Original Article

Study of Simulated Microgravity on Immunological Parameters

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Abstract

Spaceflight results in immunosuppression which is likely due to neurohumoral factors released in response to intermittent stress effects during flight. However, no major non-physiological health problems have been reported during or following spaceflight, but diseases resulting from immunosuppression could occur on long-duration missions and would include bacterial, fungal, and viral infections in addition to increased incidence of neoplasia and autoimmunity. Studies have shown impairment of cell mediated, humoral and innate immunity variables in blood samples of astronauts who have flown a space mission. This study examined the immunological parameters on healthy adults before and after simulated microgravity. 20 healthy adult male volunteer subjects were subjected to simulated microgravity for 6 hours using dry floatation tank. The immunological parameters were analyzed using the blood sample taken pre and post exposure to simulated microgravity. The parameters included: total leukocyte count, differential leukocyte count, CD4 count, CD8 count, CD4/CD8 ratio, serum IL-2 (adaptive immune cytokine) and TNF-á (innate immunity cytokine) levels. There was no statistically significant change in the CD4 count, CD8 count or CD4/CD8 ratio. However there was significant reduction in values of serum TNF-á (p=0.004) and IL-2 (p=0.0258). This experiment showed a mild lowering of cell mediated immunity and cytokines involved in innate immunity. The paper further recommends the likely countermeasures to reduce this problem and how to systematically investigate a pilot having signs of immune-suppression.

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Introduction

After several years of experimentation, it has become clear, that spaceflight can have major effects on immune parameters. The possible mechanisms by which spaceflight induces changes in immune responses include (i) exposure to microgravity, including possible direct effects and indirect effects on cells responsible for immune responses. This could include microgravity-induced changes in muscle and bone that have indirect effects on the immune system that result from alterations in levels of 1,25-dihydroxyvitamn-D3; (ii) stress induced by spaceflight; (iii) changes in circadian rhythm (sleep deprivation); (iv) changes in nutritional intake; (v) radiation encountered during spaceflight and (vi) multiple other unidentified factors [1].

The methods of carrying out research in these fields are (i) collecting samples from astronauts before and after a space mission; (ii) carrying out tests on animals who had been a part of space mission; (iii) simulating space environment for humans: head down tilt, dry floatation, parabolic flight, isolation studies mimicking long isolations precipitating social/ psychic stress; (iv) tail suspension models of animals on ground simulating microgravity (v) lymphocyte cell cultures-blastogenesis exposed to simulated microgravity [1].

We used dry floatation method to simulate gravity. A body that floats during immersion can be considered apparently weightless as floating occurs only when the weight of the body is opposed by an equal force of buoyancy. In non-rigid bodies like that human beings; weight is produced by

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deformation caused by the resultant of forces acting on the body. This deformation is detected by the mechanoreceptors and weight is felt. If sensory input from these receptors could be obliterated, it is reasonable to suppose that the resulting physiological adjustments normally made in response to such information would not be made [2].

Immune changes during space flights in excess of two weeks have been almost exclusively studied by Russian investigators in the last century. Most of these studies have compared post flight values with those obtained before flight. Studies of cosmonauts during spaceflight have shown that IgG levels were unchanged, whereas IgA and IgM levels were sometimes increased. Additionally, in-flight delayed type hypersensitivity testing demonstrated a decrease below the warning level in 1/3 of the cosmonauts tested. Pre vs. post flight analyses have often revealed a post flight decrease in: PHA (phytohaemagglutinin)-triggered lymphocyte blast transformation; the proliferation index of T-lymphocytes in the xenogeneic graft versus host reaction; the mitogen induced production of interleukin-2; the presence of certain leukocyte subsets; and cytotoxic activity of natural killer (NK) cells. Other factors that changed in an apparently random manner after flight included: production of á/ã interferon; autoimmune tests; and globulin classes [3].

We selected two markers determining the cell mediated immunity (CD4, CD8) and two cytokines (i) Interleukin -2 (IL-2) representing the adaptive immune cytokine (made principally by CD4+ T lymphocytes in response to antigen and other signals, and function to promote lymphocyte proliferation and differentiation and to activate effector cells) (ii) Tumour necrosis factor- alpha (TNF-á) representing innate immunity cytokine (produced rapidly in response to microbes and other stimuli, are made principally by macrophages, dendritic cells, and NK cells, and mediate inflammation and anti-viral defense.

Aim and Objectives

The aim of the study was to study the immunological parameters before and after the simulated microgravity experiment. Subsequently the goal was to find the significant difference between the two and analyze the pattern of variation and compare it with similar studies. The ultimate objective is to carry out immunological screening of potential astronauts during selection procedure and recommend countermeasures to up-regulate the immunity during space travel. This will aid in screening out an astronaut who could in long space mission develop slight immunosuppression making him prone to infections/ malignancies due to latent viruses.

Material and methods

20 volunteers were recruited for the study. The nature and purpose of the study and the risk involved were explained to the participants who were ascertained to be normal by history, complete physical examination. An informed written consent was taken. Inclusion criteria: (i) age: 20 to 40 years (ii) healthy males. Exclusion criteria: (i) subjects with known cardio-respiratory or chronic medical disorders (ii) individuals having any other inflammatory illness or on any drugs/medication.

The participants were asked to abstain from alcohol, caffeine and smoking for 24 hrs before testing. Each participant was exposed to the 6 hrs microgravity simulation in the form of dry supine thermo-neutral immersion. The participant wore well-fitting nylon vest and pyjama after complete micturition and then rested supine on the raised platform. All the participants were given exposure at the same time (in the morning between 0800-1400hrs). The platform was lowered hydraulically

and at the same time the water proof sheet was loosened from all the four sides to avoid support by it. The temperature in the immersion tank was kept at 35 ± 0.5 degree centigrade by thermostat control mechanism.

8 ml of venous blood sample was taken pre and post exposure to microgravity under sterile precautions. Parameters that were tested included –total leukocyte count, differential leukocyte count, CD4 count, CD8 count, CD4/CD8 ratio, serum IL-2 and TNF-á levels. Sysmex KX 21 was used for total leukocyte count, differential leukocyte count. Absolute CD4/CD8 T-cell counts were estimated by the Guava EasyCD4 System using two fluorescence parameters in combination with forward scatter (FSC) to identify cells. The reagents consisted of a monoclonal anti-human CD3 antibody conjugated to the tandem dye phycoerythrin (PE)-Cy5 and monoclonal anti-human CD4 and CD8 antibodies conjugated to PE. Serum samples for IL-2 and TNF-á were stores at -20°C and assayed together by sandwich ELISA technique. The kits of Immunotech (Beckman Coulter) were used. The coefficient of variation of both the cytokines ELISA inter-assay/intra-assay was < 6%.

Statistics

The data was, first, examined for normality using D'Agostino & Pearson Omnibus normality test. Results on continuous measurements are presented on Mean±SD. Significance was assessed using one-tailed paired t-test between the pre and post exposure of simulated microgravity at 5 % level of significance in the parameters that passed the normality test. In the parameters that did not pass normality test (in one/both of pre and post pairs), one-tailed Wilcoxon Signed Rank test (Wilcoxon Matched Pair test) with a Gaussian approximation done at 5% level of significance.

Results

The mean age of the subjects was 23.45±2.19 yrs. Every effort was made to keep the confounding variables to the minimum by doing the study protocol in same time, sample analyzed during similar time. The samples were coded and double blinding was ensured. Since there were no base population currently available in Indian scenario, the sample size was ensured to be statistically significant (n=20).

The parameters that passed normality test with pre and post exposure of simulated microgravity are shown in Table 1.

Table 1: One-Tailed Paired t test – Normality passed

Parameters	Mean ± S.D		t value	p value
	Pre	Post		
Total Leukocyte Count (/cmm)	8510 ± 1187	8390 ± 967.9	0.348	0.366
Neutrophils (%)	59.55 ± 4.80	59.70 ± 4.90	0.120	0.453
Eosinophils (%)	5.30 ± 1.17	5.60 ± 1.23	1.031	0.158
Monocyte (%)	1.95 ± 0.51	2.00 ± 0.79	0.237	0.408
CD 4 (/cmm)	984.7 ± 77.81	983.9 ± 69.55	0.130	0.449
CD 8 (/cmm)	249.1 ± 27.45	255.0 ± 32.63	1.202	0.122
CD4/CD8 ratio	3.980 ± 0.366	3.903 ± 0.417	1.061	0.151
TNF-á (pg/ml)	54.1± 5.94	48.5± 4.6	3.10	0.004*
IL-2 (pg/ml)	8.75± 1.47	6.95± 1.44	2.32	0.0258*

Table 2: One-Tailed Wilcoxon Matched Pair test (Wilcoxon signed rank test) – Normality not passed

Parameters	Mean ± S.D		W	p value
	Pre	Post		
Lymphocyte (%)	33.20±4.50	32.70±4.91	27.00	0.3001

p 0.05-0.01 */p 0.01-0.001 **/ p <0.0001 ***/ W – Sum of signed ranks

The parameters that did not pass normality test with pre and post exposure of simulated microgravity are shown in Table 2.

Discussion

As a response to intracellular microbes naive T lymphocytes are activated by antigen and co-stimulators in peripheral lymphoid organs, and proliferate and differentiate into effector cells that migrate to any site where the antigen (microbe) is present. One of the earliest responses of CD4+ helper T cells is secretion of the cytokine IL-2 and expression of high-affinity receptors for IL-2. IL-2 is a growth factor that acts on these T lymphocytes and stimulates their proliferation, leading to an increase in the number of antigen-specific lymphocytes. The functions of helper T cells are mediated by the combined actions of CD40-ligand (CD40L) and cytokines. The best defined subsets of differentiated CD4+ helper cells are the T_H1 and T_H2 subsets. Cells of the T_H1 subset secrete the cytokine IFN-ã and TNF-á which are a potent macrophage activator. The combination of CD40- and IFN-ã/TNF-á mediated activation results in the induction of microbicidal substances in macrophages, leading to the destruction of ingested microbes. T_H2 cells produce IL-4, which stimulates B cells to differentiate into IgE-secreting plasma cells, and IL-5, which activates eosinophils. A third subset of CD4+ T cells called the T_H17 are powerful recruiters of neutrophils and monocytes, and thus play major roles in several inflammatory diseases. Activated CD8+ lymphocytes differentiate into cytotoxic lymphocytes that kill cells harbouring microbes in the cytoplasm. By destroying the infected cells, CTLs eliminate the reservoirs of infection [4].

As a response to extracellular micro-organism B lymphocytes proliferation takes place which in turn differentiates into plasma cells that secrete antibodies with distinct functions. Typical globular protein antigens are not able to bind to many antigen receptors, and the full response of B cells to protein antigens requires help from CD4+ T cells. B cells ingest protein antigens into vesicles, degrade them, and display peptides bound to MHC molecules for recognition by helper T cells [5].

Alterations in immune parameters reported after spaceflight include the impairment of cell mediated, humoral and innate immunity. Various workers have reported a decrease in lymphocyte number, decreases in leukocyte blastogenesis, increases in leukocyte number, increases in numbers of B- and T-lymphocytes, decreases in monocytes, increases in helper T-lymphocytes, decreases in cytotoxic T-lymphocytes, and a decrease in the ratio of CD4 + /CD8 + lymphocytes post space flight [6]. Variability has been reported from flight to flight (i.e., not all results have been consistently seen after every spaceflight). Our study showed an insignificant lymphopenia and leucopenia. However there was a downward trend of CD4/CD8 ratio as a result of disproportionate increase in CD8 as compared to CD4. A likely explanation for such effects is the enhanced release of corticosteroids

that can induce alterations in the lymphocyte subpopulations of $CD4^+$ and $CD8^+$ cells, leading to a decrease in the ratio of $CD4^+$ to $CD8^+$ cells [7]. The change of CD4/CD8 ratio was not significant in our study. This can be explained by the probable low microgravity time exposure as compared to literature studies where the simulation has been as long as 120 days in continuation [8]. Therefore, it would seem appropriate to consider also, besides CD4/CD8 ratio, acute stress cytokines, other factors with known immunomodulatory effects e.g., catecholamines, cortisol vasopressin, dehydroepiandrosterone, prolactin, and growth hormone to be studied [9].

To date, the only studies on humoral immunity in astronauts have involved examining total serum immunoglobulin levels after the return of crew to Earth. In long-term studies, Russian scientists have reported increases in the level of serum immunoglobulins, particularly total serum IgA and IgM. The increased immunoglobulin levels observed after long-term spaceflight have been interpreted as indicating enhanced opportunity for the development of autoimmune diseases [10]. We did not include any humoral immunity marker in our study.

The effects of spaceflight on innate human immunity have been studied in a limited fashion. Alterations that have been observed include decrease in the killing of target cells by NK cells and decreased IFN-á/ã production. The production of IFN, IL-1, IL-2, and TNF-á from cultures of human peripheral blood leukocytes exposed to spaceflight was also altered, compared to controls [11, 12].

In our study we showed a significant decrease in TNF alpha. TNF-á is a pleiotropic inflammatory cytokine that possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self-regulatory properties as well. For instance, TNF- á induces neutrophil proliferation during inflammation, but it also induces neutrophil apoptosis upon binding to the TNF-R55 receptor [13]. Beneficial functions of TNF- á include its role in the immune response to bacterial, and certain fungal, viral, and parasitic invasions as well as its role in the necrosis of specific tumors. It acts as a key intermediary in the local inflammatory immune response. TNF- á is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to the site of infection. It promotes blood clotting which serves to contain the infection. Without TNF- á, mice infected with gram negative bacteria experienced septic shock [14]. The decrease in TNF in our study can be explained by increase in adrenocorticotropic hormone and catecholamines subsequent to microgravity that upregulates Th2 cytokine production via stimulation of the glucocorticoid and â2-adrenergic receptors. Through the stimulation of â2 receptors, catecholamines upregulate production of anti-inflammatory cytokines IL-6 and IL-10 [15, 16], leading to a decrease in TNF-á levels [17].

IL-2 in our study also showed a significant decrease post microgravity simulation. IL-2 is known to be secreted mainly at inflamed sites but also during stress. In addition, it seems to be positively controlled by catecholamines. IL-2, a product of the Th1 subset, is a major cytokine that stimulates proliferation of CD4 and CD8 lymphocytes; it also increases activities of NK cells and cells of the monocyte-macrophage lineage. Low IL-2 production capacity could compromise immune defenses not only against infectious agents but also against neoplastic cells during extended space missions. There are, indeed, numerous studies that include IL-2 in efforts to improve immune responses

against cancer [18]. Furthermore, reports that IL-2 protects memory T cells from apoptotic death [19] implies that insufficient amounts of the factor could endanger responsiveness to recall antigens, e.g., vaccines, including those recently approved for cervical cancer due to human papillomavirus types 16 and 18. Although the mechanisms remain unclear, ground-based investigations simulating microgravity suggest that the decreased production of IL-2 may be related to alterations in molecules needed for signal transduction, e.g., CD25 [20].

A study carried out simulating microgravity by head down tilt at 6° for 120 days was associated with increase in the evening secretion of cortisol, the subsequent loss of the diurnal rhythm, as well as the rise in the urine excretion of catecholamines. Functional and enumerative evaluation of peripheral WBCs indicated activation of the innate part of the immune system as suggested by the enhanced expression of â2-integrin adhesion molecules on circulating neutrophils and a rise in the relative and absolute number of NK cells. In contrast, relative numbers of CD4+ T lymphocytes slightly decreased, whereas CD8+ T lymphocytes increased. As a result, the ratio of CD4+ to CD8+ cells decreased, which might be associated with a compromise of the specific immune system. In parallel to the onset and duration of the hypokinesia period, plasma concentrations of IL-6 increased, whereas other inflammatory cytokines (TNF-á and IL-1) remained unchanged [8].

Crucian et al in their study used flowcytometry in blood samples of 27 astronauts at three points (one preflight, two postflight) surrounding four space shuttle missions. Absolute levels of peripheral granulocytes were significantly elevated following space flight, but the levels of circulating lymphocytes and monocytes were unchanged. Lymphocyte subset analysis demonstrated a decreased percentage of T cells, whereas percentages of B cells and NK cells remained unchanged after flight. Nearly all the astronauts exhibited an increased CD4/CD8 T cell ratio. Assessment of naive ($CD45RA^+$) vs. memory ($CD45RO^+$) $CD4^+$ T cell subsets was ambiguous, and subjects tended to group within specific missions. Although no significant trend was seen in absolute monocyte levels, a significant decrease in the percentage of the $CD14^+CD16^+$ monocytes was seen following space flight in all subjects tested. T cell ($CD3^+$) production of IL-2 was significantly decreased after space flight, as was IL-2 production by both $CD4^+$ and $CD8^+$ T cell subsets. Production of IFN-ã was not altered by space flight for the $CD8^+$ cell subset, but there was a significant decrease in IFN-gamma production for the $CD4^+$ T cell subset. Serum and urine stress hormone analysis indicated significant physiologic stresses in astronauts following space flight. Altered peripheral leukocyte subsets, altered serum and urine stress hormone levels, and altered T cell cytokine secretion profiles were all observed postflight [21].

Fuchs and Medvedev have elucidated countermeasures for ameliorating in-flight immune dysfunction [22]. The astronauts are to be advised to follow their daily exercise module and their diet should consist of sufficient quantities of antioxidant vitamins (A/E) and minerals like zinc that has immunomodulatory effects. If specific antibody responses are likely to be impaired, special immunizations could be devised and intravenous immunoglobulin infusions could be supplied. If abnormal inflammatory cytokine production occurs in model studies, pharmacological agents could be given to inhibit the production of these mediators. Agents such as thalidomide, levamisole, pentoxiphylline could be given to astronauts. If T-cell proliferation were likely to be significantly reduced, special immunizations with viral genome constructs in gene therapy could be given. There

have been studies on use of sodium and lithium salts of'y-hydroxy-13-aminobutynic acid in a dose 50 mg/kg of body weight that protects the NK cells from stress. The latest immunomodulators being experimented on various immunodeficiencies's like recombinant interferons, lactobacillus and IL-2 can also be carried for long space missions [1].

Reactivation of latent viruses in the blood cells of astronauts exposed to long-term space travel poses a special threat because of the possibility of acquiring a debilitating chronic infection or malignancy. Viruses that produce these grave problems include Epstein Barr Virus (EBV) and Human Herpes Virus 8 (HHV8). EBV is associated with human lymphomas and leiomyosarcomas, and HHV8 is associated with Kaposi sarcoma [23].

Limitations of study

Absolute cell count and few cytokines by itself do not encompass the entire gamut of humoral, cell mediated and innate immunity. Further studies including immunophenotyping of lymphocyte subsets and functional antibody/leukocyte studies should be carried out to make this aspect complete.

Recommendation: Approach to screening

The ultimate goal of all of these human studies is to determine whether astronauts in long-term space travel will experience alterations in normal immune function. A candidate should be suspected to have some kind of immunodeficiency if he/she has been admitted repeatedly with recurrent localized/ generalized infections. The quantifying criteria are (i) e"3 serious bacterial infections in a span of 2 years (pneumonia, meningitis, septicaemia, osteomyelitis, septic arthritis); (ii) Infection with organisms of low virulence; (iii) chronic sinopulmonary infection (iv) Unusual infecting agents (Pneumocystis carinii, Listeria monocytogenes); (v) incomplete response to treatment [24]. Primary immunodeficiencies (Wiskott-Aldrich syndrome, Chediak-Higashi syndrome, DiGeorge syndrome, hyper-IgE syndrome) are very rare to be encountered in astronaut population as this population is screened on an annual basis.

We propose a 3 phase screening for the candidates suspected to have impaired immunity: Phase 1: The initial screening would include complete blood count, differential counts (to rule out neutropenia, aplastic anaemia, haemolytic anaemia, and thrombocytopenia); urine, blood, spinal fluid cultures if infection is active and chest radiography. A repeated altered complete blood count would warrant an ELISA test for HIV1/2, HBsAg, anti HCV. Phase2: In case the serological tests are negative however the other tests of phase-1 show abnormalities then the following second line inclusive of skin tests and immunoglobulins should be adopted. Tuberculin test has its own drawbacks due to rampant primary tuberculosis in our country. Best screening test for delayed-type hypersensitivity would be Candida skin test or standardized panel of antigens prepared for this purpose. Subjects who do not respond should be retested using more concentrated forms of the antigens. Repetitive lack of response to DTH in the patient at risk for a T-cell defect warrants in vitro lymphocyte evaluation. The determination of lymphocyte phenotyping by flowcytometry is necessary when the measurement of specific types of T-, B-, NK-, and phagocytic cells determines the diagnosis of a primary immune defect. Quantitative serum immunoglobulins (IgG, IgA, IgM, and IgE) are carried out. Even though total serum IgG may be normal, subclass deficiency may still occur and quantitative measurements of individual subclasses can be performed. Studies (e.g., in vitro mitogen or antigen driven B-cell proliferation and immunoglobulin secretion) may be needed to delineate functional B-cell defects.

Phase-3: In case the phase 2 investigations show an aberration then the candidate should be referred to an immunologist for further investigations like functional antibody/lymphocyte/phagocyte assays and specialised complement activation tests.

Conclusion

The study concluded that there is a minor impairment of immunity on exposure to microgravity in form of lowered CD4/CD8 ratio and decreased IL-2 and TNF-á. The two goals to further pursue this study are: (1) understanding the immunopathogenesis of spaceflight alterations in immunity and (2) developing countermeasures to avoid clinical consequences of spaceflight-induced immunosuppression.

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