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**COSMOS-1989 IMMUNOLOGY STUDIES**

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## INTRODUCTION

Evidence from both human and rodent studies has indicated that alterations in immunological parameters occur after space flight [1,2]. The number of flight experiments has been small, and the full breadth of immunological alterations occurring after space flight remains to be established. Among the major effects on immune responses after space flight that have been reported are: alterations in lymphocyte blastogenesis and natural killer cell activity, alterations in production of cytokines, changes in leukocyte sub-population distribution, and decreases in the ability of bone marrow cells to respond to colony stimulating factors [6]. Changes have been reported in immunological parameters of both humans and rodents [1]. The significance of these alterations in relation to resistance to infection remains to be established.

The current study involved a determination of the effects of flight on Cosmos mission 2044 on leukocyte subset distribution and the sensitivity of bone marrow cells to colony stimulating factor-GM. A parallel study with antiorthostatic suspension was also carried out. The study involved repetition and expansion of studies carried out on Cosmos 1887.

## METHODS

Spleen and bone marrow cells were obtained from flown, vivarium control, synchronous control, and suspended rats. The



cells were stained with a series of monoclonal antibodies directed against rat leukocyte cell surface antigens [3]. Control cells were stained with a monoclonal antibody directed against an irrelevant species or were unstained. Cells were then analyzed for fluorescence using a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA).

GM-CSF was obtained from Immunex Corp. Bone marrow cells were placed in culture with GM-CSF in McCoy's 5a medium and incubated for 5 days. Cultures were then evaluated for the number of colonies of 50 cells or greater [4].



## RESULTS AND DISCUSSION

Analysis of the data has permitted the drawing of the following conclusions:

1) Bone marrow cells from flown rats showed a decrease in response to Colony stimulating factor-granulocyte/monocyte,

2) Alterations occur in the sub-populations of spleen and bone marrow cells studied,

3) The results of the suspension study indicate that there was a similar result of suspension and space flight with regard to the decreased response of bone marrow cells to CSF-GM. There is no similarity between the effects of space flight and suspension on leuckocyte sub-population distribution.

4) In joint studies with Drs. I. Konstantinova and A. Lesnyak, we have shown that natural killer cell activity is inhibited using spleen cells from flown rat, but the inhibition is dependent on the natural killer cell target used.

The data are summarized in attached manuscripts.

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Revised

## **SPACE FLIGHT ALTERS IMMUNE CELL FUNCTION AND DISTRIBUTION**

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Running Title: Space Flight and Immune Responses

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## ABSTRACT

Experiments were carried out aboard Soviet Cosmos biosatellite number 2044 to determine the effects of space flight on immunologically important cell function and distribution. Control groups included vivarium, synchronous and antiorthostatically suspended rats. In one experiment, rat bone marrow cells were examined in Moscow, U.S.S.R. for their response to recombinant murine granulocyte/monocyte-colony stimulating factor (GM-CSF). In another experiment, rat spleen and bone marrow cells were stained in Moscow with a variety of antibodies directed against cell surface antigenic markers. These cells were preserved and shipped to the United States for analysis on a flow cytometer. The results of the studies indicated that bone marrow cells from flown and suspended rats showed a decreased response to GM-CSF as compared to bone marrow cells from control rats. Of the spleen cell sub-populations examined from flown rats, only those cells expressing markers for suppressor-cytotoxic-T and helper-T cells showed an increased percentage of stained cells. Bone marrow cells showed an increase in the percentage of cells expressing markers for helper-T cells in the myelogenous population and increased percentages of anti-asialo GM-1 bearing, interleukin-2 receptor bearing, pan-T and helper-T cells in the lymphocytic population. Cell populations from rats suspended antiorthostatically did not follow the same pattern of distribution of leukocytes as cell populations for flown rats. The results from Cosmos 2044 are similar, but not identical to, earlier results from Cosmos 1887, and confirm that space flight can have profound effects on immune system components and activities.

INDEX TERMS: Microgravity; granulocyte/monocyte-colony stimulating factor, leukocyte phenotypes

## INTRODUCTION

Over the past several years, various alterations in immunological parameters have been reported after space flight (1,3-6,10,12,13,17-21). Similar changes have been observed after antiorthostatic suspension (2,7,14,16). The changes have ranged from alterations in lymphoid organ size (5) to alterations in the production of interferons (8,19,20) to alterations in lymphocyte activation (3,4).

The purpose of the immunology studies flown on Cosmos 2044 and described in this paper was to continue a systematic attempt to define the range of immunological parameters affected by space flight. The experiments were designed to allow repetition and expansion of experiments carried out on the previous Cosmos 1887 flight. This was an extremely important goal, as it opportunities to repeat immunological space flight experiments under similar conditions have been very rare in the past. In addition, an antiorthostatic suspension study was included to allow direct comparison of the effects of space flight with the effects of suspension on immunological parameters, thereby testing the efficacy of suspension as a microgravity model. This has not been able to be carried out in the past, and it was of great importance to determine the reliability of the model for the effects of space flight.

Two different areas of immunological studies were chosen. The first involved a determination of the effects of space flight on the ability of cells to respond to granulocyte/monocyte-colony stimulating factor- (GM-CSF). GM-CSF is an important regulator of the differentiation of bone marrow cells of both the monocyte/macrophage and granulocyte lineages. An alteration in the ability of cells to respond to GM-CSF can result in altered immune function (22). This is the first time a study of this nature has been done using recombinant GM-CSF.

The second set of studies involved a determination of the effect of space flight on the expression of cell surface markers of both spleen and bone marrow cells. These markers represent various immunologically important cell populations. An alteration in the percentage of cells expressing the markers can also result in an alteration of immunological function (9). The markers that were tested included T-cell markers, B-cell markers, natural killer cell markers, and interleukin-2 receptors. These studies were performed by staining the spleen and bone marrow cells of space-flown rats with fluorescein-labeled antibodies directed against the appropriate surface markers and analyzing them in a flow cytometer. The percentage of cells expressing the markers was compared with the percentage of cells expressing markers in similarly treated cell populations from control rats.

## MATERIALS AND METHODS

**Animals and space flight conditions.** Ten male specific pathogen free rats of Czechoslovakia-Wistar origin (Institute of Endocrinology, Bratislava Czechoslovakia) were flown on the Cosmos 2044 Biosputnik flight for 14 days. Flight, housing, feeding, recovery, age, weight and sacrifice conditions were as described in the overview paper in this issue of the **Journal of Applied Physiology** (8). Only tissue from 5 flown rats, numbers 6-10, were made available for our project.

**Harvest of tissues.** The rats were sacrificed from 9-11 hrs after landing, and tissue collected from 25-33 min later. After sacrifice of the rats, bone marrow cells were extruded with a needle and syringe from the left femur of each rat using RPMI-1640 medium (MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum, antibiotics (penicillin - 100 units/ml, streptomycin, 100 µg/ml, gentamicin sulfate - 50 µg/ml) and 2mM glutamine. One-third of the spleen of each of five rats was dissociated into individual cells and placed into supplemented RPMI-1640 medium. All of the samples were placed into transporter vials, held at 4°C, and transported to Moscow (17). Samples reached the laboratory and analytical work began approximately 24 hr after removal of the tissue from the rats.

Synchronous control, vivarium control, and antiorthostatically tail-suspended rats were treated as described in the overview paper in this issue of the **Journal of Applied Physiology** (8). Briefly, synchronous control rats were housed and treated as flight rats for the duration of the flight, but without microgravity and radiation that occurs during flight, vivarium control rats were housed in standard caging in a vivarium, and suspended rats were suspended antiorthostatically by the tail for the duration of the flight. Tissues were removed

and treated as described for the flight tissue. In addition, a normal rat was sacrificed and tissue harvested and analyzed to serve as an internal control for our assay procedures.

**Experimental procedures.** Upon arrival at the laboratory in Moscow, the cells were centrifuged, washed in RPMI-1640 medium, and counted in a hemocytometer using trypan blue dye exclusion for determination of viability. For colony stimulating factor assays,  $1 \times 10^5$  bone marrow cells were suspended in McCoy's 5a medium (MA Bioproducts, Walkersville MD) supplemented with 10% fetal bovine serum and antibiotics and containing 3% agar (15). Included in the medium was 0.1 ml of 1,000 units of recombinant murine CSF-GM (a gift of Dr. Steven Gillis, Immunex Research and Development Corp, Seattle, WA). The GM-CSF was lot 620-028-5 and was of specific activity of at least  $5 \times 10^7$  units/mg protein. Five replicate cultures were set up for each sample. The suspended cells were then placed in 35 mm tissue culture dishes and incubated in a 37°C incubator with 5% CO<sub>2</sub> (15). After the appropriate incubation period (5 days), 10 microscope fields on each slide were evaluated for the number of colonies (aggregates of 50 cells or more) formed (17).

For the cell surface antigenic markers, the following procedure was carried out (9). One  $\times 10^6$  bone marrow or spleen cells were placed in a microcentrifuge tube. The cells were resuspended in FTA buffer (BBL Microbiology Systems, Cockeysville, MD) with 0.1% sodium azide and 0.5% normal mouse serum to decrease background staining. The cells were centrifuged for 1.5 min at 1,000 x g. Supernatants were removed and cells resuspended. Five  $\mu$ l of the appropriate antibody was added to each cell suspension. All antibodies were obtained from Accurate Chemical and Scientific, Westbury, NY except for anti-asialo GM-1, which was obtained from Wako Fine Chemicals, Dallas, TX. The cells were allowed to incubate at 4°C for 25 min. The antibodies used were as follows:

1. Anti-asialo GM-1 (anti-natural killer cell antigen)
2. OX-39 (anti-interleukin-2 receptor)

3. OX-1 (anti-pan leukocyte marker)
4. W3/25 (anti-helper T-cell)
5. OX-8 (anti-suppressor T-cell)
6. OX-12 (anti-rat IgG Fab')
7. W3/13 (anti-pan T-cell)
8. OX-4 (anti-polymorphic Ia)
9. Anti-rabbit IgG
10. No antibody added.

All antibodies were fluorescein-tagged directly except for anti-asialo GM-1 and OX-39. For these two antibodies, the following indirect technique was carried out. Cells tagged with these antibodies were resuspended in 1 ml of FTA buffer and centrifuged for 1.5 m at 1,000 x g. The cells were resuspended in residual buffer and 5 µl of a second, fluorescein-conjugated antibody and 25 µl of fetal bovine serum were added. The second antibody for anti-asialo GM-1 was anti-rabbit IgG and for OX-39 was anti-mouse IgG. Incubation for these samples was at 4°C for 25 m.

At this point, the following procedure was carried out for all cells tagged with all of the antibodies. One ml of lysing solution (8.26 g ammonium chloride, 1.00 g potassium bicarbonate, 37 mg of tetrasodium EDTA, brought to 1 l with distilled water at pH 7.4) was added to each sample and the cells were allowed to incubate at room temperature for 6 min to lyse erythrocytes. The cells were then centrifuged at 1,000 x g for 1.5 min, and then resuspended in 1 ml of FTA buffer. Cells were again centrifuged and fixed by resuspending in 0.5 ml of 1% paraformaldehyde. The cells were then placed at 4°C and transported to the United States.

The cells were analyzed upon return to the United States for fluorescence, an indicator of presence of the antigen, using a FACSCAN flow cytometer (Becton-Dickinson Cytometry

Systems, Mountain View, CA) with Consort 30 software. Lymphocytic and myelogenous regions were gated on three-part differentials using forward vs. side scatter plots. Negative gates were set using unstained samples for direct stains and second antibody as the negative control for indirect stains. Fluorescein isothiocyanate fluorescence greater than 530 nm was detected using the 488 nm line of an argon ion laser for excitation.

**Statistical Analysis.** A Student's T test was carried out for statistical analysis of the CSF-GM data, and an ANOVA using transformed data was carried out for statistical analysis of the cell surface antigenic marker data. Alpha was set *a priori* at  $P \leq 0.05$ .

## RESULTS

### **Effect of space flight and suspension on the response of bone marrow cells to GM-CSF**

At the time of commencement of the culture, there were no differences in viability as determined by trypan blue viability staining or in percent viable yield of cells (Percent viable cells x number of cells) among any of the groups. Bone marrow cells from flown rats have a reduced capacity to respond to CSF-GM compared to bone marrow cells from vivarium control rats (Table 1). Cells from synchronous control rats showed some degree of decreased responsiveness compared to cells from vivarium control rats; however, the reduction in responsiveness of cells from flight rats was greater than that of cells from synchronous control rats (Table 1). Cells from suspended rats also showed a pattern of reduced response to GM-CSF as did cells from flown rats (Table 1).

### **Effect of space flight on the percentage of cells expressing cell surface antigenic markers**

For spleen cells, higher percentages of cells staining with antibodies directed against helper T-cell antigenic markers (W3/25) and suppressor-cytotoxic T-cell antigenic markers were observed from flight animals (Table 2). These changes were beyond increases observed in synchronous controls compared to vivarium controls. For OX-1, the pan-leukocyte marker, there was a statistically significant difference in values from flight animals compared to those from synchronous control animals, but the change is very small and not likely to be biologically meaningful. There were some small, but statistically significant, differences in levels of unstained cells or anti-rabbit IgG stained cells after flight or synchronous control or suspension treatment. These small changes did not affect our results with other markers as

these differences were subtracted (gated) from appropriate experimental values during the flow cytometric analysis (see Materials and Methods). Cells from suspended animals showed a pattern of staining different from that of cells from the flown rats (Table 2). No other changes in expression of antigenic surface markers were observed.

For bone marrow cells, lymphocytic and myelogenous cell populations were analyzed differentially as described in Materials and Methods. In the lymphoid cell population, increases in the percentages of cells staining with antibodies directed against asialo GM-1 markers, helper T-cell markers (W3/25), pan-T-cell markers (W3/13) and interleukin-2 receptors (OX-39) increased after flight compared to both synchronous and vivarium controls (Table 3). Cells from suspended animals produced a different pattern of markers compared to cells from flight animals (Table 3).

For the myelogenous population of bone marrow cells, the population of cells staining with antibodies directed against the helper T-cell marker (W3/25) was increased compared to both synchronous and vivarium controls (Table 4). Cells from suspended animals produced a different pattern of markers compared to cells from flight animals (Table 4).

## DISCUSSION

The results of the current study again show that space flight can profoundly affect immune responses. This supports several previous studies (1,6,10-12,18-21) as well as our findings on the previous Cosmos 1887 flight (17). This study has allowed an attempt at confirmation of a previous study and an attempt at validation of the suspension model for the effects of space flight on immune responses.

In the present series of experiments, the ability of bone marrow cells from rats exposed to space flight to respond to GM-CSF was severely compromised compared to vivarium controls. Cells from synchronous controls showed some inhibition of responsiveness to CSF-GM compared to cells from vivarium controls, but this decrease in responsiveness was not sufficient to account for the severe inhibition seen in cells from the flight animals. The cells from the synchronous control animals may have shown reduced responsiveness to GM-CSF because of stress factors induced by the synchronous treatment. Since viabilities and yields of the bone marrow cells were not different in any group, it is unlikely that alterations in viability contributed to the results observed. This result is consistent with our studies carried out during the Cosmos 1887 flight, which showed that bone marrow cells from flight animals were compromised in their ability to respond to CSF-M (17). It extends the previous finding to demonstrate that the response to a recombinant DNA-derived cytokine with broader biological activity affecting both monocyte and granulocyte cell populations in the bone marrow (GM-CSF) is also compromised by space flight.

The results of the leukocyte phenotyping experiments involving the determination of the percentage of cells stained by an antibody directed against cell surface antigenic markers also confirm earlier reports indicating that the distribution of these cell populations is altered

by space flight (11,17). In the current series of experiments spleen cells from flown rats showed increased percentages of cells staining with the marker carried by helper-T and suppressor-cytotoxic-T cells. This is similar to results observed after the Cosmos 1887 flight (17). The proportion of cells staining with the marker for interleukin-2 receptor bearing cells did not increase after this flight, as they did after the Cosmos 1887 flight, indicating that differences in flight conditions could affect the nature and range of the immunological changes induced. In the Cosmos 1887 flight, the animals remained on the ground for two days prior to sacrifice and analysis of cell populations (17).

The proportion of cells staining with antibodies directed against certain cell surface markers (e.g. W3/25, OX8) appear to be higher than expected in control animals. This may be due to the antibodies not being totally specific to the antigens being tested (e.g. helper T-cells, suppressor T-cells). In any case, alterations in the population(s) of cells carrying the markers do occur after space flight, indicating that space flight does alter the distribution of leukocyte sub-populations.

The results of the bone marrow staining with antibodies were also of interest. For this experiment, due to availability of additional bone marrow cells, we were able to stain the bone marrow cells with the full repertoire of antibodies. This was not possible after the Cosmos 1887 flight (17). In the bone marrow myelogenous cell population, the percentage of cells staining with the helper T-cell marker was increased after flight, but the percentage of cells staining with the marker for Ig+ was not increased as it was after the Cosmos 1887 flight. In the bone marrow lymphocytic cell population, the percentage of cells staining with antibodies directed against asialo GM-1, interleukin-2 receptor, pan-T, and helper T-cell antigens was increased after flight. Although results were not identical with those observed after Cosmos 1887 (17), they were similar. The results of both flight studies suggest that immunological parameters are altered after space flight.

The GM-CSF and leukocyte phenotyping experimental results, along with those obtained from the previous Cosmos 1887 flight (17), could provide an interesting scenario for the effects of space flight on immune responses. Alterations induced by space flight on precursor populations in the bone marrow as evidenced by changes in leukocyte phenotypes in the myelogenous population, could render these cells non-responsive to their normal stimuli after return to earth. Therefore, GM-CSF might not be able to stimulate bone marrow cells from the flight animals because of a defect induced in the cells during space flight. Further support for this possibility will require additional flight studies.

The use of a suspended rat control run in parallel with flight animals allowed a first-time direct comparison of the effects of flight and the effects of suspension on immunological parameters. It appeared that the effects of suspension and spaceflight were similar with regard to GM-CSF reactivity of bone marrow cells. There was, however, no agreement between space flight effects and suspension effects on the distribution of leukocyte subsets. This is consistent with our previous findings that suspension is useful for modeling effects of space flight of functional immune responses (7,14), but not adequate for modeling the effects of space flight on the distribution of cell populations (Berry, Murphy, Taylor, Smith, and Sonnenfeld, manuscript in preparation).

The current study presents additional data to confirm that space flight affects certain parameters of the immune system. This study has several advantages over our previous Cosmos 1887 study. There was no delay in sacrifice of the animals after return to earth, decreasing the possibility of re-adaptation to earth gravity playing a role in our results. In addition, our controls on the antibody staining of spleen and bone marrow cells were very clear and definitive for experiments carried out on this flight, adding confidence to the interpretation of our data. The availability of additional cells allowed for expansion of the CSF-GM and leukocyte phenotyping studies.

The data from the suspension study are also of interest. The ability to carry out the same experiment in parallel using cells from flown and suspended animals is helping to determine the advantages and limitations of this model in simulating the effects of space flight on the immune response,

The results of the current study suggest and confirm that several profound alterations in parameters that play important roles in the regulation of immune responses occur as a result of space flight. These results support the previous studies, and suggest that the effects of space flight on immune responses and resistance to infection should be an area of continued and expanded study.

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TABLE 1

## EFFECT OF SPACE FLIGHT ON THE RESPONSE OF BONE MARROW CELLS TO GM-CSF

Mean Number of Colonies ( $\pm$  Standard Error) in 10 Microscope Fields¶

<u>Flight</u>	<u>Synchronous</u>	<u>Vivarium</u>	<u>Suspension</u>
1.3 $\pm$ 0.4*	7.4 $\pm$ 0.9*	24.0 $\pm$ 15.3	12.4 $\pm$ 3.6*

¶ Mean of 10 replicate cell cultures of each of 5 rats

\* Statistically significant difference from vivarium control

TABLE 2

EFFECT OF SPACE FLIGHT ON THE PERCENTAGE OF SPLEEN CELLS EXPRESSING  
CELL SURFACE MARKERS

Antibody	<u>Mean ± Standard Deviation of % of Cells Expressing Marker†</u>			
	<u>Flight</u>	<u>Synchronous</u>	<u>Vivarium</u>	<u>Suspension</u>
No antibody added	2.7 ± 1.2*	1.1 ± 1.0	0.6 ± 1.4	1.0 ± 1.0
OX-1(pan-leukocyte)	95.6 ± 4.6*	96.5 ± 4.6	98.4 ± 0.9	98.4 ± 1.2
W3/13 (pan T-cell)	52.0 ± 20.6	30.5 ± 5.8	41.0 ± 7.1	39.8 ± 8.2
W3/25 (helper T-cell)	71.6 ± 6.2*	44.1 ± 6.5	49.4 ± 3.4	56.2 ± 3.8
OX-8 (suppressor T-cell)	58.4 ± 17.8*	39.3 ± 10.9	45.8 ± 4.0	44.6 ± 5.4
OX-4 (Ia)	40.7 ± 7.2	36.1 ± 9.9	41.8 ± 2.1	64.2 ± 9.6*
OX-39 (interleukin-2 recep.)	28.5 ± 4.9	21.3 ± 2.6	19.8 ± 2.1	48.7 ± 9.6*
Anti-IgG Fab'	60.3 ± 7.6	51.9 ± 3.5	62.4 ± 5.7	65.9 ± 8.0
Anti-Asialo GM-1 (NK cell)	31.7 ± 4.7	34.7 ± 12.5	18.9 ± 0.8	53.7 ± 12.2
Anti-rabbit IgG	0.8 ± 2.9*	0.4 ± 2.7*	1.9 ± 2.1	0.1 ± 1.4*

† Mean of five different rats

\* Statistically significant difference from vivarium control

TABLE 3

EFFECT OF SPACE FLIGHT ON THE PERCENTAGE OF THE LYMPHOCYTIC POPULATION  
OF BONE MARROW CELLS EXPRESSING CELL SURFACE MARKERS

Antibody	<u>Mean ± Standard Deviation of % of Cells Expressing Marker</u>			
	<u>Flight</u>	<u>Synchronous</u>	<u>Vivarium</u>	<u>Suspension</u>
No antibody added	0.6 ± 1.0	1.7 ± 0.9*	0.5 ± 1.0	0.8 ± 1.3
OX-1 (pan-leukocyte)	75.0 ± 13.3	80.2 ± 0.5	82.6 ± 0.8	82.3 ± 0.4
W3/13 (pan T-cell)	75.2 ± 5.8*	47.9 ± 17.2*	26.8 ± 9.7	30.6 ± 1.4
W3/25 (helper T-cell)	72.0 ± 4.5*	36.3 ± 6.0	33.5 ± 14.3	32.1 ± 1.5
OX-8 (suppressor T-cell)	54.8 ± 16.3	57.0 ± 2.9	51.0 ± 17.5	43.9 ± 16.0
OX-4 (Ia)	48.1 ± 17.8*	39.2 ± 8.1	27.6 ± 3.1	40.6 ± 3.7
OX-39 (interleukin-2 recep.)	36.8 ± 5.1*	25.1 ± 7.1	20.7 ± 7.2	27.7 ± 2.0
Anti-IgG Fab'	49.3 ± 17.5	51.8 ± 13.3	44.2 ± 2.6	68.1 ± 12.3*
Anti-Asialo GM-1 (NK cell)	37.2 ± 2.9*	25.3 ± 9.1*	17.2 ± 5.0	29.8 ± 2.8*
Anti-rabbit IgG	13.6 ± 1.9*	4.0 ± 2.8	5.4 ± 4.9	3.7 ± 2.7

¶ Mean of five different rats

\* Statistically significant difference from vivarium control

TABLE 4

EFFECT OF SPACE FLIGHT ON THE PERCENTAGE OF THE MYELOGENOUS  
POPULATION OF BONE MARROW CELLS EXPRESSING CELL SURFACE MARKERS

Antibody	Mean $\pm$ Standard Deviation of % of Cells Expressing Marker $\ddagger$			
	Flight	Synchronous	Vivarium	Suspension
No antibody added	1.2 $\pm$ 1.0	1.6 $\pm$ 1.6	1.1 $\pm$ 1.4	0.9 $\pm$ 0.8
OX-1 (pan-leukocyte)	97.4 $\pm$ 2.5	97.4 $\pm$ 1.3	97.5 $\pm$ 2.1	98.3 $\pm$ 1.5
W3/13 (pan T-cell)	85.0 $\pm$ 11.3*	78.4 $\pm$ 3.2	71.1 $\pm$ 1.6	77.1 $\pm$ 1.6
W3/25 (helper T-cell)	69.4 $\pm$ 14.7*	31.4 $\pm$ 5.9	38.1 $\pm$ 12.9	35.7 $\pm$ 7.9
OX-8 (suppressor T-cell)	23.8 $\pm$ 13.0	16.6 $\pm$ 3.0	16.5 $\pm$ 5.7	22.3 $\pm$ 5.7
OX-4 (Ia)	31.6 $\pm$ 12.8	22.0 $\pm$ 13.5	6.2 $\pm$ 3.9	13.2 $\pm$ 4.8
OX-39 (interleukin-2 recep.)	67.2 $\pm$ 23.7	55.6 $\pm$ 12.1	62.5 $\pm$ 13.0	46.3 $\pm$ 6.9
Anti IgG Fab'	26.2 $\pm$ 30.0	32.0 $\pm$ 18.7	22.1 $\pm$ 9.1	54.3 $\pm$ 19.6
Anti-Asialo GM1 (NK cell)	87.2 $\pm$ 6.2	75.0 $\pm$ 10.6	71.8 $\pm$ 10.4	58.6 $\pm$ 2.8
Anti-rabbit IgG	3.0 $\pm$ 2.8	2.0 $\pm$ 3.5*	4.5 $\pm$ 2.9	0.7 $\pm$ 1.3*

$\ddagger$  Mean of five different rats

\* Statistically significant difference from vivarium control

## EFFECT OF SPACE FLIGHT ON NATURAL KILLER ACTIVITY

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## ABSTRACT

The effects of space flight on Soviet biosatellite number 2044 of rat immune cell function were determined. Control groups included vivarium, synchronous and antiorthostatically suspended rats. The effects of flight on the ability of natural killer cells to lyse two different target cell lines was determined. Spleen and bone marrow cells obtained from flight rats showed significantly inhibited cytotoxicity for YAC-1 target cells compared to cells from synchronous control rats. This could be due to exposure of the rats to microgravity. Antiorthostatic suspension did not affect the level of cytotoxicity from spleen cells of suspended rats for YAC-1 cells. On the other hand, cells from rats flown in space showed no significant differences from vivarium and synchronous control rats in cytotoxicity for K 562 target cells. Binding of natural killer cells to K-562 target cells was unaffected by space flight. Antiorthostatic suspension resulted in higher levels of cytotoxicity from spleen cells for  $^{51}\text{Cr}$ -labelled K 562 cells. The results indicate differential effects of space flight on function of natural killer cells.

INDEX TERMS: Microgravity, natural killer cells

## INTRODUCTION

Immune responses have been shown to be altered after space flight (1,3-6,11,14,15,19-23). Among the immune responses that have been altered have been the activity of natural killer cells against tumor cell targets (12). Natural killer cells are an important defense mechanism against viral infections and tumors (10). The compromised natural killer cell activity after space flight could lead to altered resistance; however, the nature of the effects of space flight on natural killer cell activity has not been characterized fully.

The current study was carried out to determine the full range of effects of space flight on natural killer cell activity. In addition, an antiorthostatic suspension study was included to allow comparison of the effects of suspension on natural killer activity with the effects of flight. This allowed direct comparison of flight effects with suspension effects, permitting a test of the validity of the model.

In order to carry out this study, two different target cell lines were used. The first, YAC-1 is a murine T-cell lymphoma. The second, K 562 is a human chronic myelogenous leukemia line. These are standard targets for natural killer cells. The experiments were designed to determine if splenocytes and bone marrow cells isolated from rats flown in space differed in their ability to lyse and bind the individual target cell lines compared to synchronous and vivarium ground controls. Additional experiments included the determination of the ability of spleen and bone marrow cells and from rats suspended antiorthostatically to lyse and bind the target cells lines, and a comparison of the effects of space flight with the effects of suspension.

## MATERIALS AND METHODS

**Animals and space flight conditions.** Ten male specific pathogen free rats of Czechoslovakia-Wistar origin (Institute of Endocrinology, Bratislava Czechoslovakia) were flown on the Cosmos 2044 Biosputnik flight for 14 days. Flight, housing, feeding, recovery, age, weight and sacrifice conditions were as described in the overview paper in this issue of the **Journal of Applied Physiology** (9). Tissue from rats number 6-10 were used in our project.

**Harvest of tissues.** After sacrifice of the rats, bone marrow cells were extruded with a needle and syringe from the left femur of each rat using RPMI-1640 medium (Flow Laboratories, United Kingdom) supplemented with 10% fetal bovine serum, antibiotics (penicillin - 100 units/ml, streptomycin, 100 µg/ml, gentamicin sulfate - 50 µg/ml) and 2mM glutamine. One-third of the spleen of each of five rats was dissociated into individual cells and placed into supplemented RPMI-1640 medium. All of the samples were placed into transporter vials, held at 4°C, and transported to Moscow (19). Samples reached the laboratory and analytical work began approximately 24 hr after removal of the tissue from the rats, within 32 hrs of landing.

Synchronous control, vivarium control, and antiorthostatically tail-suspended rats were treated as described in the overview paper in this issue of the **Journal of Applied Physiology** (9). Tissues were removed and treated as described for the flight tissue. In addition, a normal rat was sacrificed and tissue harvested and analyzed to serve as an internal control for our assay procedures

**Natural killer (NK) cell assays.** YAC-1 or K 562 target cells were maintained in supplemented RPMI-1640 medium (Flow Laboratories, United Kingdom) with 10% fetal

bovine serum. Three types of natural killer cell assays were then carried out to fully explore possible flight-induced alterations in natural killer cell activity.

For the first assay, on the day of the assay approximately  $5 \times 10^6$  target cells were labeled for 60 min with 10  $\mu\text{Ci}$  of  $^3\text{H}$ -uridine in 3 ml of supplemented RPMI-1640 medium with 20 mM HEPES buffer added (complete medium). The cells were washed three times to remove exogenous  $^3\text{H}$ -uridine and diluted to a final concentration of  $1 \times 10^5$  target cells/ml of complete medium supplemented with 1mg/ml of bovine pancreas ribonuclease (17). One-tenth ml of the target cells were placed in wells of round-bottomed 96-well plastic microtiter plates. Spleen and bone marrow cells were first pretreated at  $37^\circ\text{C}$  with 2  $\mu\text{g/ml}$  of actinomycin-D. Sixty minutes later, these effector cells were washed three times, diluted to a final concentration of  $2 \times 10^6$  cells in 1 ml of complete RPMI-1640 medium, and 0.1 ml was added to each well. Thus,  $1 \times 10^4$  target cells were co-cultured with  $2 \times 10^5$  effector cells in a total volume of 0.2 ml/well. The cells were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  for 14 hours. At that time, the level of radioactive counts was determined using a liquid scintillation counter. The formula used to calculate cytotoxic index was

$$\text{cytotoxic index} = \left( 1 - \frac{\text{Experimental CPM}}{\text{Control CPM}} \right) \times 100$$

For the second assay, on the day of the assay, the target cells were labeled for 70 minutes with 200  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  (Amersham Searle) and washed three times to remove exogenous  $^{51}\text{Cr}$ . The target cells were then plated in 96 well tissue culture dishes at a concentration of  $1 \times 10^4$  cells per well. Spleen cells isolated from rats in each of the various groups were added to different wells to allow final effector:target ratios of 100:1, 50:1, and 25:1 (10). Control wells containing only labeled target cells were also prepared to test for spontaneous  $^{51}\text{Cr}$  release. The plates were centrifuged at 600 rpm for 4 min and then incubated for 4 hr at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . After incubation, the supernatant fluids were harvested

and counted using a gamma counter. The mean percent specific cytotoxicity was calculated as follows:

$$\text{mean \% specific cytotoxicity} = \frac{^{51}\text{[Cr]} \text{ cpm, Experimental-Spontaneous (medium)}}{\text{Maximum (H}_2\text{O) - Spontaneous}} \times 100$$

For the third type of assay, the single cell assay, agarose was prepared by melting 10 ml of agarose (0.7% in RPMI-1640 medium with HEPES buffer) in boiling water and then cooling to 40°C in a water bath prior to adding the cells. Effector and target cell (K-562 cells only) conjugates were formed by mixing equal numbers of each cell type in a total volume of 0.2 ml of RPMI-1640 medium with 10% fetal bovine serum in "V" shaped 8 ml centrifuge tubes. The cells were kept in a 37°C water bath for 8 min and spun at 1,000 rpm for 5 min. After addition of agarose, the cells were resuspended gently and placed into 24-well tissue culture plates (total volume of one well was 2 ml). After the agarose solidified, 0.5 ml of RPMI-1640 medium with 10% fetal bovine serum was added. Plates were incubated at 37°C in 5% CO<sub>2</sub> for 4 hrs. At the termination of the incubation, the medium was aspirated and 1.5 ml of trypan blue dye were added for 5 min. The agarose was next washed 3 times with medium. The number of cell conjugates was counted microscopically after addition of 2.5% formaldehyde. The percentage of lymphocytes forming conjugates was determined by counting the number of natural killer cells bound to target cells out of a population of 800 counted natural killer cells.

**Statistical analysis.** A Student's T test was carried out for statistical analysis of the radioactive incorporation data. The data obtained from the single cell assay were analyzed by means of the Mann-Whitney "U" test.

## RESULTS

### **Effect of space flight and suspension on natural killer cell activity.**

Spleen and bone marrow cells from flown, suspended and control rats were tested for natural killer cell activity against the two target lines, YAC-1 and K-562. Spleen cells from flight rats showed a decreased natural killer cell activity against YAC-1 target cells labeled with  $^3\text{H}$ -uridine or  $^{51}\text{Cr}$  compared to spleen cells from synchronous control rats (Tables 1 and 2). When the natural killer cell activity of spleen cells against K-562 cells was determined, there was no effect of space flight on killing of either  $^3\text{H}$ -uridine or  $^{51}\text{Cr}$ -labelled cells (Tables 3 and 4). There was no difference in natural killer activity of spleen cells from synchronous control and vivarium control rats.

An analysis of natural killer cell activity from bone marrow cells against YAC-1 target cells labelled with  $^3\text{H}$ -uridine also pointed to a significant decrease in the flight group activity compared to the activity of synchronous control rats (Table 1). When K-562 labeled with  $^3\text{H}$ uridine were tested for natural killer cell activity, there was no inhibition of activity in cells from flight rats compared to cells from synchronous control rats (Table 3). There was no difference in natural killer cell activity of spleen cells from synchronous control and vivarium control rats.

There was no difference in the binding of the natural killer cells from spleen and bone marrow from flight and synchronous control rats to K-562 target cells (Table 5). Binding of cells from these two groups, however, was decreased when compared to binding of cells from the vivarium control rats (Table 5).

Spleen and bone marrow cells from suspended rats showed no difference in natural

killer cell activity using  $^3\text{H}$ -uridine labelled target cells (Tables 1 and 3). When  $^{51}\text{Cr}$ -labelled K-562 target cells were used, increased activity of splenic natural killer cells was observed compared to synchronous and vivarium controls (Table 4). No other differences were noted (Table 3).

## DISCUSSION

The results of the present study indicate that natural killer cell activity can be affected profoundly by space flight. This confirms the results of previous studies on humans (11,12) and Cosmos 1887 studies on rats (13).

An environment similar to that aboard the biosputnik space craft, but without microgravity and radiation found in space, was created on the ground for the synchronous control animals. Flight studies have suggested that the effects of radiation are minimal on natural killer cells (11-13). Therefore, the synchronous control played a major role in evaluating the role of microgravity in the effects of the space flight on natural killer cell responses. In the current study, a statistically significant inhibition of natural killer cell activity of bone marrow and spleen cells against some targets was observed after flight.. The space capsule environment for rats, with its isolated and unfamiliar surroundings, may induce stress for the rats whether in flight or on the ground. Therefore, it is likely that the stress of being in the capsule environment was a major contributory factor for the decreased ability of natural killer cells from both the flight and synchronous control rats to bind and form conjugates with target cells. On the other hand, it is less likely that stress, and more likely that microgravity, played some role in the decreased ability of natural killer cells to kill target cells observed in cells obtained from the flight rats but not from the synchronous control rats.

The current studies raise some other interesting new issues. The results of the present experiments indicate differential effects of space flight on natural killer cell activity based on the target cell. Space flight inhibited rat natural killer cell activity against YAC-1 target cells, but not against K 562 target cells. This may be due to differences in the actual target cell and the sensitivity of each target cell to rat natural killer cells. Another possibility is that there are

sub-populations of natural killer cells responsible for cytotoxicity against each type of target cell with different sensitivities to space flight (10).

The explanation for this differential effect of space flight remains to be established, nevertheless, the current finding is an important one. It supports previous findings with cytokines (6) and leukocyte sub-population distribution (19) that there are differential effects of space flight on immunological parameters. Not all immunological parameters are affected equally by space flight.

Another interesting result of the current study derives from the antiorthostatic suspension control. Previous studies have indicated that antiorthostatic suspension is a useful model for the effects of weightlessness on some functional immune responses such as cytokine production (16,18) and resistance to viral infection (7). However, this is the first study that has permitted a simultaneous comparison of space flight with antiorthostatic suspension using matched animals. The results of the current study suggest that antiorthostatic suspension is not a good model for studying the effects of microgravity on natural killer cells activity. The effects of antiorthostatic suspension did not match those of space flight for natural killer cell activity against either target cell. Comparisons of antiorthostatic suspension and space flight for other functional immunological parameters, such as interferon production (16,18) and the ability to respond to colony stimulating factor (Sonnenfeld, *et al.*, this volume) have indicated it is a useful model for those parameters. Therefore, antiorthostatic suspension has proven to be a useful model for the study of some immunological parameters, but not for others.

The current study supports previous results indicating that space flight induces serious alterations in certain immunological parameters. The full nature of these changes and their significance regard to resistance to infection remain to be established.

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TABLE 1

EFFECT OF SPACE FLIGHT ON NATURAL KILLER CELL ACTIVITY AGAINST  $^3\text{H}$ -URIDINE  
LABELLED YAC-1 TARGET CELLS

Effector:	<u>Mean Cytotoxic Index <math>\pm</math> Standard Deviation</u>				<u>P</u>			
Target Ratio								
and Source	<u>1. Flight</u>	<u>2. Synchronous</u>	<u>3. Vivarium</u>	<u>4. Suspension</u>	<u>1 vs 2</u>	<u>1 vs 3</u>	<u>1 vs 4</u>	<u>2 vs 3</u>
20:1 Spleen	53 $\pm$ 4.1	81 $\pm$ 4.7	74 $\pm$ 2.8	74 $\pm$ 1.2	<0.001	<0.001	<0.001	<0.05
20:1 Bone								
Marrow	26 $\pm$ 9.9	73 $\pm$ 3.0	64 $\pm$ 3.7	73 $\pm$ 4.8	<0.001	<0.001	<0.001	<0.05

¶ Mean of values from 5 different rats

TABLE 2

EFFECT OF SPACE FLIGHT ON SPLENIC NATURAL KILLER CELL ACTIVITY AGAINST  
<sup>51</sup>[Cr]-LABELLED YAC-1 TARGET CELLS

Effector:	Mean % Lysis ± Standard Deviation¶				P		
	1. Flight	2. Synchronous	3. Vivarium	4. Suspension	1 vs 2	1 vs 3	1 vs 4
100:1	24 ± 2.0	40 ± 0.7	37 ± 1.0	39 ± 3.5	<0.001	<0.001	<0.001
50:1	14 ± 1.5	25 ± 0.9	26 ± 0.9	29 ± 2.3	<0.001	<0.001	<0.001
25:1	7 ± 1.8	16 ± 0.7	16 ± 1.9	14 ± 2.4	<0.001	<0.001	<0.001

¶ Mean of values from 5 different rats

TABLE 3

EFFECT OF SPACE FLIGHT ON NATURAL KILLER CELL ACTIVITY AGAINST  $^3\text{H}$ -URIDINE  
LABELLED K-562 TARGET CELLS

Effector:	Mean Cytotoxic Index $\pm$ Standard Deviation¶				P			
Target Ratio								
and Source	1. Flight	2. Synchronous	3. Vivarium	4. Suspension	1 vs 2	1 vs 3	1 vs 4	2 vs 3
20:1 Spleen	55 $\pm$ 4.3	52 $\pm$ 2.4	51 $\pm$ 4.3	45 $\pm$ 2.0	>0.05	>0.05	<0.05	>0.05
20:1 Bone								
Marrow	61 $\pm$ 1.1	49 $\pm$ 7.9	35 $\pm$ 3.4	41 $\pm$ 1.4	<0.05	<0.001	<0.001	<0.05

¶ Mean of values from 5 different rats

TABLE 4

EFFECT OF SPACE FLIGHT ON SPLENIC NATURAL KILLER CELL ACTIVITY AGAINST  
<sup>51</sup>[CR]-LABELLED K 562 TARGET CELLS

Effector: Target Ratio	Mean % Lysis ± Standard Deviation¶				P		
	1. Flight	2. Synchronous	3. Vivarium	4. Suspension	1 vs 2	1 vs 3	1 vs 4
100:1	11 ± 3.0	14 ± 0.8	13 ± 0.8	26 ± 1.3	>0.050	>0.100	<0.001
50:1	6 ± 1.5	7 ± 0.7	5 ± 0.8	16 ± 1.7	<0.010	>0.050	<0.001
25:1	3 ± 0.8	3 ± 0.7	5 ± 0.7	9 ± 1.4	>0.050	>0.050	<0.010

¶ Mean of values from 5 different rats

TABLE 5

EFFECT OF SPACE FLIGHT ON TARGET-BINDING ABILITY OF SPLEEN AND BONE  
Marrow Cells TO K-562 TARGET CELLS

Effector Cell Source	Mean % Target-Binding Cells¶			P		
	1. Flight	2. Synchronous	3. Vivarium	1 vs 2	1 vs 3	2 vs 3
Spleen	4.4	3.9	12.1	>0.05	<0.05	<0.05
Bone Marrow	4.5	4.7	12.3	>0.05	<0.05	<0.05

¶ Mean of values from 5 different rats