SPACEFLIGHT ASSOCIATED APOPTOSIS

Albert T. Ichiki¹, Linda A. Gibson², and Zuhair Allebban³
University of Tennessee Medical Center at Knoxville
Knoxville, Tennessee, U.S.A.

ABSTRACT

Lymphoid tissues have been shown to atrophy in rats flown on Russian spaceflights. Histological examination indicated evidence for cell degradation. Lymphoid tissues from rats flown on the Spacelab Life Sciences-2 mission were analyzed for apoptosis by evidence of fragmented lymphocytes, which could be engulfed by macrophages, or DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. Apoptosis was not detected in the thymus and spleen collected inflight or from the synchronous ground control rats but was detected in the thymus, spleen and inguinal lymph node of the flight animals on recovery. These results indicate that the apoptosis observed in the lymphatic tissues of the rats on recovery could have been induced by the gravitational stress of reentry, corroborating the findings from the early spaceflight observations.

INTRODUCTION

Apoptosis is a genetically controlled process where cells commit suicide following activation by environmental or developmental stimuli. Apoptosis is essential for maintaining homeostasis and normal development where the elimination of the dying cell is not associated with the leakage of cytoplasmic materials which could induce an inflammatory response. Following the induction of apoptosis, the cell undergoes a series of morphological changes resulting

¹Professor ²Research Associate ³Assistant Professor
Tel: 423 544-9467 Fax: 423 544-9460
E-mail: atichiki@utkux.utcc.utk.edu

in the ultimate fragmentation of the cell. The detection of nuclear DNA fragmentation has been considered to be the hallmark for apoptosis.

Rats flown on early space flights were observed to have marked, but temporary, retrogressive changes involving the atrophy of the thymus and spleen on return to gravity. There was massive accumulation of lymphocyte nuclear debris in the cortex of the thymus. We considered these observations to be suggestive of apoptosis. We were able to probe for evidence of apoptosis in the lymphoid tissues from rats flown on the SLS-2 mission, a 14-day flight. A most momentous event was that we were able to examine the thymus and spleen for apoptosis from rats killed inflight on flight day 13 to compare with the thymus and spleen of the rats killed on recovery and 9 days post landing. The inguinal lymph nodes from rats killed on R+0 and R+9 were also examined. Our evidence for apoptosis was the phagocytosis of apoptotic bodies as observed by histological examination and by DNA fragmentation using the TUNEL assay.

MATERIALS AND METHODS

Six rats were killed inflight on day 13 and the spleens were fixed in 10% neutral buffered formalin (NBF), and the thymus tissues were fixed in Bouin's solution. The tissues from the same number of synchronous ground control rats killed at the same time were obtained and fixed. Ten flight and ten ground control rats were killed on landing and five flight and five ground control rats were killed 9 days post land-
ing. The tissues were weighed and fixed in NBF. The tissues were embedded in paraffin and 5 μm sections were prepared. Sections stained with hematoxylin and eosin were examined by light microscopy.

The DNA strand breaks were labeled with Apoptag™ reagents obtained from ONCOR, Inc., Gaithersburg, MD, after the tissue sections were deparaffinized. The vendor's protocol was followed. Cells positive for the incorporation of dUTP by *in situ* terminal deoxynucleotidyl transferase stained brown. Appropriate controls were included with each run.

**RESULTS**

The weights of the thymus and spleens from the flight animals were less than the synchronous ground control rats, but this was not considered to be statistically significant.

Light microscopic examination of the stained sections from the rats killed in space did not have any differences as compared to the synchronous ground controls. On R+O, there was evidence of lymphocyte fragmentation or apoptotic bodies which were or were not engulfed by the resident macrophages in the cortex of the thymus, white pulp of the spleen, and the inguinal lymph node. These observations showed evidence for morphological changes associated with apoptosis.

DNA fragmentation, detected by TUNEL, occurred only in the tissue sections from the flight rats on R+O. There was no evidence for DNA fragmentation in the thymus and spleens from the rats killed in space nor their synchronous ground controls. Also, there was no evidence for DNA fragmentation in the control rats on R+O or the flight and ground control rats on R+9.

**DISCUSSION**

The apoptosis observed in the lymphoid tissues of the flight rats on R+O indicates the magnitude of all the environmental forces associated with reentry and readaptation to gravity. Mechanical stresses have been shown to affect cell function⁶, so it could be possible that the intensity of the combined forces could be effective in inducing apoptosis in the rats. The corollary to these findings would be that the forces associated with launch and the adaptation to microgravity could also be effective in inducing apoptosis in the rat. Apoptosis is a temporal process where the cells, which were programmed for death, were efficiently cleared away so the process of repair could commence. This clearing process is rapid since we were unable to show any evidence of apoptosis by R+9. This observation agrees with the transient, retrogressive changes in the lymphatic tissues.

It was reported that p53, the gene product of a tumor suppressing gene detected by Western blot, was significantly augmented in the skin of the flight rats on R+O as compared to the synchronous ground control rats⁷. The skin was obtained from the same rats we used in our study. p53 can enhance apoptosis when there is genotoxic damage or if there are DNA strand breaks caused by radiation⁸. If p53 was the modulator of apoptosis in the flight rats, the intensity of the forces associated with reentry and readaptation to gravity must have been of a magnitude to cause DNA strand breaks.

It was further reported that p53 remained in a detectable range in the skin of flight and synchronous ground control rats on R+9 even though apoptosis was not detected in the lymphoid tissues examined. It was unfortunate that p53 was not determined in the skin from rats killed in space. It
would have been possible to correlate the level of p53 expression with the induction of apoptosis.

In summary, apoptosis was detected in lymphoid tissues in rats flown in space at the time of recovery, but not on flight day 13 or 9 days post recovery. We suggest that the intensity of the forces associated with reentry and readaptation to gravity resulted in DNA strand breaks which could initiate p53-dependent apoptosis.


REFERENCES


Lymphatic tissue changes in rats flown on Spacelab Life Sciences-2


Department of Medical Biology, University of Tennessee Medical Center at Knoxville, Knoxville, Tennessee 37920; and Institute for Biomedical Problems, Moscow 123007, Russia

LYMPHATIC TISSUES have been examined from the animals flown on Russian Cosmos orbiting satellites and on National Aeronautics and Space Administration (NASA) shuttle flights. A sample of spleen lymphatic tissue from animals flown on the 9-day Spacelab Life Sciences-1 (SLS-1) mission was examined histologically, whereas, in this report on the 14-day SLS-2 mission, thymus, spleen, and inguinal lymph node were examined. In the SLS-1 mission (1) and our studies from this SLS-2 mission, peripheral blood lymphocytes were counted along with other blood elements. The Russian Cosmos series, very early in spaceflight studies, included morphological examination of lymphatic tissue and peripheral blood lymphocytes as well as many investigations on immune function (6–8).

In NASA and the Russian Cosmos research, nearly all of the studies on lymphatic tissues and peripheral blood lymphocytes took place after recovery. All of the results support the conclusion that marked but temporary retrogressive changes took place in thymus, spleen, lymph node, and peripheral blood lymphocytes on return to Earth's normal gravitational field (1 G). In the present study, we did not observe retrogressive tissue

changes in lymphatic tissues obtained during the in-flight tissue collection on the SLS-2 mission.

The retrogressive change noted by Russian and American investigators was a transient loss of lymphatic tissue and atrophy. This was explained as a reaction to the stresses associated with return to 1 G. The gravitational stress of reentry is the phrase used to explain the morphological and physiological changes (7).

The present results, obtained from SLS-2, confirm earlier work and add new data points for help in describing the gravitational stress of reentry. Furthermore, it was shown in an initial study of the retrogressive changes that the process was one of induced apoptosis. The retrogressive change in lymphatic tissue and reduced lymphocytes in the peripheral blood after exposure to spaceflight could be a marker for the gravitational stress of reentry.

MATERIALS AND METHODS

Animals and experimental groups. The overall plan of the study as it related to lymphatic tissues and bone marrow is shown in Fig. 1 as an outline of the various groups of rats in both flight and ground control circumstances for SLS-2. From a pool of 55 male Sprague-Dawley rats (Taconic Farms, Indianapolis, IN), 30 rats (8–9 wk old) were chosen to become either flight rats or ground controls. The 30 rats were then randomly divided into the flight group (n = 15) or ground controls (n = 15). The flight rats were housed singly in a rodent animal-holding facility. The ground controls were placed in a rodent animal-holding facility ground simulator. Both flight and ground control animals were placed on flight food bars and water ad libitum. Temperature, humidity, and the 12:12-h light-dark cycle were the same for the flight and ground controls. The experimental plan called for the flight and ground control rats to be divided into three groups of five animals (groups A–C). Another group of rats, group D (n = 6), was killed during flight on flight day (FD) 13. Thymus, femur, and spleen were obtained. The number of specimens collected is indicated to the right of the name of each group (Fig. 1). The animals were killed, and tissue collection was started 4–5 h after landing. It took 2–3 h to collect the specimens. On the day of return (R+0), groups B and C and their ground controls were exsanguinated through cardiocentesis and immediately decapitated. One femur and inguinal fat pad with lymph node were removed. The thymus and spleen were removed, weighed, and then sectioned. Group A flight and control rats were held for 9 days after landing. At that time, the same protocol and tissue collection methods were followed as on R+0. All procedures were approved by the Animal Care and Use Committees of both the University of Tennessee Medical Center and NASA Ames Research Center.

Tissue preparation. All tissues were placed in fixative, 10% neutral-buffered Formalin, after collection, except for the FD13 thymus, which was fixed in Bouin's solution and

One of a series of articles that describes research conducted on dedicated life sciences missions flown on the US space shuttle.
obtained from Dr. A. S. Kaplansky. The fixed tissues were
held and shipped at room temperature to the home labora-
tory, where the femur was decalcified by using Baxter's
decalcifying solution (McGaw Park, IL). The tissues were
embedded in paraffin, and 5-μm sections were prepared.
Hematoxylin and eosin staining was performed on all slides.
The slides were examined with light microscopy. Two indepen-
dent microscopists reviewed the slides without reference to
the experimental or control groupings.

In situ terminal deoxynucleotidyltransferase assay. Tissue
sections (5 μm) of thymus, spleen, and inguinal lymph node
of flight and control rats were cut and mounted from previously
prepared paraffin blocks. The tissues were deparaffinized and
subjected to the procedure of labeling DNA strand breaks by
using a deoxynucleotidyltransferase (TdT) commercial kit
(ApopTag, no. S7100, Oncor, Gaithersburg, MD). The protocol
was provided by the vendor. Digoxigenin-conjugated deoxury-
dine 5'-triphosphate (d-dUTP) is incorporated into DNA
strand breaks by exogenous TdT. The incorporated d-dUTP is
then detected with peroxidase-labeled digoxigenin antibod-
ies, followed by the addition of diaminobenzidine. The speci-
mens were then washed with distilled water and counter-
stained with methyl green, dehydrated, mounted, and
examined by using a light microscope. Because of apoptotic
changes, cells will stain brown. Positive and negative control
sections from every block were included with each run.

RESULTS

With the use of light microscopy, the only groups
showing significant tissue changes that varied from the
ground controls were the flight groups B and C on R + 0.
This finding applied to the thymus, splenic white pulp
(the lymphatic tissue component), and to the inguinal
lymph node.

The FD13 tissues (Fig. 2A) and group A tissues of
flight animals examined 9 days after recovery (R + 9)
were comparable to those of ground controls. FD13-
fixed pieces of thymus were part of a separate SLS-2
study of the spaceflight to investigate the stress ques-
tion carried out by Kaplansky and others (for details
see A. S. Kaplansky, I. A. Popova, G. N. Durnova, W.
Hinds, L. M. Kurkina, I. V. Zaboloskaya, and V. N.
Vorobyova, unpublished observations). That study fo-
cused on thymus and adrenal glands. None of the
rather extensive retrogressive changes found on R + 0
was observed in the FD13 and the R + 9 groups.

The retrogressive changes seen in flight animals of
groups B and C killed on R + 0 consisted of a scattered
presence of macrophages filled with densely stained
nuclear bodies throughout the cortex of the thymus
(Fig. 2B). The term “tingible bodies” has been used to
refer to this observation. The degree of change was
variable to some extent from specimen to specimen, but
every thymus in these groups contained this notable
excess of tingible bodies throughout the cortical region.

In the splenic white pulp and the inguinal lymph
node of this same group of flight rats, a similar variable
change was shown. DNA fragmentation was observed in the thymus,
spleen, and lymph node by an in situ incorporation of
d-dUTP with exogenous TdT in the macrophages. This
was a critical criterion for the tingible bodies engulfed
by the macrophages (Fig. 2, A and B).
Fig. 2. A: absence of densely staining nuclear bodies (tingible bodies) in thymus cortex (a, c) and splenic white pulp (e, g) of flight day (FD) 13 flight rats as well as in thymus cortex (b, d) and splenic white pulp (f, h) of comparable ground control animals. Hematoxylin and eosin, magnification ×1,000; methyl green counterstaining for apoptotic bodies, magnification ×400. B: densely staining nuclear bodies (tingible bodies) in macrophages in thymus cortex (a), splenic white pulp (e), and inguinal lymph node (i) of flight rats on R+0 compared with their respective ground controls (b, f, j); hematoxylin and eosin, magnification ×1,000. Brown staining of alkaline phosphatase shows tingible bodies are apoptotic in thymus, spleen, and inguinal lymph node (c, g, k) of R+0 flight rats compared with absence of apoptotic bodies in their respective ground controls (d, h, l); counterstaining with methyl green, magnification ×400.
The weight of the thymus at the time of recovery (R+0) in the pooled group of 10 rats from flight groups B and C (Fig. 1) did not differ significantly from the weight of the thymus in the ground control animals (Fig. 3). However, the mean thymus weight was ~70 mg less in the flight animals compared with the ground control population. The difference was significant at the 0.1 level (Table 1). Similarly, 9 days after recovery (R+9 in Fig. 1), the mean thymus weight of the flight rats was ~64 mg less than that of the ground control groups. The difference, however, was not statistically significant (Table 1). Flight rats had a period of 8 h or so after recovery when movement was difficult because of transient muscle weakness to access the usual food and water sources. However, this was not great enough to account for a mean drop in body weight through ~5 days after recovery (Fig. 4).

Spleen weight of the pooled flight animals (groups B and C) was also less on the day of recovery than that of the comparable pooled ground control group (Fig. 3). The difference was ~98 mg. The reduced weight was significantly less at the 0.01 level (Table 1). On R+9, spleen weights of the flight animals were still ~30 mg less on average than the mean for its ground control group. The smaller mean spleen weight was not statistically significant (Table 1). Here, too, the transient body weight loss in the flight group could explain the slight reduction in mean spleen weight.

**Table 1. Normalized thymus and spleen weight in flight and control groups**

<table>
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<tr>
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<th>Normalized Thymus Wt, g</th>
<th>Normalized Spleen Wt, g</th>
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<tr>
<td></td>
<td>Flight</td>
<td>Control</td>
</tr>
<tr>
<td>R + 0</td>
<td>0.1996*</td>
<td>0.2261</td>
</tr>
<tr>
<td>R + 9</td>
<td>0.1543‡</td>
<td>0.1668</td>
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*Significant at 0.1 level (P = 0.0812); †significant at 0.01 level (P = 0.0089); ‡not statistically significant. R + 0, day of return.

The head of one femur and proximal end of the shaft were removed for examination by light microscopy in all the groups, as indicated in Fig. 1. All of the flight specimens on FD13, R+0, and R+9 were comparable to their equivalent ground controls.

**DISCUSSION**

In the two NASA missions, SLS-1 and SLS-2, lymphatic tissues were obtained for examination on R+0 and at 9 days after landing. SLS-1 was a 9-day mission, and SLS-2 was a 14-day mission. The extensive studies in the Cosmos series on lymphatic tissue included R+0 and 2, 6, and 22 days later for histological examination of tissue by light microscopy and a variety of tests (8). The flight duration of the Cosmos series varied from 5 to 22 days in microgravity. The NASA SLS-1 study was limited to a single cross section of the spleen. On reexamination, the SLS-1 flight rats on R+0 did show slight differences from the ground control group in the thymus-dependent area of the splenic white pulp. There was an increase in macrophages containing more than one or two tingible bodies such as one might see in a ground control animal. Tingible bodies in macrophages in the germinal centers, when present in spleen lymphatic tissue, were not considered different in flight and control animals on either SLS-1 or SLS-2. The germinal centers are quite variable in numbers and may not be present in a given specimen.

Both SLS-1 and SLS-2 animals had a transient reduction in peripheral blood lymphocytes on R+0. The values returned to normal levels by R+1 on SLS-1 (1) and R+2 in this study (data not shown). These observations fit well with transient retrogressive changes in lymphatic tissues seen on SLS-2.

Konstantinova and Fuchs (8) summarized the anatomical changes in rats flown in the Cosmos series and the physiological interpretations of the Russian investigators. In general, the animal studies supported the studies on cosmonauts. The morphological studies of
Durnova et al. (5, 6), Kaplansky et al. (7), and others between 1976 and 1986 of lymphatic tissues showed the transient regressive changes characteristic of acute stress within a few hours of recovery. They concluded that acute stress developed on landing and was not present earlier (7). Accelerations and shock overloads were not, in themselves, the cause of the acute stress-related changes in spleen and thymus of the flight animals. Duration of flight was not a factor, although the regressive change was less pronounced in a 7-day microgravity experiment compared with ones of longer duration. The reaction to the shift from weightlessness to 1 G was the principal cause of the transient stress-related changes in lymphatic tissues (7).

The transient increase in tingible bodies in macrophages in lymphatic tissues on R+0 of the flight rats and the associated transient atrophy at later intervals noted by the Russian investigators can be thought of as a marker for the return to the Earth's 1-G gravitational field. The gravitational stress of reentry, as indicated by transient changes in lymphatic tissues, has been repeatedly demonstrated (6). Of special interest is the extensive work on tingible bodies engulfed by macrophages in the thymus in many laboratories totally unrelated to spaceflight research, which refers to the entire process as apoptosis and to the tingible bodies as apoptotic bodies (10). Apoptosis is considered to be an active regulatory response by inducible cells to a specific inducing stimulus. This is accompanied by morphological changes, including marked condensation of nuclear chromatin with shrinkage of the nucleus and the formation of dense chromatin masses (apoptotic bodies) and subsequent phagocytosis of the apoptotic cells (3, 4). The most common biochemical feature of apoptosis is the fragmentation of DNA into single or multiple internucleosomal fragments of 180–200 base pairs or multiples thereof caused by Ca²⁺/Mg²⁺-dependent endonucleases (3, 4). Cells often undergo apoptosis as a response to environmental stimuli. Immature thymocytes, for example, respond to stress with an increase in glucocorticoid hormones. Apoptosis may also occur in the absence of cytokines, such as interleukin-2, tumor necrosis factor-α, and colony-stimulating factors (9, 13, 14). The vulnerability of cells, (e.g., thymocyte or lymphocyte) to apoptosis might be related to the sensitivity of these cells to various biological signals. Apoptosis, resulting from the selection for molecules of the major histocompatibility complex, was demonstrated in the thymus of mice by dense aggregates of apoptotic cells with DNA breaks engulfed by macrophages (12). It is known also that the phenomenon of thymus involution is related to stress. The identification of apoptotic bodies in the lymphatic tissues, and only in flight animals that were killed shortly after landing, is of particular importance for studies of the mechanism of apoptosis such as 1) recognition of preapoptotic cell population; 2) early biochemical events in DNA degradation; 3) detection of gene(s) that are involved in apoptosis; and 4) identification of the environmental or physical factor(s) that trigger apoptosis and the types of cells that were involved in this phenomenon. Serova (11) reported a two- to threefold increase in corticosteroid concentration in blood plasma and adrenals of flight animals compared with control animals. The results of the present studies fulfill a critical criterion of apoptosis in the mononuclear cells with fragmented DNA that was engulfed by the local macrophages. Apoptosis was detected only in the flight animals that were killed on R+0. We suggest that the transient alteration in the local or steroid level or cytokine levels in flight animals due to gravitational stress of reentry into Earth's 1-G gravitational field resulted in the profound changes in the thymus, spleen, and inguinal lymph node. These studies will enhance our understanding of the factors that induce apoptosis and also could be useful in developing a strategy to inhibit or reverse any detrimental effects.

This study was supported by National Aeronautics and Space Administration contract NAG 2-494, no. 1092. We acknowledge the following for their dedication: National Aeronautics and Space Administration Ames Research Center personnel at Moffett Field, CA; Hangar 2 personnel at Kennedy Space Center, FL, and the National Aeronautics and Space Administration. We also thank, from the University of Tennessee Medical Center at Knoxville, David Gerard and Brett Brunson for help with histological techniques and Drs. Jay Wimalasena and Tzu-Hao Wang for apoptosis evaluation.

Address for reprint requests: A. Ichiki, Univ. of Tennessee Medical Center at Knoxville, Graduate School of Medicine, Box 2, 1924 Alcoa Hwy., Knoxville, TN 37920.

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REFERENCES


Effects of spaceflight on rat erythroid parameters


University of Tennessee Medical Center at Knoxville, Knoxville, Tennessee 37920

Allebban, Z., L. A. Gibson, R. D. Lange, T. L. Jago, K. M. Strickland, D. L. Johnson, and A. T. Ichiki. Effects of spaceflight on rat erythroid parameters. J. Appl. Physiol. 81(1): 117-122, 1996.—Hematologic studies were performed on 21 ground control rats and 21 rats flown during the Spacelab Life Sciences-2 14-day mission. Group A (n = 5) was used to collect blood in flight and 9 days postflight, group B (n = 5) was injected with recombinant human erythropoietin (rhEpo), group C (n = 5) received saline as a control, and group D (n = 6) was killed in flight and tissues were collected. Results indicated no significant changes in peripheral blood erythrocyte counts between flight and ground control rats. The nonadherent bone marrow on flight day 13 showed a lower number of recombinant rat interleukin-3 (rrIL-3)-responsive and rrIL-3 + rhEpo-responsive blast-forming unit erythroid (BFU-e) colonies in flight rats compared with ground control rats. On landing day, a slight increase in the number of rhEpo + rrIL-3-responsive BFU-e colonies of flight animals compared with ground control rats was evident. Nine days postflight, bone marrow from flight rats stimulated with rhEpo alone or with rhEpo + rrIL-3 showed an increase in the number of colony-forming unit erythroid colonies and a decrease in BFU-e colonies compared with ground control rats. This is the first time that animals were injected with rhEpo and subsequently blood and tissues were collected during the spaceflight to study the regulation of erythropoiesis in microgravity.

Spaceflight anemia

Spaceflight anemia has been observed during both the American [Gemini, Apollo, Skylab, and Spacelab Life Sciences-1 (SLS-1)] and Russian (Apollo-Soyuz test project) orbital spaceflights. The most consistent hematologic finding during the orbital flights has been a reduction in red blood cell mass (RBCM) and plasma volume (PV) (3, 5, 9, 10, 16, 23). Hematologic studies carried out on rats flown aboard Russian and American spaceflights have produced conflicting results (5), (7, 13, 18, 21, 24-26), and it is not known whether the rat is a valid model to study the changes that occur in humans during spaceflight. The cause of the decrease in RBCM in humans is multifactorial, such as a decrease in the levels of serum erythropoietin (Epo) and insufficient protein intake, and differs qualitatively and quantitatively from flight to flight. Gazenko et al. (5) and Ilyin et al. (7) reported that animals flown on Cosmos 936 and 605 flights had no significant differences in the values of hemoglobin (Hb), hematocrit (Hct), and red blood cell (RBC) counts. Isotope studies that include RBCM and PV measurements were not performed. Our results from the studies performed on SLS-1 confirmed these findings and also showed no changes in serum Epo levels (24). Vacek et al. (25, 27) and the results from our studies on SLS-1 (24) and Cosmos 2044 (15, 20) indicated a decrease in the number of Epo-responsive total bone marrow progenitors in flight animals compared with ground control animals.

SLS-1 was the first dedicated flight that studied the effects of spaceflight on the physiology of humans and used the rat as an animal model. Hematologic studies on Spacelab Life Sciences-2 (SLS-2) allowed the expansion of the experiments conducted on SLS-1 to include two unique features: 1) the in-flight injection of recombinant human Epo (rhEpo) into rats to examine the effect of exogenous rhEpo on the responsiveness of erythropoietic progenitor cells at microgravity and 2) the in-flight collection of blood samples, serum, and tissues to evaluate the changes in erythroid parameters in peripheral blood and bone marrow samples. Our objectives were to assess peripheral blood and bone marrow erythroid parameters from tissues collected preflight, in flight, on landing, and postflight.

Materials and Methods

Animals and Experimental Groups

Fifty-five male Sprague-Dawley rats, weighing 86 ± 2.2 g (Taconic Farms, Indianapolis, IN), were received at Kennedy Space Center, FL. They were premonitored for 24 days before launch and weighed at regular intervals, and their food and water intake also were monitored to determine their growth and eating patterns. Thirty rats with consistent growth and eating and drinking patterns were chosen and randomly selected as flight or ground controls. Three days before launch (L = 3, where L = x is no. of days before launch), these rats weighed 235 ± 3.2 g. The flight rats were housed singly in a Rodent Animal Holding Facility (RAHF), and the ground control rats were placed in a RAHF ground simulator. Both flight and ground control animals were given flight food bars and water ad libitum. The temperature, humidity, and the 12:12-h light-dark cycle were the same for the flight and ground control rats. The flight and ground control rats were divided into three groups of five: groups A, B, and C (Table 1). The low number of rats in each group was due to the limited rat housing on the shuttle. Group B rats were injected intravenously with 200 U rhEpo on flight day 9 (FD9). Group C was the control group and was injected with saline. Another group of rats, group D (n = 6), was killed by decapitation during flight on flight day 13 (FD13) and the serum from the "stump" blood and bone marrow from the femur were obtained. Four to 5 h after landing on the day of return (R+0, where R = x is return day + x days), groups B and C flight rats and their ground controls were exsanguinated through cardiac puncture and immediately decapitated and tissues were obtained. Group A flight and ground control rats were held for 9 days after landing. At that time the same protocol and tissue collection methods were followed as those on R+0. All procedures were approved by the Animal Care and Use Committees of both the University of Tennessee Medical Center and National Aeronautics and Space Administration Ames Research Center.

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**Table 1. Experimental design summary of erythroid parameters studied during SLS-2 spaceflight**

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<th>Blood Draws</th>
<th>Injections</th>
<th>Tissue Collection</th>
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<tr>
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<td>Group A flight (5) and control (5) rats</td>
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<tr>
<td>L-3</td>
<td>FD6, FD7, and FD14</td>
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<td>R + 2 and R + 8</td>
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<td>Group B flight (5) and control (5) rats</td>
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<td>L-3</td>
<td>FD9 and FD10</td>
<td>rhEpo</td>
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<td>R + 0</td>
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<td>R + 0</td>
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<td></td>
<td>Group C flight (5) and control (5) rats</td>
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<tr>
<td>L-3</td>
<td>FD9 and FD10</td>
<td>Saline</td>
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<td>R + 0</td>
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<td>R + 0</td>
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<tr>
<td></td>
<td>Group D flight (6) and control (6) rats</td>
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<td>FD13</td>
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Nos. in parentheses are no. of rats. SLS-2, Spacelab Life Sciences-2; L-3, 3 days before launch; R + x, return day + x days; FD, flight day; rhEpo, recombinant human erythropoietin.

**Hematology**

Blood was collected via the rat tail vein as part of the scheduled blood sampling. The first phlebotomies were performed on all of the animals on L-3. In-flight blood samples and ground control samples were taken on FD6 (~120-140 µl), FD7 (~210 µl), FD9 (~120-140 µl), FD10 (~210 µl), and FD14 (~120-140 µl). Blood samples were obtained on R + 0 (~210 µl) from all of the animals and then on R + 2, R + 4, R + 6 (~120-140 µl each day), and R + 8 (~210 µl) on group A (flight and ground control animals).

**Blood Cell Counts**

Automated blood cell counts were performed on 20 µl of rat blood by using the TOA Sysmex F-800 microcell counter (TOA Electronics, Kobe, Japan). The in-flight blood samples were held under refrigeration until R + 0. The ground control samples were treated similarly. All samples were analyzed twice and the results averaged.

**Reticulocyte Counts**

Two methods were utilized in determining reticulocyte counts.

*Flow cytometry.* Peripheral blood (~30 µl) was removed from each rat and shipped at temperatures of 2–8°C to the Knoxville laboratory. Each sample was thoroughly mixed, and 10 µl were pipetted into 1 ml of thiazole orange, mixed, and incubated for 30 min. The samples were mixed and analyzed by a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). A software program, ReticCount, also by Becton-Dickinson, was utilized to analyze the results of 10,000 events.

*Manual.* The reticulocyte slides were prepared shortly after blood collection from both in-flight and ground control rats. Equal amounts of blood and new methylene blue-N were allowed to stain for a minimum of 3 min, after which standard smears were prepared. The slides were fixed for 10 min in absolute methanol, returned to Knoxville, and counterstained by using a Dif-Quik (Baxter Healthcare, Miami, FL) staining procedure. A total of 3,000 (1,000 by each of 3 technicians) RBCs were counted and percents were averaged.

**Erythroid Colony Assay**

The cultures were carried out according to the method described by Ogawa (19) with some modifications. One femur was removed from each of the flight and ground control rats; the bone marrow was flushed into a 15-ml conical tube that contained 10 ml of Iscove’s modified Dulbecco medium, penicillin-streptomycin, and fetal calf serum; and the tube was shipped back on ice to the Knoxville laboratory. The bone marrow suspension was then counted and divided. One-half of the bone marrow suspension was utilized as total marrow to duplicate SLS-1 studies and was cultured in premade methylcellulose and spleen cell-conditioned media (no. HCC-3230 and no. HCC-2100, respectively, Terry Fox Laboratories, Vancouver, BC, Canada) either in the absence of rhEpo or with 3 doses of rhEpo (0.25, 0.50, and 1.0 U/ml). The other one-half of the bone marrow suspension was incubated in plastic culture flasks to obtain the nonadherent marrow cells and after centrifugation was incubated without rhEpo, with rhEpo alone (0.25 U/ml), with rhEpo + recombinant rat interleukin-3 (rIL-331), or with rIL-3 alone (6 U/ml). A late progenitor, the colony-forming unit erythroid (CFU-e), was enumerated on day 2 of culture for all assays by using 2,7-diaminofluorene to stain the Hb-containing colonies (28). The early progenitor, blast-forming unit erythroid (BFU-e), was determined on day 7 for all assays.

**Bone Marrow Differential**

The bone marrow smears were prepared shortly after collection of the bone marrow, both in flight and on the ground, on FD13, R + 0, and R + 9. The “paint brush” technique was used to make bone marrow preparations for differential counting (1, 6). The slides were air-dried, fixed in absolute methanol for 10 min, and stained by using Wright and Giemsa stains. A 200-cell differential count was performed. Cells were classified as follows: small lymphocytes, large lymphocytes, plasma cells, reticulum cells, mature myeloid (metamyelocytes, bands, segmented forms), immature myeloid (myeloblasts, promyelocytes, myelocytes), eosinophils, tissue basophils, mature erythroid (polychromatophilic and acidophilic normoblasts), and immature erythroid (pronormoblasts and basophilic normoblasts).

**Serum Epo Level**

On dissection days (R + 0 and R + 9), a cardiocentesis was performed. Stump blood was collected in flight on FD13. All blood was allowed to clot and then centrifuged to obtain the serum samples. The serum was immediately frozen, transported to Knoxville, and stored at ~70°C. All samples were run at the same time (120 days postflight). Epo was measured by using a commercial radioimmunoassay kit (Diagnostic Systems Laboratories, Houston, TX). The range for normal rats is 16–24.3 mU/ml (2, 22), and the cross-reactivity of human Epo with rat Epo is 87% (as per vendor’s insert).

**Statistical Analysis**

Statistical analyses were performed by the biostatistician at the University of Tennessee Medical Center. Three statistical tests were utilized: analysis of variance, Wilcoxon, and median.

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1. rIL-3 was a generous gift from Dr. Andrew J. Hapel of the John Curtin School of Medical Research, Canberra City, Australia.
RESULTS

The body weights of flight and ground control animals are presented in Fig. 1. The L+0 group, which represented the flight and ground control rats on the day of launch, weighed 283.6 ± 7.3 g. There was no significant difference between flight and ground control animals. However, the flight animals grew slightly faster than did ground controls in flight and until R+2, when the flight animals grew at a slightly slower rate than did ground controls (data not shown).

There were no significant differences in RBC counts and Hb and Hct values of flight and ground control rats within any of the groups on any given day (data not shown). These values were increased for both groups during the postflight period. This increase was probably due to the growth of the rats during this period. The reticulocyte counts that were measured by both manual and flow cytometry methods indicated that there were no statistical differences observed between flight and ground control animals of any groups on any given day of the flight (data not shown).

The effects of rhEpo and spleen-conditioned medium on total bone marrow collected preflight, in flight, at landing, and postflight are shown in Fig. 2. On FD13, the average number of nucleated bone marrow cells was 9.6 × 10^7/femur (range 5.88–13.8 × 10^7) for flight animals and 7.9 × 10^7/femur (range 6.3–10.56 × 10^7) for ground control animals. The numbers of CFU-e and BFU-e colonies of group D flight animals were fewer than the numbers of colonies from ground control animals at the three doses of rhEpo. The average viability of bone marrow cells was decreased by ~20%.

On R+0, group B had an average number of bone marrow nucleated cells of 1.72 × 10^7/femur (range 1.36–2.16 × 10^7) for flight animals and 1.82 × 10^7/femur (range 1.14–2.94 × 10^7) for ground control animals. Group C flight rats showed a decrease in the number of CFU-e colonies at the three doses of rhEpo compared with ground control animals. The numbers of BFU-e colonies in group B flight and ground control animals were higher than those of group C flight and ground control rats, respectively. The numbers of BFU-e colonies in groups B and C flight animals were markedly lower compared with the ground control animals at all rhEpo doses. Again, the numbers of BFU-e colonies in both ground control and flight rats of group B were increased compared with both flight and
spaceflight and rat erythroid parameters

on r+0, both groups B and C demonstrated no significant differences in the number of CFU-e colonies between flight and ground control animals in their response to either rhEpo alone or a combination of rhEpo and rrIL-3. The numbers of rhEpo-responsive and rhEpo + rrIL-3-responsive CFU-e colonies of group B ground control and flight animals were higher than those in group C. No BFU-e colonies were observed in either flight or ground control animals of groups B and C after stimulation with rhEpo alone. There were no significant differences in the number of BFU-e colonies between flight and ground control animals of both groups B and C when stimulated with rrIL-3 alone.

on R+9, there was a significant increase (P < 0.05) in the number of CFU-e colonies of flight rats compared with ground control rats after stimulation with rhEpo or a combination of rhEpo and rrIL-3. The number of BFU-e colonies of flight rats was significantly lower (P < 0.05) compared with ground control rats after stimulation with either rrIL-3 or a combination of rrIL-3 and rhEpo.

Bone marrow differentials showed that on FD13 the number of immature erythroid cells of flight animals was slightly increased compared with ground control animals, whereas the number of mature erythroid cells was decreased compared with ground control animals (Fig. 4). At R+0, the number of immature erythroid cells of group C flight animals was significantly lower (P < 0.1) compared with ground control animals, and on R+9 the number of immature erythroid cells was still significantly lower compared with ground control animals (P < 0.05). No significant differences were

ground control rats in group C, respectively. At R+9, the numbers of CFU-e and BFU-e colonies of flight animals were increased compared with those of the ground control rats at all levels of rhEpo except the BFU-e at 1.0 U/ml of rhEpo as shown in Fig. 2.

The results of nonadherent bone marrow cells stimulated with rhEpo, rrIL-3, and their combination are presented in Fig. 3. On FD13 in group D, there were very few rhEpo-responsive CFU-e colonies in either flight or ground control animals, and there was no difference between the two groups. The number of BFU-e colonies was lower in flight rats in response to rrIL-3 and significantly lower (P < 0.05) in response to a combination of rrIL-3 and rhEpo compared with ground control animals.

Fig. 3. Effect of recombinant rat interleukin-3 (rrIL-3), rhEpo, and their synergism on number of CFU-e (top) and BFU-e (bottom) colonies of nonadherent bone marrow cells of SLS-2 flight and ground control animals. Bone marrow was obtained in flight (FD13) and on R+0 and R+9. Values are means ± SE. *Values of flight rats were significantly decreased compared with those of control rats (BFU-e), and values of control rats were significantly decreased compared with those of flight rats (CFU-e), P ≤ 0.05.

Fig. 4. Bone marrow cell differential mean percentages of immature and mature as well as myeloid progenitors of both ground control and flight groups. Bone marrow smears were prepared in flight (FD13) and on R+0 and R+9. Values of flight rats were significantly decreased compared with control rats, †P ≤ 0.1; *P ≤ 0.05.
observed for any of the lymphocytes, reticulum cells, eosinophils, and tissue basophils between flight and control animals.

The Epo serum levels are presented in Fig. 5. On FD13, there was no significant difference between flight and ground control rats. However, on R+0 the comparison between flight and ground control rats of both groups B and C did show differences. There was no significant difference in the Epo serum levels between flight and ground control rats on R+9.

**DISCUSSION**

The results of the current studies demonstrate that spaceflight can profoundly affect rat bone marrow progenitor cells but has minimally observed effect on peripheral blood erythroid parameters. These results are consistent with SLS-1 and other previously published results (20, 24). The body weights of flight and ground control rats of group A indicated that the growth pattern of SLS-2 rats mimicked those in SLS-1 rats (24).

It has been reported that on R+0 there was a decrease in the number of reticulocytes in humans and animals exposed to microgravity (3, 5, 11, 17). In the present studies, there were no statistical differences between flight and ground control rats in reticulocyte values at FD6, FD7, FD9, FD10, and FD14. Also, on R+0 and R+9, no statistical difference between flight and ground control rats was seen. These findings are comparable to our SLS-1 results (24).

After the in-flight injection of group B rats with rhEpo on FD9, no difference in peripheral blood reticulocyte counts was demonstrated. This may be due to the time the reticulocyte counts were measured after Epo injections.

On FD13, a decrease in the numbers of CFU-e and BFU-e colonies of total bone marrow of the flight rats that were responsive to a combination of rhEpo and mouse spleen cell-conditioned medium may be an indication that a decrease in bone marrow progenitors' responsiveness to exogenous erythropoietic stimuli is a contributing factor of spaceflight anemia. At landing, total bone marrow cells obtained from groups B and C demonstrated a marked decrease in the numbers of CFU-e and BFU-e colonies of flight animals compared with ground control animals. These results are similar to those of SLS-1 (24) and those reported by the Russians (25-27). However, experiments on Skylab-3 (SL-3) indicated that bone marrow obtained from flight animals on R+0 showed an increase in the numbers of CFU-e and BFU-e colonies in response to 0.02 and 1.0 U/ml of Epo (12). On SL-3, nonrecombinant Epo was used and the animals were not available for study until ~12-18 h after landing. During this SLS-2 study, on R+9, the increase in the number of CFU-e colonies of flight animals was a possible response to an unexpected increase in the number of Epo-responsive erythroid progenitor cells during the recovery period. This increase possibly was due to the animals getting older or to simple stress.

On FD13, the nonadherent bone marrow progenitors stimulated with rhEpo demonstrated fewer rhEpo-responsive CFU-e colonies in both flight and ground control animals; this may be due to the lack of endogenous and exogenous synergistic growth factors in the culture medium. Stimulation with rrIL-3 or a combination of rrIL-3 and rhEpo also showed a decrease in the number of BFU-e colonies of flight animals, similar to the results obtained from using total bone marrow cells. This is another indication of a decrease in erythroid progenitor responsiveness to exogenous stimuli during spaceflight. On landing, the slight increase in the numbers of CFU-e and BFU-e colonies of group B compared with those of group C may be due to in-flight rhEpo injection.

In the present study, although there may have been statistical differences in Epo serum levels between flight and ground control rats on R+0, it probably was not a physiological difference. These results are different from our previous studies that were carried out during SL-3, Cosmos 2044, and SLS-1 (12-15). Spaceflight is a controlled “insult” that may affect positive and negative regulators of erythropoiesis. It has been reported that in vivo suppression of CFU-e response to exogenous Epo was mediated by macrophages or monokines such as tumor necrosis factor-α (8). It is possible that some cytokines could alter the interaction of Epo with its responsive cells by downregulating the Epo receptor on the erythroid progenitors, altering Epo binding, or internalization. However, the local bone marrow macrophages and the locally released cytokines (interleukin-9, stem cell factor, interleukin-11) were not evaluated and might play an important role in regulating erythropoiesis.

The harvesting of bone marrow cells in space on FD13 and the phlebotomy of the rats several times in flight were important in differentiating between hematologic changes due to spaceflight and those due to the gravitational stress of reentry to Earth's 1 G.
We acknowledge the following individuals for their dedication to this project: the space shuttle transport EE-8 crew; the National Aeronautics and Space Administration (NASA) Ames Research Center personnel at Moffett Field, CA; Laurie Dubrovin, experiment support scientist, for work on project no. E012; Hangar L personnel at Cape Canaveral, FL; Dryden payload receiving facility personnel at Edwards Air Force Base, CA; and Dr. Sharon Donnelly for statistical analysis, Dr. Joseph E. Fuhr for flow cytometry services, and Sara Bailey and Timothy Doyle for technical assistance, all at the University of Tennessee Medical Center at Knoxville.

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Address for reprint requests: Z. Allebban, Univ. of Tennessee Medical Center at Knoxville, Graduate School of Medicine, Rm. R219, 1924 Alcoa Highway, Knoxville, TN 37920.

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REFERENCES


Effects of spaceflight on rat peripheral blood leukocytes and bone marrow progenitor cells


Graduate School of Medicine, University of Tennessee Medical Center at Knoxville, Knoxville, Tennessee

Abstract: The white blood cell (WBC) elements and the bone marrow myeloid progenitor cell populations were analyzed to ascertain adaptation to microgravity and subsequent readaptation to 1 G in rats flown on the 14-day Spacelab Life Sciences-2 (SLS-2) mission. Bone marrow cells were harvested from one group of rats killed inflight (FD13) and blood was drawn from three other groups at various times. The WBC level was normal on FD14 with the exception of neutrophilia. On FD13, numbers of colony-forming units-granulocyte (CFU-G), CFU-GM, and CFU-M from flight animals were decreased compared with ground controls when incubated with recombinant rat interleukin-3 (rrIL-3) alone or in combination with recombinant human erythropoietin (rhEpo). On recovery (R + 0), flight rats had decreased numbers of total leukocytes and absolute numbers of lymphocytes and monocytes with elevated neutrophils compared with control rats. They had lower numbers of CD4, CD8, CD2, CD3, and B cells in the peripheral blood but no differences in spleen lymphocytes. J. Leukoc. Biol. 60: 37-43; 1996.

Key Words: microgravity · interleukins · lymphocytes

INTRODUCTION

Circulating leukocytes in the peripheral blood are unidirectionally and homeostatically controlled from the progenitor cells in the bone marrow to the storage pool of differentiated leukocytes by a complex network of cytokines and growth factors. When the homeostasis of peripheral blood leukocytes in healthy animals is dramatically altered, a potent insult has occurred. Changes in the leukocyte levels have been attributed to secondary responses to stress, extreme physical conditions, infection, or many other factors [1]. There have been many observations that spaceflight greatly affected the homeostasis of circulating leukocytes in animals, which ultimately altered immune and nonimmune cell functions, as reviewed by Lesnyak et al. [2].

Rats flown on the Russian Cosmos or U.S. space shuttle missions have all returned to earth with altered levels of white blood cells, suggesting that an insult affected homeostasis. Contrary data have been reported regarding the effects of spaceflight on the peripheral white blood cell populations. Rats flown on Cosmos 936 and 1129 had decreased levels of leukocytes, which included both lymphocytes and neutrophils, whereas the levels of leukocytes and eosinophils were increased in rats on Cosmos 605 [3-6]. The differences could be influenced by the rat strain, the time the animals were bled after landing, the availability of water and food, or many other factors. Bone marrow cells obtained from rats flown aboard Cosmos 1987 and 2044 had decreased number of myeloid colonies when stimulated with macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) compared with ground controls [7-9]. However, a limitation of all of these studies was that they did not determine whether these changes occurred in the circulating white blood cell elements and bone marrow progenitor cells independently or simultaneously.

Our studies of rats flown on the Spacelab Life Sciences-1 (SLS-1) mission, the first of a series of spaceflights dedicated to the study of life sciences in space, showed a significant decrease in the total WBC counts and the absolute number of lymphocytes and monocytes on landing [10]. This decrease was transient, because the WBC levels rebounded to preflight values within 24 h. The rats had recovered from the insult, which we considered to be the combined effects of spaceflight, reentry, and the return to gravity. Until this SLS-2 mission, the second flight in the series, analyses were performed prior to the flight, on recovery, and at intervals following recovery. SLS-2 was unlike all other previous spaceflight missions, because one group of rats was killed and dissected by the astronauts in

Abbreviations: ANOVA, analysis of variance; CFU, colony-forming unit; FACS, fluorescence-activated cell sorter; FD, flight day; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IgG, immunoglobulin G; II-3, interleukin-3; IMDM, Iscove's modified Dulbecco's medium; LFA-1, lymphocyte function-associated antigen-1; M-CSF, macrophage colony-stimulating factor; PE, phycoerythrin; R, recovery; RAIF, rodent animal holding facility; rhEpo, recombinant human erythropoietin; rrIL-3, recombinant rat II-3; SLS-1, Spacelab Life Sciences-1; VLA-4, very late activation antigen-4; WBC, white blood cell.

Reprint requests: Albert T. Ichiki, Graduate School of Medicine, University of Tennessee Medical Center at Knoxville, 1924 Alcoa Highway, Knoxville, TN 37920.

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space. The tissues, bone marrow cells, and stump blood specimens were available for analysis shortly after the shuttle landed.

Although it was not the objective of this study to evaluate the immunity of the rats flown in space, we considered the immunophenotyping of spleen and peripheral blood lymphocytes a part of the assessment of the effects of spaceflight on leukocytes. There are contradictions in the published immunophenotyping data for spleen lymphocytes from rats flown in space, which could be attributed to the same reasons as the differences in the hematology data [2, 7, 10–12]. In addition, it is possible that there were differences in reagents and method of analysis. Peripheral blood T cell subsets and B cells were significantly decreased on R + 0 in rats flown on the SLS-1 mission because of the decreased lymphocyte level on recovery.

It was the objective of the SLS-2 study to determine the effects of spaceflight on the hematopoietic system. We probed the red cells and their bone marrow progenitors [13] and the lymphoid tissues [14]. This paper focuses on the white blood cell elements and the bone marrow myeloid progenitor cells and how spaceflight and landing affected them.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were obtained from Taconic Farms (Indianapolis, IN). The food and water consumption was monitored and recorded. Body weights were taken at regular intervals in order to establish the normal growth pattern, and rats that did not show a consistent growth pattern were not chosen as either flight animals or synchronous ground controls. Fifteen rats, dedicated for hematological studies, were randomly placed in the rodent animal holding facility (RAHF) [15] 24 h prior to launch. An equal number of synchronous ground control rats were placed in simulated RAHF cages at the same time and were flown from Kennedy Space Center, Florida, to the Dryden Receiving Facility, California, the day after launch. They were maintained under conditions similar to those of the flight rats, that is, the same light and dark cycle, humidity, temperature, and housing in simulated RAHF cages. The RAHF temperature ranged between 24 to 32°C throughout this 14-day flight. Both food, which was the same as for the flight rats, and water were available ad libitum. The flight and ground controls were divided equally into three groups of five: A, B, and C (Fig. 1). On flight day 9 (FD9), group B rats were injected intravenously with 200 U (0.3 mL) of recombinant human erythropoietin (rhEpo; Amgen, Thousand Oaks, CA), which was one of the hematology experiments, to determine its effects on erythropoiesis. Group C rats were injected with the same volume of saline, and group A rats were not injected. The data for rats in group A and group C were combined as a single group because the volume of saline injected into the group C rats was not considered to be of significance. Although we did not expect rhEpo to affect the leukocyte compartment of the rats in group B, we maintained it as a separate group for data analysis in order to be consistent with our study of the red blood cell compartment of the rats flown on this mission. We also obtained tissue specimens from six rats from another group (group D), which were killed on FD13. All procedures were approved by the Animal Care and Use Committees of both the University of Tennessee Medical Center and NASA Ames Research Center.

Hematology

The phlebotomies were performed on all of the animals 3 days prior to launch, L-3. Group A had blood drawn on FD6, 7, 14, R + 0, R + 2, 4, 6, and 8 with tissue collection on R + 9. Group B had blood draws on FD9, 10, and R + 0 with tissue collection on R + 0. Group D (n = 6) had stump blood collection inflight on FD13.

White blood cell count

The WBC count was obtained as part of the complete blood cell count. The blood cell counts were performed using the TOA Sysmex T-800 with 20 μL of blood. These measurements were made on site shortly after the acquisition of the blood samples. The inflight blood samples were held under refrigeration until the day of landing. All samples were analyzed two times and the average computed. The control samples followed the same time line.

Peripheral blood differentials

Blood smears were prepared shortly after blood collection for all time points. Ten microliters of whole blood was added to 5 μL of 0.9% isotonic saline. This method was utilized as a precaution in case the rats became dehydrated, causing the peripheral blood to increase in viscosity. After landing, the smears were fixed in absolute methanol, shipped to Knoxville, and stained with Wright-Giemsa stain. A total of 300 leukocytes were counted for differential determinations.

Peripheral blood and spleen immunophenotyping

The staining of the peripheral blood and spleen lymphocytes was performed according to methods previously described [10]. The inflight materials were kept under refrigeration until the day of landing and then processed. Again, the controls samples had the same storage and processing. The peripheral blood or spleen lymphocytes were washed and stained with the following combination of fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs): PE-anti-CD45 and FITC-anti-myeloid antigen (for lymphocyte gating), FITC-anti-immunoglobulin G1 (IgG1) and PE-anti-IgG2 (for nonspecific binding), FITC-anti-CD4 and PE-anti-CD8, FITC-anti-CD3 and PE-anti-CD45RA, and FITC-anti-CD2, which were all purchased.

Fig. 1. Time line of activities for hematology experiments from the SLS-2 mission.
from PharMingen, San Diego, CA. The erythrocytes were lysed with florescence-activated cell sorter (FACS) Lysing Solution (Becton Dickinson, Mountain View, CA). The leukocytes were fixed with 1% formaldehyde on site. The fixed cells were shipped back to Knoxville and enumerated on the FACSscan flow cytometer (Becton Dickinson).

**Bone marrow colony assay**

The cultures were carried out according to methods previously described [16] with some modifications [17, 18]. On R + 0 and R + 9, one femur was removed from each of the flight and control rats and the bone marrow cells were flushed into a 15-mL conical tube containing Iscove’s modified Dulbecco’s medium (IMDM) and antibiotics (1% Pen/Strep solution), then shipped on ice back to Knoxville as well as the bone marrow cells collected on FD13. The bone marrow cell suspension, 1 × 10^6 cells/mL, was incubated in a 75-mm² plastic flask for 2 h to obtain a preparation of nonadherent hematopoietic progenitor cells. Recombinant rat interleukin-3 (rrIL-3) was used to stimulate the myeloid progenitor cells, since IL-3 is a multipotent hematopoietic growth factor that promotes the survival and proliferation of all hematopoietic progenitor cells [18, 19]. The nonadherent progenitor cell preparations, 2 × 10^5/mL, were incubated with rrIL-3 alone, rhEpo alone, and a combination of rhEpo and rrIL-3. The colonies were counted on day 3 and 7 of incubation. The criterion for a colony was that it consisted of more than 50 cells. Morphologically, the CFU-G colonies were more compact than the CFU-M or CFU-GM colonies. The ultimate identity of the cells in the individual colonies was based on the morphological and cytochemical characteristics. Colonies were plucked with a Pasteur pipette, suspended in IMDM medium, cytocentrifuged, and stained with Giemsa or Naphthol AS-D chloroacetate esterase and alpha-Naphthyl acetate esterase (Sigma Diagnostics, St. Louis, MO). Granulocytes had bright red granules and macrophages had black granules. A colony was considered to be mixed if it had less than 90% of a predominant cell type.

**Statistical analysis**

Statistical analyses were performed by the biostatistician at the University of Tennessee Medical Center. Three statistical tests used to compare group means or medians were analysis of variance (ANOVA), Wilcoxon, and Median. The final analysis used for each group was determined by how the data fit the most assumptions of each test.

**RESULTS**

**White blood cell counts**

Peripheral WBC counts were determined preflight, inflight on FD14, on recovery (R + 0), R + 2, and R + 8 (Fig. 2). There were no differences in total WBC levels between the flight and ground control rats on FD14. There was a significant decrease in total WBC level in the flight rats compared with the ground control rats on R + 0 (P ≤ 0.05 for groups AC and B). The total WBC count for group A flight rats was the same as for the ground controls by R + 2 and R + 9.

Peripheral blood smears obtained from the rats inflight did not indicate any differences in the percent lymphocytes and neutrophils between the flight and ground control rats (data not shown). The absolute counts of the peripheral blood lymphocytes, neutrophils, and monocytes are shown in Figure 3. On FD14 the neutrophil level for the flight animals was significantly elevated (P ≤ 0.05) compared with the ground controls. On the other hand, there were no differences in the number of lymphocytes and monocytes. On R + 0, the neutrophil count for the flight animals, combined as group AC, was significantly elevated compared with the ground control rats; however, no significant differences were discerned between the group B flight and ground control rats. Also, the number of lymphocytes and monocytes was decreased significantly in the flight groups compared with the ground control groups. No differences in the number of lymphocytes, neutrophils, and monocytes in the group A flight rats were discerned on R + 2 and on R + 8 compared with the ground controls. These data show that neutrophilia occurred in the rats inflight in the blood specimens obtained on FD14; however, neutrophilia, lymphopenia, and monocytopenia were detected on R + 0. These conditions returned to normal by R + 2.

**T and B cell surface antigen expression**

The T cell subsets and B cells were enumerated for both peripheral blood and spleen lymphocytes to determine the effects of spaceflight and recovery. The immunophenotyping results of the peripheral blood lymphocytes on specimens obtained on FD14, R + 0, and R + 8 were expressed as the number of antibody-reactive cells/µL (Fig. 4). On FD14, no differences were noted between the flight group and the ground control rats. The data from R + 0 showed a significantly lower number of CD2, CD3, CD4, CD8, and B cells for the flight animals in the combined groups A and C (P ≤ 0.05). Only the CD3 and B cell levels were significantly depressed for group B rats, which may be due to the smaller n value. No differences were

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1 rrIL-3 was a generous gift from Dr. Andrew J. Hapel of The John Curtin School of Medical Research, Canberra, Australia.

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![Fig. 2. Effect of spaceflight on total white blood cells/L peripheral blood. Each group (A, B, C) included five rats. Values are ± SEM. *Statistically significant, P ≤ 0.05.](image-url)
LYMPHOCYTES

- Flight
- Ground Control

NEUTROPHILS

MONOCYTES

Fig. 3. Effect of spaceflight on number of lymphocytes, neutrophils, and monocytes per microliter of peripheral blood. Values represent means ± SEM. *Statistically significant, P ≤ 0.05.

discerned between the flight and ground control group on R + 8. Spaceflight did not affect the percentages of T cell subsets or B cells (data not shown). The profound decrease in lymphocytes at recovery was reflected in the decreased numbers of T cell subsets and B cells. This condition returned to normal by R + 8.

The spleen lymphocyte immunophenotyping results for specimens obtained on FD13, R + 0, and R + 9 were expressed as percent antibody-reactive cells (Fig. 5). No differences in percent reactive cells were noted between the flight and ground control spleen T cell subsets and B cells on FD13. On R + 0, the flight rats in group B had a higher percentage of CD3+ lymphocytes but a lower percentage of CD4+ cells (P ≤ 0.05). On R + 9, the percent CD8 was lower (P ≤ 0.05) and percent B cells was higher for the flight animals than the ground controls. The immunophenotyping data indicated that spaceflight did not affect the percent spleen T cell subsets and B cells, but there was some modulation on R + 0.

Enumeration of bone marrow myeloid progenitor cells

The responsiveness of nonadherent bone marrow nucleated cells from the flight or ground control rats was evaluated the number of myeloid progenitor cells stimulated by rrIL-3 or rhEpo or both in vitro identified as CFU-G, CFU-M,

Fig. 4. Effect of spaceflight on the absolute number of lymphocyte subsets per microliter of peripheral blood. Rats were bled on FD14, R + 0, and R + 8. Values are ± SEM. *Statistically significant, P ≤ 0.05.
or CFU-GM (Fig. 6). On FD13, R + 0 (group C rats that were stimulated with rIL-3 only) and R + 9, flight rats had fewer CFU-GM colonies when cultured with either rIL-3 alone or in combination with rhEpo compared with ground controls. On R + 0, the number of CFU-G colonies in group C flight rats was significantly lower (P ≤ 0.05) than in ground controls in response to rIL-3 alone. On R + 9, the number of CFU-G colonies was significantly lower (P ≤ 0.05) than in ground controls after stimulation with rIL-3. No significant difference in the number of CFU-M colonies was observed between flight and control rats at any time point during the flight.

DISCUSSION

One of the objectives of the animal studies on the SLS-2 mission was to determine adaptation to microgravity and readaptation to 1 G. We approached this study by assuming that launch and reentry were two potent insults to the homeostasis of the leukocytes. Adaptation or readaptation was considered the result of recovery. This was possible only because we were able to compare blood, spleen, and bone marrow cell specimens obtained inflight for the first time with specimens obtained postflight at recovery and 9 days later. The range of values of the peripheral blood leukocyte populations, T and B lymphocytes in the peripheral blood and spleen, and the colony-forming progenitor cells in the bone marrow was established with rats bled prior to launch. Variations of some of these values were observed in the flight rats as well as in the synchronous ground controls. Without further studies, we do not have any explanation for the variations observed in the ground control rats. Every effort was made to simulate the conditions of flight, other than the effects of spaceflight.

The inflight WBC results showed a significant elevation of the neutrophil count without an increase in the overall leukocyte count, suggesting that the rats could be experiencing stress neutrophilia, because, other than being in microgravity, they were not exposed to extreme physical conditions [1]. If launch was considered a strong insult to the leukocyte compartment, the neutrophilia reflected that the rats had not fully adapted to microgravity, even though they had been in space for 14 days. On the other hand, the decrease in the number of CFU-GM and CFU-G of flight animals could reflect the decrease in the responsiveness of specific lineages of myeloid progenitors to rIL-3. The observations suggest that the leukocyte compartment had not fully adapted to microgravity by FD14.

The postflight WBC results indicated a significant decrease in the number of leukocytes, specifically the lymphocytes and monocytes, but an increase in the number of neutrophils. Along with the neutrophilia, there was an apparent leukopenia, which corroborated our observations from the previous SLS-1 mission [10]. How soon the rats are bled after recovery appears to be critical for the true reflection of the stresses associated with the reentry to gravity on leukocytes and lymphocyte subsets. In both SLS missions, blood was drawn within 8 h of recovery [10, 13]. The leukocyte level recovers very quickly, as observed in the SLS-1 flight, where the WBC level was back to ground control levels by R + 1 [10]. On the SL3 mission, the flight rats apparently experienced only mild lymphopenia and neutrophilia where the blood draws were 12 to 18 h after recovery [20]. Rats flown on Cosmos 936 and 1129 had decreased leukocyte levels because these rats were bled between 7 to 13 h after landing [4, 5, 21]. The leukocyte and eosinophil levels were increased in rats on Cosmos 605; however, there was no specific time mentioned when the blood draws were made [6].

The temporary lymphopenia and monocytopenia on recovery, which were not observed inflight, appear to reflect Ichiki et al. Spaceflight and rat leukocyte parameters 41
the response to reentry. It is possible that the mechanical stresses of reentry and readaptation to 1 G could affect cell function [22], resulting in the expression of adhesion molecules [11]. Grove et al. [11] reported that the splenocytes of rats flown on a 10-day spaceflight had increased expression of the integrins lymphocyte function-associated antigen-1 (LFA-1), and β, decreased reactivity to monoclonals from two different clones of L-selectins, and no differences in reactivity to intercellular adhesion molecule-1 (ICAM-1). We speculate that the peripheral blood monocytes and lymphocytes could have adhered to endothelial cells following induction of the expression of the integrin very late activation antigen-4 (VLA-4), which is specific for monocytes and lymphocytes but not neutrophils [23]. The neutrophilia could be due to an imbalance by the demargination of the dynamic balance between the circulating and marginal neutrophil pools [24].

The peripheral blood lymphocyte subsets were depressed only on R + 0, which corroborated the results from the SLS-1 mission [10]. In both missions, the decrease was attributed to the lower number of lymphocytes and not to any changes in the percentage of antibody-binding cells (data not shown). In the present study, only the percentage of CD3 was significantly elevated in the spleen lymphocytes on R + 0 from the rats in group B but not group C. We did not observe any differences in spleen T cell subsets and B cells in rats flown in the SLS-1 mission [10]. However, the spleen lymphocytes in flight rats on Cosmos 1887 had higher percentages of cells expressing the pan T antigen, CD8, and IL-2R [7] and on Cosmos 2044 higher percentages of CD4 and CD8 cells [9]. Rats flown on the 4-day STS-41 mission were reported not to have any differences in the percentages of T cell subsets and B cells between flight and ground control [12] but increased CD8, CD4, and kappa-bearing B cells after the 10-day STS-51
mission [11]. Contrary results were also observed in the immunophenotyping of lymph node lymphocytes, where no differences were reported [12], while increases in CD2 and a decrease in CD5 cells were noted [11].

In the present study, there were no differences in the number of CFU-M between the flight and control rats in response to rIL-3, which did not corroborate the results previously reported by Sonnenfeld et al. [7–9]. This may be due to various factors: time the bone marrow was cultured after flight, culturing of an enriched preparation of bone marrow progenitor cells, and stimulation of colony formation with rat specific recombinant IL-3. These are possible factors that can influence our results compared with those of previously reported studies.

This study suggests the compartmentalization of the bone marrow progenitor cells and the peripheral blood leukocytes by the responses to the insults of spaceflight. The inflight results showed that the rIL-3-responsive CFU-G and CFU-GM progenitor cells were decreased compared with the ground control in spite of an increase in the absolute neutrophil count. Meanwhile, on recovery, the decrease in peripheral blood monocytes may not have been entirely due to the decrease in the number of rIL-3-responsive CFU-M and CFU-GM progenitors. Peripheral blood monocytes are long-living cells and they rebounded within 48h after the dramatic decrease on R + 0.

The response to the insults of spaceflight by bone marrow progenitor cells and the circulating elements indicated that the homeostasis of the leukocyte compartment could be altered to compensate for the effects of spaceflight. Despite this alteration, the levels of both the peripheral blood leukocyte and myeloid progenitor cells returned to the control 1 0 levels by R + 2 and R + 0, respectively.

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