test Project, LMSTP). Saliva, blood, and urine were collected from each crewmember participating in the LMLSTP studies. Saliva samples was collected every Monday, Wednesday, and Friday, and blood and urine were collected only once before and once after these studies. Of the 8 crewmembers, included in the study, EBV DNA was detected in 24% of the saliva samples. These data suggest increased shedding of EBV before, during, and after the chamber test (Appendix 10). CMI responsiveness was found to be reduced during the chamber isolation phase.

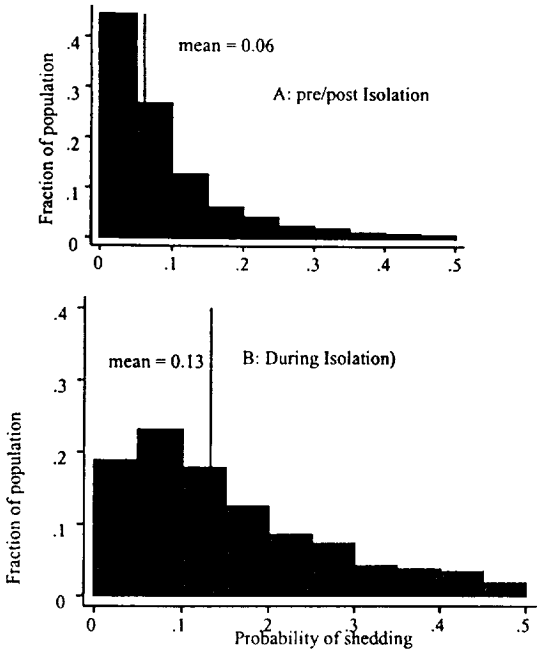

Figure 4. EBV shedding probability before and after isolation (A) and during isolation (B) in Antarctica.

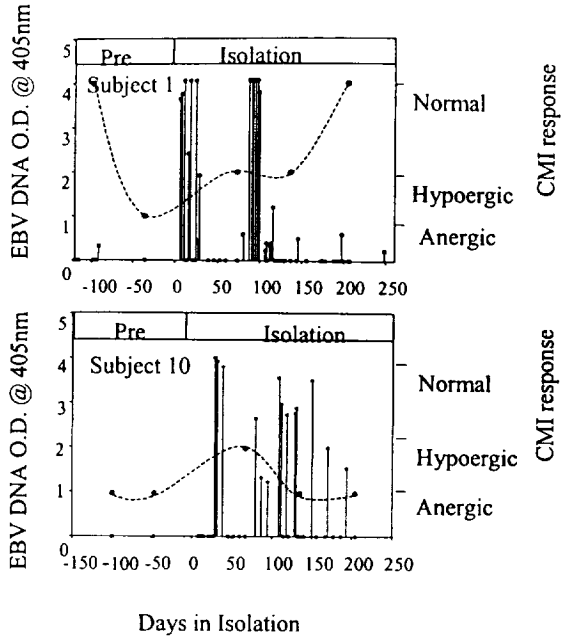

Figure 5. Viral shedding patterns and CMI status of two Antarctic expiditioners before and during winter isolation

In addition to EBV, we have done preliminary studies on the reactivation of HSV types 1&2, HHV 6, and CMV from the saliva samples collected from space shuttle crew members . HSV types 1&2 were detected in 2-3% pre and post flight and 8% in inflight saliva samples. HHV6 was detected in 29% of preflight, 2% inflight and 15 % postflight samples. CMV was not isolated in any of the saliva samples collected from crewmembers.

Preliminary studies were conducted on the reactivation and shedding of CMV in urine samples from astronauts before and after short space flight. Urine samples (127) were collected from 62 crewmembers from 12 separate space missions. Urine samples were collected 10 and 3 days before flight and within 2-3 hours after landing of the space shuttle. CMV DNA was detected in 11.81% of the urine samples and 20.96% of the crewmembers studied. Urine samples were also collected from 50 healthy control subjects (31 male, and 19 female). CMV DNA was not detected in any of the control subjects.

As we know space flight can cause changes in the immune system resulting in increased antibody titers to latent viruses. Therefore, we examined sera from 29 astronauts for EBV Viral Capsid Antigen (VCA) and Early Antigen (EA) before and after space flight. The VCA antibody titer was significantly higher prior to the flight compared to baseline (annual physical). These results have been submitted to Journal of Clinical Microbiology (Appendix 7a). We have shown increased levels of stress hormones, activated (CD23 expressing) B cells, viral (EBV) load in B-cells and IL-10 in astronauts with evidence of EBV reactivation. These results suggest diminished immunity prior to space flight resulting from stress-induced enhancement of Th2 cytokine synthesis. These data will be submitted to J. Leukocyte Biology (Appendix 7b).

Preliminary studies using PCR have measured the presence of JCV in multiple urine samples from 30 healthy individuals in Houston, Texas. Virus shedding was found to be age-dependent: of those age 20–29 years, 16.6% had at least one virus-positive urine specimen; those 30–39 years, 28.6% were positive; and those ≥40 years, 58.8% were positive. Overall, 13/30 (43.3%) were positive for JCV excretion at least once. Seven individuals were found to be frequent shedders, having 3 or more positive urine samples over 6 months time; six of those seven (85.7%) were over 40 years old.

Preliminary assays have been carried out on urine samples collected in 1998 from Antarctic expeditioners. Eight of 30 subjects (26.7%) had one or more urine samples positive for JCV. Viral shedding was also age-dependent in this group; 5 of 11 persons (45.4%) over age 40 were positive at least once. Interestingly, of 4 individuals found to be frequent shedders of JCV, 2 (50%) were under age 40. These preliminary observations suggest that the stresses associated with Antarctic isolation may increase the frequency of JCV excretion by younger subjects. Additional samples from Antarctica subjects will need to be analyzed to substantiate these preliminary findings.

Expected results:

The successful completion of these studies will answer the specific aims and the critical questions (see pages 1-2). The data will allow us to determine the role of stress associated with space flight. The clinical significance of our findings will be determined through determination of infectious virus in body fluids and comparisons with acute and chronic virally infected patients. If crewmembers are exposed to unacceptable risks from viral reactivation, countermeasures must be developed and tested. Countermeasures available would include stress reduction, stress management training, and antiviral pharmaceuticals.

In addition to determining the significance of viral shedding in astronauts, we may gain new insights into the reactivation of latent viruses in the general population. In addition to increasing the scientific knowledge of the latent viruses, products such as the CMV primers for CMV diagnostics, the new assay for EBV infected B cells, and others would be expected to benefit the private sector.

References

1. Adler, S. P. 1991Pediatr Infect Dis J **10:** 584.
2. Azzi, A., De Santis, R., Ciappi, S., Leoncini, F., Sterrantino, G., Marino, N., Mazzotta, F., Laszlo, D., Fanci, R. and Bosi, A. J. NeuroVirol. 2:411–416, 1996.
3. Arthur, R.R. and Shah, K.V. Prog. Med. Virol. 36:42–61, 1989.
4. Bagg, J. 1991. J Oral Pathol Med **20:** 465.
5. Berger, J.R. and Concha, M. J. NeuroVirol. 1:5–18, 1995.
6. Black PH 1994. Chemother. 38:1-6.
7. Black PH 1994 . 38:7-12.
8. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. *J Clin Microbiol.* 1990;28(3):495–503.
9. Bratt, G., Hammarin, A.L., Grandien, M., Hedquist, B.G., Nennesmo, I., Sundelin, B. and Seregard, S. 1999. AIDS 13:1071–1075.
10. Brouchaert P; Libert C; Everaerdt B, Takahashi N, Cauwel A, Fiers W. 1993. Immunology 187:317-29.
11. Butel, J.S. 1994. In Encyclopedia of Virology, vol. 3, pp. 1322–1329. Academic Press, San Diego,
12. Butel, J.S. and Lednicky, J.A. J. Natl. Cancer Inst. 91:119–134, 1999.
13. Butel, J.S., Tevethia, S.S. and Melnick, J.L. Adv. Cancer Res. 15:1–55, 1972.
14. Butel, J.S., Arrington, A.S., Wong, C., Lednicky, J.A. and Finegold, M.J. J. Infect. Dis. 180:884–887, 1999.
15. Butel, J.S., Jafar, S., Wong, C., Arrington, A.S., Opekun, A.R., Finegold, M.J. and Adam, E. Human Pathol., in press.
16. Cohen S, Kaplan JR, Cunnick JE, Manuck SB, Rabin BS. 1992. Psychol. Sci. 3: 301-4.
17. Chomczynski P and Sacchi N. 1987. *Anal Biochem.* 162:156–159.
18. Cole, C.N 1996. In Fields Virology, 3rd ed., pp. 1997–2025. Lippincott-Raven, Philadelphia,
19. Coleman, D.V., Gardner, S.D. and Field, A.M. Human polyomavirus infection in renal allograft recipients. Br. Med. J. 3:371–375, 1973.
20. Davis-poynter N J and Farrell H E. 1996. 74: 513-522.
21. Dunn 1988. 43: 429-435.
22. Fan, H. Y., Chen, S.Y., Rosenberg, N & Sugnden, W. 1991. American Society for Microbiology, Washington, DC.
23. Fleming, S. D., Rosenkrans, C. F., Jr., and Chapes, S. K. 1990. Aviat Space Environ Med **61:** 327.
24. Frisque, R.J. JC and BK viruses. In *Encyclopedia of Virology*, vol. 2, pp. 752–757. Academic Press, San Diego, 1994.
25. Gershon, A. A., Steinberg, S. P., and Schmidt, H. D. 1991. Varicella-Zoster virus, Amercian Society for Microbiology, Washington DC, 838 pp.
26. Glaser R, Kennedy S, Lafuse WP, Bonneau RH, Speicher CE etal 1990. Arch. Gen.Psychiatr. 47:707-12.
27. Glaser, R., and Kiecolt-Glaser, J. K. 1997. Ann Behav Med **19:** 78.
28. Glaser, R., Pearson, G. R., Jones, J. F., Hillhouse, J., Kennedy, S., Mao, H. Y., and Kiecolt-Glaser, J. K. 1991. Immun **5:** 219.
29. Glaser R., Pearson GR, Bonneau RH, Esterling, BA, Atkinson C, Kiecolt-Glaser J.K.1993. 12 (6): 435-442.
30. Glaser, R., Kutz, L. A., MacCallum, R. C., and Malarkey, W. B. 1995. Neuroendocrinology **62:** 356.
31. Glaser R, Kutz LA, MacCallum RC, Malarkey WB. *Neuroendocrinology* 1995; 62(4):356–361.

32. Glaser, R., Kiecolt-Glaser, J. K., Marucha, P. T., MacCallum, R. C., Laskowski, B. F., and Malarkey, W. B. 1999. Arch Gen Psychiatry **56:** 450.
33. Gosselin, J., Flamand, L., D'Addario, M., Hiscott, J., Stefanescu, I., Ablashi, D. V., Gallo, R. C., and Menezes, J. 1992. J Immunol **149:** 181.
34. Goudsmit, J., Wertheim-van, D.P., van Strien, A. and van der Noordaa, J. J. Med. Virol. 10:91–99, 1982.
34b. Hara, K., Sugimoto, C., Kitamura, T., Aoki, N., Taguchi, F. and Yogo, Y. Archetype JC. J. Virol. 72:5335–5342, 1998.
35. Hawkins, W. R., and Ziegleschmid, J. F. 1992. Clinical aspects of crew health, NASA, Washington DC, 43 pp.
36. Hillhouse, J. E., Kiecolt-Glaser, J. K., and Glaser, R. 1991. Stress-associated modulation of the immune response in humans., CRC Press, Boca Raton FL, 3 pp.
37. Hodinka, R. L., and Friedman, H. M. 1991. Human cytomegalovirus, American Society for Microbiology, Washington DC, 820 pp.
38. Horvath, C.J., Simon, M.A., Bergsagel, D.J., Garcea, R.L. and Ringler, D.J. Am. J. Path. 140:1431–1440, 1992.
39. Jabaaij L, Grosheilde PM, Heijtink RA, Duivenvoorden HJ, Ballieux RE. 1993. J. Psychosom. Res. 37 : 361-69.
40. Jafar, S., Rodriguez-Barradas, M., Graham, D.Y. and Butel, J.S. J. Med. Virol. 54:276–284, 1998.
41. Jordan, M. C. 1986. W.B. Saunders Company, Philadelphia, 1311 pp.
42. Kelley S., Hertzman C, Marion S., Hershler R. 1995. Ottawa: Natl. popul. Health Surv. Stat. Can.
43. Kelly S, Hertzman, Daniels M 1997. Annu Rev. Public Health 18: 437-62.
44. Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T., and Morishima, T. 1999. J. Clin. Microbiol. **37:** 132-136.
45. Klein RS, Flanigan, Schuman P, Smith D, Vishov D 1999. J. Clin. Immunology, January, 93-98.
46. Kniker WT, Anderson CT, Roumiantzeff M. 1979. Ann Allergy 43:73-79.
47. Kohl, S. 1985. J. Infect. Dis. **152:** 435.
48. Krynska, B., Del Valle, L., Croul, S., Gordon, J., Katsetos, C.D., Carbone, M., Giordano, A., and Khalili, K. Proc. Natl. Acad. Sci. USA 96:11519–11524, 1999.
49. Laghi, L., Randolph, A.E., Chauhan, D.P., Marra, G., Major, E.O., Neel, J.V. and Boland, C.R. Proc. Natl. Acad. Sci. USA 96:7484–7489, 1999.
49b. Landry, M.L. and Hsiung, G.D. Primary isolation of viruses. In Specter, S. and Lancz, G., *Clinical Virology Manual*, 2nd ed., pp. 43–69. Elsevier, New York, 1992.
50. Leach, C.S., Chen, J.P., Crosby, W., Johnson, P.C., Lange, R.D., Larkin, E., Tavassoli, M. (1988) Regulation of Erythropoiesis. New York; PMA publishing Corp.
51. Lednicky, J.A., Stewart, A.R., Jenkins, J.J. III, Finegold, M.J. and Butel, J.S. Int. J. Cancer 72:791–800, 1997.
51b. Lednicky, J.A., Wong, C. and Butel, J.S. Virus Res. 35:143–153, 1995.
52. Lennette, E. T. 1991. Epstein-Barr virus, American Society for Microbiology, Washington DC, 847 pp.
53. Lin N, Ensel W.M. 1989. Am. Social.Rev.54: 382-99.
54. Lin, J.C., Lin, S.C., de, B.K., Chan, W.P., Evatt, B.L. 1993. Blood 81 :3372-3381.
55. Markowitz, R.B., Thompson, H.C., Mueller, J.F., Cohen, J.A. and Dynan, W.S. J. 1993. Infect. Dis. 167:13–20.
56. Marshall, G.S., Stout G.G., Knights M.E. et al 1994. J. Med. Virol.
57. Meehan, R. T., Neale, L. S., Kraus, E. T., Stuart, C. A., Smith, M. L., Cintron, N. M., and Sams, C. F. 1992. **76:** 491.

58. Meehan, R. 1987. Adv Exp Med Biol **225:** 273.
59. Miller, C.L., Lee, J.H., Kieff, E., Burkhardt, A.L., Bolen, J.B., Longnecker, R. (1994) Infect. Agents and Dis. **3**: 128-36.
60. Mishra, S. K., Segal, E., Gunter, E., Kurup, V. P., Mishra, J., Murali, P. S., Pierson, D. L., Sandovsky-Losica, H., and Stevens, D. A. 1994. J Med Vet Mycol **32:** 379.
61. Monaco, M.C.G., Atwood, W.J., Gravell, M., Tornatore, C.S. and Major, E.O. J. Virol. 70:7004–7012, 1996.
62. Muller HK, Lugg DJ, Quinn D. 1995a. Immunol Cell Biol 73:316-320.
63. Muller HK, Lugg DJ, Ursin H, Quinn D, Donovan K. 1995b. Pathology 27:186-190.
64. Oren, I., and Sobel, J. D. 1992. Clin Infect Dis **14:** 741.
65. Oxman, M. N. 1986. Herpes stomatitis, W.B. Saunders Co., Philadelphia, 752 pp.
66. Pagano, J. S., and Lemon, S. M. 1986. The herpesviruses, W.B. Saunders Co., Philadelphia, 470 pp
67. Payne, D.A., Mehta S.K, Tyring. S. K., Stowe R. P. and Pierson D. 1999. 70: No. 12, 1211-1213.
68. Payne D, Hoskins S, Schouten H, van Vleuten H, Tyring S. *J Virol Methods* 1995;52:105–110.
69. Payne D, Tyring S. Enhancing PCR sensitivity. In: Burke JF, Rickwood D, eds. *PCR: Essential Techniques.* Chichester, UK: John Wiley & Sons; 1996:10–13.
70. Pavan-Langston, D. 1990. Trans Am Ophthalmol Soc **88:** 727.
71. Petry LJ and WeemsLB 2nd, Liningstone JN 2nd 1991.J. Fam. Pract. 32: 481-86.
72. Pierson, D. L. 1983. Shuttle Medical Report : Summary of Medical results from STS 1, STS 2, STS3, and STS 4 (NASA TM 58252, pp 49-52). Houston TX.
73. Rand, K. H., Hoon, E. F., Massey, J. K., and Johnson, J. H. 1990. Arch Intern Med **150:** 1889.
74. Reichlin S. 1993. N. Engl. J. Med.329: 1246-53.
75. Rencic, A., Gordon, J., Otte, J., Curtis, M., Kovatich, A., Zoltick, P., Khalili, K. and Andrews, D. Proc. Natl. Acad. Sci. USA 93:7352–7357, 1996.
76. Riddler, S.A., Breinig, and McKnight.1994.Blood **84:** 972-984.
77. Rowe, D.T., Qu, L., Reyes, J., Jabbhour, N., Yunis, E., Putnum, P., Tado, S. and Green, S. 1997. J. Clin. Microbiol. **35:** 1612-1615.
78. Shah, K.V. Polyomaviruses. 1996. In Fields Virology, 3rd ed., pp. 2027–2043. Lippincott-Raven, Philadelphia.
79. Shah, K. and Nathanson. 1976.N. Am. J. Epidemiol. 103:1–12.
79b.Shah, K.V. Papovaviruses. In Specter, S. and Lancz, G., *Clinical Virology Manual*, 2nd ed., pp. 455–472. Elsevier, New York, 1992.
80. Simon, M.A., Ilyinskii, P.O., Baskin, G.B., Knight, H.Y., Pauley, D.R. and Lackner, A.A. Am. J. Path. 154:437–446, 1999.
81. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. *Science* 1988; 239:487–491.
82. Shearer, W.T. 1999. J Allergy Clin Immunol 103:26-28.
83. Shirakawa T and Morimoto K. 1991. Allergy 46: 561- 69.
84. Solomon, G. F. 1987. J. Neurosci. Res. 18: 1.
85. Stein, T. P., and M. D. Schluter. 1994. *Am. J. Physiol.* 266: E448-E452.
86. Sumaya, C.V. (1977) J. Infect. Dis. **135**: 374-80.
87. Sumaya, C.V. (1986) Ped. Infect. Dis. **5**: 337-342.
88. Sundsfjord, A., Flaegstad, T., Flø, R., Spein, A.R., Pedersen, M., Permin, H., Julsrud, J. and Traavik, T. J. Infect. Dis. 169:485–490, 1994.
89. Taylor, G. R. 1987. Adv Exp Med Biol 225: 259.

90. Taylor, G. R., and Janney, R. P. 1992. J Leukoc Biol **51:** 129.
91. Taylor, G. R., Kropp, K. D., Henney, M. R. et al.1977. Apollo-Soyuz test Project Medical report (NASA SP-412) Washington DC: NASA
92. Taylor, G. R., Graves, R. C., Brockett, R. M., et al. 1977. Medical microbiological analysis of US crewmembers, U.S. Govt. Printing Office, Washington DC, 237 pp.
93. Theorell T, Orth-Gomer K., Eneroth P. 1990. Psychosom Med. 52: 511-16.
94. Ursin H, Mykletun R, Tonder O, Vaernes R, Relling G et al. 1984. Scand. J. Psychol. 25: 340-47.
95. Vassend O, and Halvorsen R. 1987. Scand, J. Psychol. 28: 233-41.
96. Youngner, J. S. 1986. Persistent viral infections, W. B. Saunders Co., Philadelphia, 103 pp.

96b. Yao, Q.Y., Rickinson, A.B. and Epstein, M.A. Int. J. Cancer 35:35–42, 1985.
97. Wald, A., Zeh, J., Barnum, G., Davis, L. G., and Corey, L. 1996. Ann Intern Med **124:** 8.
98. Waldrop, S.L, Pitcher, C.J., Peterson, D.M., Maino, V.C., Picker, L.J. (1997) J. Clin. Invest. **99**: 1739-50.
99. Weiner, L.P., Herndon, R.M., Narayan, O., Johnson, R.T., Shah, K., Rubinstein, L.J., Preziosi, T.J. and Conley, F.K. NEJM 286:385–390, 1972.
100. Williams DL, Climie A, Muller HK, Lugg DJ. 1986. J Clin Lab Immunol 20:43–49.
101. Workman, E. A., and Lavia, M. F. 1996. Stress and immunity: A behavioral medicine perspective, CRC Press, Boca Raton FL, 69 pp.

Appendix

Table A

Herpes viruses and Polyomavirus PCR, DNA Sequencing, and Probe Oligonucleotides

HSV1, HSV2		HSV011	CAG TAC GGC CCC GAG TTC GTG A
			Biotin-GTA GAT GGT GCG GGT GAT GTT
CMV		MIE-D1	TGT CCT CCC GCT CCT C
		MIE-D2B	Biotin ATG AAG GTC TTT GCC CAG TA
HHV6	HHV6-1		GTT CCA GGC GGC ATG AAT TC
		HHV6-2B	Biotin-ACA CGG CCT CTC TAC ATC AC

SV40 Regulatory Region Oligonucleotides	Reference positions[a]	temp.[b]	Size (bp), PCR product [SVSph21-N	SV21-N	SV2X21-N][c]
RA1: 5'-AATGTGTGTCAGTTAGGGTGTG-3'	nt 266-245				
RA2: 5'-TCCAAAAAAGCCTCCTCACTACTT-3'	nt 5195-5218	63(52)	245	317	386
RA3: 5'-GCGTGACAGCCGGCGCAGCACCA-3'	nt 358-336				
RA4: 5'-GTCCATTAGCTGCAAAGATTCCTC-3'	nt 5119-5142	63(52)	413	485	554

SV40 VP1 Carboxy Proximal Oligonucleotides	Reference positions[a]	temp.[b]	Size (bp), PCR product[d]
LA1: 5'-GGGTGTTGGGCCCTTGTGCAAAGC-3'	nt 2251-2274		
LA2: 5'-CATGTCTGGATCCCCAGGAAGCTC-3'	nt 2545-2522	63(52)	294
LA3: 5'-CAGCAGTGGAAGGGACTTCCCAG-3'	nt 2336-2358	(N/A)	(N/A; VP1 probe)

SV40 T-ag Amino-Proximal Oligonucleotides	Reference positions[a]	temp.[b]	Size (bp), PCR product[d]
SV.for3: 5'-TGAGGCTACTGCTGACTCTCAACA-3'	nt 4476-4453		
SV.rev: 5'-GCATGACTCAAAAAACTTAGCAATTCTG-3'	nt 4372-4399	63(52)	105

SV40 T-ag Carboxy Terminal Oligonucleotides	Reference positions[a]	temp.[b]	Size (bp), PCR product[e]
TA1: 5'-GACCTGTGGCTGAGTTTGCTCA-3'	nt 3070-3049		
TA2: 5'-GCTTTATTTGTAACCATTATAAG-3'	nt 2630-2652	60(52)	441

JCV Regulatory Region Oligonucleotides	Reference positionf	temp.	Size (bp), PCR productf
JC1: 5'-CCTCCACGCCCTTACTACTTCTGAG-3'	nt 5086-51105		
JC2: 5'-AGCTGGTGACAAGCCAAAACAGCTCT-3'	nt 272-2475	60(52)	316

BKV Regulatory Region Oligonucleotides	Reference position[g]	temp.	Size (bp), PCR product
BK1: 5'-GGCCTCAGAAAAAGCTTCCACACCCTT-ACTACTTGA-3'	nt 50-854	60(52)	335
BK2: 5'-CTTGTCGTGACAGCTGGCGCAGAAC-3'	nt 415-391		

Human A gamma-hemoglobin gene	Reference position[i]	temp.	Size (bp), PCR product
AG1: 5'-CTCAGACGTTCCAGAAGCGAGTGT-3'	nt 1252-1229		
AG2: 5'-AAACGGCTGACAAAAGAAGTCCT-3'	nt 876-897	60(52)	379

[a] Reference nucleotide positions in SV40 reference strain 776 (SV40-776).
[b] PCR annealing temperature (°C) under stringent conditions and non-stringent conditions (number in parenthesis).
[c] Artificial SV40 control templates (34).
[d] pSV21-N and other full-length SV40 genomes that have been currently tested.
[e] pSV21-N and other SV40-776-derived plasmids.
[f] pJC-MAD-1(Plasmid clone of JCV reference strain MAD-1).
[g] pBK-DUN (Plasmid clone of BKV reference strain Dunlop).
[h] GenBank Human A-gamma globulin gene sequence, accession # M32724.