FINAL REPORT

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"SLS-2 Involvement".

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(NASA-CR-199367) SLS-2 INVOLVEMENT
Final Report (Carolinans Medical Center) 27 p

Unclas
The purpose of this study is to support Russian space flight experiments carried out on rats flown aboard Space Shuttle Mission SLS-2. The Russian experiments were designed to determine the effects of space flight on immunological parameters. The Russian experiment included the first in-flight dissection of rodents that allowed the determination of kinetics of when space flight affected immune responses.

The support given the Russians by this laboratory was to carry out assays for immunologically important cytokines that could not readily be carried out in their home laboratories. These included assays of interleukin-1, interleukin-6, interferon-gamma and possibly other cytokines. All data will be made available to the Russian investigator, Dr. Andre Lesnyak of the Institute of Biomedical Problems.
METHODS, RESULTS AND DISCUSSION

In the first six months of this study, we have received supernatant fluids from cells from rats flown aboard the Space Shuttle mission SLS-2. Samples received include supernatants from controls, rats flown in space and dissected in space and rats flown in space and dissected upon return to earth.

Production of interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor-alpha, tumor necrosis factor-beta, interferon-alpha, interferon-gamma was examined. Also, the activity of natural killer cells was examined. The data are included in detailed in the appended tables and manuscript. In summary, changes in immunological parameters were shown to be occurring in cells obtained from in-flight dissections of rats. This is the first time this has been demonstrated. For some immunological parameters, these alterations persisted after flight, but for others there was a return to normal values after flight.

For the first time, it has now been demonstrated that changes in immune response occur during flight, and not just immediately after flight. This could indicate that microgravity could play a role in the alterations in immune responses induced by space flight.
1. Lesnyak, A., Sonnenfeld, G., Avery, L., Konstantinova, I., Rykova, M., Meshkov, D., and Oriova, T.


(Appended to this report).
Spleen and bone marrow cell cultures stimulated with 1μg/ml of ConA and assayed for levels of IL-4.

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**adapt. ctrl and basal samples were mislabelled and therefore were not used.**

** rec.ctrl did not have sample #2, but had two #3, they were labelled 3a and 3b and used.**

** adap. did not have sample #1 **
### SLS-2 BSP Rat Experiment

**Spleen and bone marrow cell cultures stimulated with 1ug/ml of LPS and assayed for levels of IL-6**

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EFFECT OF SLS-2 SPACE FLIGHT ON IMMUNOLOGICAL PARAMETERS OF RATS

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ABSTRACT

During the Space Shuttle SLS-2 mission rats were dissected in space and biosamples were returned to the Earth for analysis. Immunological studies addressed the kinetics of T-lymphocyte proliferative responses, cytotoxic activity of natural killer cells, and cytokine production. Experiments were performed using spleen and bone marrow of rats dissected before flight, during flight, immediately after recovery (R) or 14 days after recovery (R + 14), as well as respective control animals. Each group consisted of 5 Sprague-Dawley male rats. It was demonstrated that T-lymphocyte activity of rats dissected in flight was significantly decreased when compared to the controls. This was observed during 48-, 72- and 96-hour cultivation and stimulation with the following mitogenic stimuli: concavalin A (Con A) 0.1, 1.0, and 10.0 mg/ml, phytohemagglutinin (PHA) 2.5 mg/ml, and interleukin-2 (IL-2) 1 U/ml. The proliferative response returned to normal only in PHA stimulated cultures after an extended 96-hour cultivation. The cell proliferation rate in rats dissected immediately after recovery did not decrease, while that in rats dissected at R+14 increased. The activity of spleen natural killer cells was reduced in response to $^{51}$Cr-labeled target cells during flight (YAC-1 and K-562) and after flight (YAC-1). At R+14, their activity returned to normal. Another technique employed to measure natural cytotoxicity, using $^3$H-uridine labeled target cells and RNase, did not reveal any differences between control and experimental groups. In bone marrow, the activity of natural killer cells did not vary significantly. The production of interleukin-1 (IL-1), IL-2, tumor necrosis factor - alpha (TNF-alpha) and tumor necrosis factor - beta (TNF-beta) in spleen cell cultures of the flight rats was reduced. At R+0, IL-1 and TNF-beta remained lowered, while TNF-alpha was increased. At R+0, interferon-alpha (IFN-alpha) and interferon-gamma (IFN-gamma) were diminished. In summary, cell-mediated immunity in rats was drastically suppressed during flight. The time course
variation of immune parameters after flight suggests that the changes may truly indicate a response of the immune system to space flight conditions that could increase over time.

Space flight, T-lymphocytes, natural killers, immunity parameters, cytokines
INTRODUCTION

It has been demonstrated that exposure of animals and humans to microgravity caused, as a rule, immune alterations detected immediately after flight [3-7, 9, 10, 12, 14, 15]. Immune changes mainly consisted of decreases in the proliferative activity of T-lymphocytes [4-6, 8-10], cytotoxic activity of natural killer cells [6, 9-11], and production of cytokines [2, 6, 9-11]. Spaceflight was also found to have an impact on the distribution of immunocompetent cells [6, 8, 12, 14, 15] and bone marrow cell sensitivity to the colony forming factors [12, 13].

SLS-2 was the first flight in which rats were dissected in orbit and biosamples returned to the Earth for analysis. This made it possible to obtain unique data about cell-mediated immunity of rats during space flight without having to consider landing stresses.

The purpose of this experiment was to assess spaceflight effects on cell-mediated immunity in orbit and the pattern of its recovery after flight.

The objectives of the experiment were:
1. To study spaceflight effects on the kinetics of lymphocyte proliferation,
2. to assess the activity of natural killer cells,
3. to investigate the production of cytokines, i.e., interleukin-1 (IL-1), interleukin-2 (IL-2), interferons- (IFN) alpha and gamma, and tumor necrosis factors- (TNF) alpha and beta.
MATERIALS AND METHODS

Biosamples were obtained from 15 Sprague-Dawley male rats, which weighed approximately 300 g on the dissection day and were flown for 14 days on SLS-2. Twenty ground-based control animals were kept in an environment mocking that in orbit with respect to the diet and water consumption, air temperature and humidity, and light-dark cycle.

The experimental animals formed the following groups:

- Basal control group (BC) 5 rats
- Group dissected in flight (F) 5 rats
- Ground-based control group to F rats (FC) 5 rats
- Flight rats dissected immediately after recovery (R+0) 5 rats
- Ground-based control group to R+0 rats (RC+0) 5 rats
- Flight rats dissected 14 days after recovery (R+14) 5 rats
- Ground-based control group to R+14 rats (RC+14) 5 rats

Spleen and bone marrow cells from the rats were used for our experiments. Cell suspensions were prepared at the dissection site and shipped at +4 °C to NASA Ames Research Center. Arrival time at the Ames Research Center was about 12 hr after termination of the dissections. Immediately upon receipt, cells were cultured. Cell viability measured by trypan blue dye exclusion was not less than 80 - 90%.

Cell suspensions were stored and shipped in McCoy's 5A (Sigma) culture media supplemented with L-glutamine, HEPES-buffer, fetal bovine serum, and antibiotics. Lymphocyte proliferation activity and natural killer cytotoxicity were measured using RPMI-1640 media (Sigma) that also contained L-glutamine, fetal bovine serum, and antibiotics. Cytokine production was assessed on Iskove media (Sigma) containing L-glutamine and antibiotics.

Parameters measured in the experiment.
Proliferative activity of Concavalin A (Sigma) (Con A 0.1, 1.0, and 10.0 mg/ml), phytohemagglutinin (Sigma) (PHA 2.5 mg/ml), and interleukin-2 (Sigma) (IL-2, 1.0 U/ml) stimulated spleen T-cells was measured in terms of DNA synthesis (\(^3\)H-thymidine incorporation during 18 hours) after 48, 72 and 96 hours of cultivation at 37 °C in humidified 5% CO\(_2\) air. The cell concentration was 0.2 \(\times\) 10\(^5\) cells/well of a round-bottom culture plate in each of two arrays. After incubation, cell residues were placed onto filters using a cell harvester, and their radioactivity was measured by means of a liquid scintillation counter. Results were expressed as disintegrations per minute (dpm).

Activity of natural killer cells found in spleen and bone marrow was assayed on YAC-1 and K-562 cultured target cells labeled with \(^{51}\)Cr and \(^3\)H-uridine. The effector: target ratios used were: 100: 1, 50: 1, 25: 1, 12: 1, 6: 1, 3:1, and 1.5: 1 (10,000 target cells per well). In \(^3\)H-uridine tests, effector cells were treated with actinomycin D (Sigma) (5 mg/ml) for 1 hour at 37 °C and the reaction conducted in the presence of ribonuclease A (Sigma) containing culture media to prevent label re-utilization. Cell cultures were incubated for 4 hours in \(^{51}\)Cr tests and for 16 hours in \(^3\)H-uridine tests in humidified 5% CO\(_2\) air at 37 °C. After incubation in cultures containing \(^3\)H-uridine labeled target cells, residues were transferred onto filters using a cell harvester, and their radioactivity was measured by means of a liquid scintillation counter. Results were expressed as a cytotoxicity index equal to \((1 - \text{dpm in experiment} / \text{dpm in control}) \times 100\). In cultures containing \(^{51}\)Cr labeled target cells, the supernatant fluid was collected, and its radioactivity was measured by means of a gamma-counter. Results were expressed as a cytotoxicity index equal to \((\text{dpm in experiment} - \text{dpm in control}) / (\text{maximum dpm in supernatant} - \text{dpm in control})\) \times 100.

Cytokines were assessed in supernatant fluids of spleen and bone marrow cells cultivated in 24-well flat-bottom plates in humidified 5% CO\(_2\) air at 37 °C at a
concentration of $5 \times 10^6$ cells / 1.5 ml/ well. After incubation, supernatant fluids were frozen and stored at -20 °C until testing.

**Interleukin-1 (IL-1).** Spleen and bone marrow cells were incubated for 24 hours in the presence of E. coli lipopolysaccharide (LPS) (Sigma) (1 mg/ml) in humidified 5% CO$_2$ air at 37 °C. Supernatant activity was measured in 72-hour cell cultures of C3H/HeJ murine thymus (2 month old males). Serial supernatant dilutions (two per dilution) were added to the wells of a 96-well plate containing $1.5 \times 10^6$ thymus cells. The reaction was measured in terms of $^3$H-uridine incorporation during the last 18 hours of incubation. Cell residues were transferred onto filters using a cell harvester, and their radioactivity was measured by means of a liquid scintillation counter. Results were compared with standard IL-1 (Interleukin-1 alpha, Cytokine Science Inc.) and expressed as U/ml.

**Interleukin-2 (IL-2).** Spleen cells were incubated for 48 hours in the presence of Con A (1 mg/ml). Interleukin-2 activity was measured in CTLL-2 cell culture for 48 hours in humidified 5% CO$_2$ air at 37 °C. Supernatant fluids were serially diluted (two per dilution). The reaction was measured in terms of $^3$H-uridine incorporation during the last 18 hours of incubation. Cell residues were transferred onto filters using a cell harvester, and their radioactivity was measured by means of a liquid scintillation counter. Results were compared with standard IL-2 (T-cell growth factor, Sigma) and expressed as U/ml.

**Interferon-alpha (IFN-alpha).** Spleen cells were incubated for 24 hours in the presence of poly-I:C (polynosinic-polycytidylic acid, Sigma) (25 mg/ml) and DEAE-dextrane (Sigma) (50 mg/ml).

**Interferon-gamma (IFN-gamma).** Spleen cells were incubated for 48 hours in the presence of Con A (1 mg/ml).

Interferon activity was measured in cultured murine L-cells with respect
to the suppression of the cytopathic effect of murine encephalomyocarditis virus. Supernatant fluids diluted with the test cell culture were incubated for 24 hours in humidified 5% CO₂ air at 37 °C. Results were expressed as ln titer (units/ml).

**Tumor necrosis factor - alpha (TNF-alpha).** Spleen cells were cultivated for 24 hours in the presence of LPS (10 ng/ml) and muramyl dipeptide (MDP, 10 mg/ml) (Adjuvant peptide, Sigma).

**Tumor necrotic factor - beta (TNF-beta).** From a spleen cell suspension, a nonadherent fraction was isolated by incubating the suspension in Petri dishes (60 min) in humidified 5% CO₂ air at 37 °C. Nonadherent cells were cultivated for 48 hours in the presence of Con A (1 mg/ml).

TNF-alpha and TNF-beta activities were measured with respect to their cytopathic effect on L929 cells. Cells were incubated for 14 hours with serially diluted supernatant fluids containing TNF-alpha and TNF-beta; then dead cells were identified, and viable cells were stained with crystal violet. The titer of TNF was the reciprocal last dilution of test sample that decreased viability by 50%. Results were compared with standard TNF-alpha and TNF-beta (Sigma).

The resultant data were treated statistically to determine mean arithmetic (M) and standard error (m). The difference between flight and control groups of rats was evaluated using the Student's t-test (t) and nonparametric Wilcoxon-Mann-Whitney test (U).
RESULTS

Proliferative activity.

Basal controls showed a high level of T-lymphocyte proliferation during different cultivation times and with different mitogen concentrations used.

The proliferative activity of spleen T-lymphocytes sacrificed in flight (Figure 1) was reduced in both unstimulated cultures and in cultures stimulated with various concentrations of Con A, PHA and IL-2 during 48-, 72- and 96-hour incubation. The response returned to the control level only in PHA stimulated cultures cultivated for 96 hours.

In R+0 animals, spontaneous proliferative activity decreased slightly in 48- and 72-hour cultures.

At R+14, 48-hour cultures showed increases in spontaneous proliferative activity and in responses to stimulation with Con A (0.1 and 1.0 mg/ml) and IL-2, when compared to the controls; 72-hour cultures showed a higher response to PHA stimulation; 96-hour cultures had the same level of proliferative activity as controls.

Functional activity of natural killers.

Spleen cells

Test with $^{51}$Cr-labeled target cells.

In cell cultures with YAC-1 (Figure 2) of F and R+0 rats, the functional activity of natural killer cells was decreased in the case of most effector:target ratios used. At R+14, cytotoxic activity was fully recovered. In cell cultures with K-562 (Figure 3) of F rats, cytotoxicity index was significantly decreased at ratios of 1: 100, 1: 50, 1: 12 and 1: 6. No marked differences were seen in R+0 and R+14 groups.

Test with $^3$H-uridine labeled target cells.
In the test with $^3$H-uridine labeled target cells changes were smaller. In cell cultures with YAC-1, the cytotoxicity index was lowered in F rats at a ratio of 1:3 and in R+0 rats at ratios of 1:3 and 1:1.5. The cytotoxicity index was, on the contrary, increased in R+14 rats at a ratio of 1:1.5. In cell cultures with K-562, functional activity was decreased in F rats at a ratio of 1:50 and in R+0 rats at ratios of 1:100 and 1:50. The cytotoxicity index in R+0 rats was increased at a ratio of 1:1.5. At R+14, no changes were detected.

**Bone marrow cells.**

Test with $^{51}$Cr labeled target cells.

In YAC-1 and K-562 cell cultures no significant differences were found between flight and control animals. The only exception was a decreased cytotoxicity index in R+0 rats identified in K-562 cultures at ratios of 1:25 and 1:6.

Test with $^3$H-uridine labeled target cells.

No significant differences were observed with respect to YAC-1 and K-562.

**Cytokines (Table 1).**

**Interleukin-1**

The ability of spleen cells to produce IL-1 was diminished in F and R+0 rats. No differences were seen in R+14 rats. IL-1 activity in supernatant fluids of bone marrow cells was low, being very similar in flight animals and their respective controls.

**Interleukin-2**

The ability of spleen cells to produce IL-2 remained high in basal controls. This parameter was not determined in F rats and was not detectable in their controls. In R+0 and R+14 rats, the parameter did not differ from the controls.
Interferon-alpha.

IFN-alpha activity in supernatant fluids of spleen cell cultures of F and R+0 rats remained very close to that in the controls, and in R+14 rats it was decreased.

Interferon-gamma.

IFN-gamma production by spleen cells was high in basal controls. The parameter was not determined in supernatant fluids of F rats or their controls. The parameter in R+0 rats was the same as in the controls, and in R+14 rats it was lowered.

Tumor necrotic factor - alpha.

TNF-alpha production by spleen cells was decreased in F rats and increased in R+0 rats, when compared to their controls. At R+14, no significant changes were observed.

Tumor necrotic factor - beta.

TNF-beta production by spleen cells was decreased in F and in R+0 rats, when compared to their controls. At R+14, no significant changes were found.

DISCUSSION

Our investigations demonstrated significant changes in immune responses of rats, when tissues were harvested during flight. The rats dissected in orbit showed a marked decline of the spleen lymphocyte capability to proliferate in response to mitogenic stimulation during all cultivation times. The data obtained for the first time ever by in vitro cell cultivation techniques that indicated a significant decrease of the functional activity of T-lymphocytes in space flight could yield difficulties in the formation of a specific immune response. The fact that flight rat cells recovered their capability of responding to one of the mitogens used, i.e. PHA, when the incubation time was increased
to 96 hours, gave evidence that the changes could be transient. This was supported by
the observation that the proliferative activity of spleen stimulated cells of flight rats
dissected at R+0 was very similar to that of controls. Additionally, IL-2 activity was
reduced only in flight rats, which was consistent with a proliferation rate decrease
observed at that time. Changes detected in R+0 and R+14 rats were
in agreement with immunological observations on SLS-1 rats (A. Lesnyak,
G. Sonnenfeld, unpublished data).

Measurement of cytotoxic activity also yielded interesting data. The function of
spleen natural killers was significantly inhibited. The test with $^{51}$Cr- labeled target
cells demonstrated a significant decrease of the cytotoxicity index of F rats in relation to
both YAC-1 and K-562 cells, and that of R+0 rats in relation to YAC-1 cells. The
parameter returned to normal at R+14. The test with $^{3}$H-uridine labeled target cells
showed less substantial changes in spleen natural killers. Bone marrow natural killers
varied insignificantly. Cosmos rat experiments revealed a decreased activity of spleen
natural killers in the test with $^{51}$Cr- labeled target cells [11].

The production of cytokines also varied significantly in space. IL-1 activity in
cultured spleen cells was diminished in F and R+0 rats. TNF-alpha and TNF-beta in
cultured spleen cells was also lowered in F rats. At R+0, TNF-beta remained decreased
while TNF-alpha increased. In F rats, IFN-alpha remained unaltered. At R+0, IFN-
alpha and IFN-gamma production was identical to that in the controls and at R+14 it was
reduced. It should be noted that similar changes in the cytokine production were found in
the SLS-1 rat experiment (A. Lesnyak, G. Sonnenfeld, unpublished data). However,
Cosmos experiments yielded different results [8]. At R+0, IL-1 production was
increased and IL-2 decreased. IFN-gamma activity was also reduced in both Cosmos [8]

In summary, SLS-2 experiments provided new data about spaceflight effects on
immune responses. Proliferative activity and natural cytotoxicity of spleen cells, as
well as IL-1, IL-2, TNF-alpha and TNF-beta production were decreased in orbit. IFN-alpha remained unaltered. After flight most immune parameters returned to normal. At R+0, spontaneous lymphocyte proliferation, natural cytotoxicity in the \( ^{51}\text{Cr}}\)-YAC-1 test, and IL-1 and TNF-beta production remained at a lower level; however, TNF-alpha was increased. At R+14, proliferative activity tended to increase and interferon production to decrease.

No significant changes were found in cultured bone marrow cells. In our opinion, the discrepancy between Cosmos and SLS results can be attributed to differences in flight conditions. Those would include a less stressful SLS landing profile, earlier animal dissection after SLS flight, and differences between Wistar and Sprague-Dawley rat strains. These assumptions require experimental verification.

Of crucial importance is the fact that immunological studies were performed on animals dissected in orbit for the first time ever. Changes seen can be qualified as significant immune alterations. However, immune data can be properly interpreted only in relation to biochemical, morphological and hormonal observations. In spaceflight, the suppression of immunocompetent cell populations was selective rather than total. At R+14, readaptation of the immune system was not yet complete, indicating that 14 days was an insufficient time period for all the immune parameters to return to normal. In any case, it is now clear that immune alterations observed in space can be attributed directly to spaceflight conditions.
ACKNOWLEDGMENT

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CAPTIONS TO FIGURES

Figure 1. $^3$H-thymidine incorporation into mitogen stimulated spleen cell cultures (from left to right: no stimulation, Con A -- 0.1 mg/ml, Con A -- 1.0 mg/ml, Con A -- 10.0 mg/ml, PHA -- 2.5 mg/ml, and IL-2 -- 1 U/ml).

Figure 2. Cytotoxic activity of spleen natural killer cells toward $^{51}$Cr-labeled target cells YAC-1 (from left to right effector: target ratio -- 1: 100, 1: 50, 1: 25, 1: 12, 1: 6, 1: 3, 1: 1.5).

Figure 3. Cytotoxic activity of spleen natural killer cells toward $^{51}$Cr-labeled target cells K-562 (from left to right effector: target ratio -- 1: 100, 1: 50, 1: 25, 1: 12, 1: 6, 1: 3, 1: 1.5).
TABLE 1. Production of cytokines by spleen cells of SLS-2 rats (M± m)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BC</th>
<th>F</th>
<th>FC</th>
<th>R+0</th>
<th>RC+0</th>
<th>R+14</th>
<th>RC+14</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1, U/ml</td>
<td>1.5±0.64</td>
<td>0.8±0.11</td>
<td>1.1±0.14</td>
<td>1.5±0.37</td>
<td>2.8±0.28</td>
<td>2.4±0.52</td>
<td>3.5±0.46</td>
</tr>
<tr>
<td>IL-2, U/ml</td>
<td>53.5±11.04</td>
<td>0.0±0.00</td>
<td>0.6±0.24</td>
<td>33.5±9.57</td>
<td>34.6±8.82</td>
<td>61.2±7.39</td>
<td>52.6±8.99</td>
</tr>
<tr>
<td>IFN-alpha, titer ln</td>
<td>4.17±0.321</td>
<td>4.0±0.410</td>
<td>3.78±0.60</td>
<td>3.75±0.460</td>
<td>3.92±0.470</td>
<td>3.77±0.210</td>
<td>4.92±0.377</td>
</tr>
<tr>
<td>IFN-gamma, titer ln</td>
<td>3.60±0.194</td>
<td>-</td>
<td>-</td>
<td>3.31±0.188</td>
<td>3.47±0.175</td>
<td>3.19±0.192</td>
<td>3.75±0.189</td>
</tr>
<tr>
<td>TNF-alpha, ng/ml</td>
<td>15.2±2.53</td>
<td>6.0±0.90</td>
<td>12.1±3.01</td>
<td>55.4±8.30</td>
<td>24.0±6.52</td>
<td>73.5±33.71</td>
<td>126.9±26.10</td>
</tr>
<tr>
<td>TNF-beta, ng/ml</td>
<td>68.4±11.42</td>
<td>11.0±4.50</td>
<td>32.2±7.83</td>
<td>15.0±2.43</td>
<td>103.4±35.92</td>
<td>135.1±44.7</td>
<td>356.6±20.01</td>
</tr>
</tbody>
</table>

Note: BC -- basal controls;
F and FC -- rats sacrificed on flight day 13 in orbit and on the ground;
R+0 and RC+0 -- flight and control animals dissected immediately after flight;
R+14 and RC+14 -- flight and control animals dissected 14 days after flight.
DPM

Flight group
Control group

48 hrs
72 hrs
96 hrs
Time of incubation