File Name: E012.DOC

REGULATION OF ERYTHROPOIESIS IN RATS

DURING SPACE FLIGHT

FINAL REPORT

Principal Investigator

Robert D. Lange, M.D.
The University of Tennessee Medical Center at Knoxville, TN 37920

Co-Investigators

A.T. Ichiki and J.B. Jones

Co-Authors

Z. Allebban, L.A. Gibson, C.E. Irwin, and C.C Congdon

Technical Assistants

L. Payne and E. Amaba

Statistical Analysis

P. Wright and E. Walker

This study was funded by NASA contract NAG 2-494, Experiment No. E012.

TABLE OF CONTENTS

1. Introduction
2. Chapter 1 - Regulation of erythropoiesis in rats during space flight
3. Chapter 2 - Effects of space flight on rat spleen weight and histology
4. Chapter 3 - Effects of space flight on rat peripheral blood leukocytes
5. Chapter 4 - Effects of microgravity on rat spleen and peripheral blood lymphocytes
6. Chapter 5 - Summary, conclusions and recommendations for future studies
7. Bibliography
8. Acknowledgments
9. Appendix A - Materials and Methods
10. Appendix B - Extra charts and tables (chapters 1-4)
11. Appendix C - Raw data (chapters 1-4)

INTRODUCTION

One of the old gospel hymns is entitled "There is Power in the Blood." From antiquity, this precious fluid has been vital to life and, indeed, the early Hebrews were prohibited from eating blood (Genesis 7:4, RSV Bible).

One of the most consistent findings observed in men exposed to orbital space flights has been a decrease in the total circulating number of red blood cells (red blood cell mass - RBCM) (1,2). Figure 1 shows the RBCM changes in astronauts on SpaceLab 2,3, and 4 flights. It should be noted that the deficit was repaired soon after the astronauts' return to Earth's gravity.

The RBCM is the product resulting from the production versus the destruction of red blood cells. Normally functioning bone marrow has the capacity to increase its production up to 6- to 8-fold. Originally, it was thought that the decrease in the RBCM in astronauts was due to an increased destruction of red blood cells due to the use of 100% O_2 at a partial pressure of 258 mm Hg. On the Skylab and Cosmos flights, however, hyperoxia was not present but a decrease in RBCM was still noted. It then became apparent that weightlessness was, in some manner, leading to erythropoietic suppression. The implication of hemolysis cannot be completely forgotten, however, since animal studies aboard Cosmos 782 and 936 (4,5), as well as in astronauts aboard SpaceLab 1, indicated that hemolysis may still have a role in the pathogenesis of the anemia of space flight.

Accompanying the decrease in the RBCM is a decrease in plasma volume. As a consequence, little change is noted for such parameters as hematocrit (Hct), red blood cell (RBC) count, and hemoglobin (Hgb), since their measurements are a result of their concentration in the plasma. The decrease in these RBC parameters, seen immediately following a return from orbit, is probably due to an increase in the plasma volume of the astronauts.

The feedback circuit that links red blood cell production is portrayed in Figure 2 (adapted from a diagram developed by Erslev, 6). Surrounding the diagram are the measurements which were made on Space Life Sciences-1 (SLS-1) to investigate particular components of

erythropoiesis. The starting point in the development of RBC's begins at the top of the diagram with multipotential stem cells becoming committed stem cells of the erythroid series.

Figure 3 portrays a model of hematopoiesis (modified from one developed by Quesenberry and Levitt) (7) that is useful in considering stem cell problems. Current thought is that hematopoietic tissues contain pluripotential stem cells, which give rise to lymphoid, megakaryocytic, erythroid, and granulocytic committed stem cells. Existing evidence suggests that cells which form lymphoid and spleen colony cells (colony forming unit-spleen -- CFU-S) are derived from a common cell. In the mouse, the erythroid and megakaryocytic cell lines apparently have a common ancestral cell but some develop separately committed cells. Several committed cell types can be grown *in vitro* by appropriate culture methods. Colony forming unit-megakayrocyte (CFU-Mk) is the cultured precursor cell of platelets. The earliest erythroid cells are termed burst forming units-erythroid (BFU-E) and they give rise to colony forming units-erythroid (CFU-E).

The granulocytic precursors develop into at least four separate clonal systems: the granulocyte-monocyte (CFU-GM), granulocytic (CFU-G), monocytic (CFU-M), and eosinophilic (CFU-Eos) colonies.

Several hormones and cytokines effect various phases of stem cell activity. One such hormone we studied is erythropoietin (Epo), which acts on erythroid committed cells to cause differentiation leading to mature RBC's. A burst promoting activity (BPA) acts to enhance differentiation of BFU-E. Production of platelets is thought to be regulated by thrombopoietin. A heterogeneous group of substances stimulate the *in vitro* solid phase colony formation of leukocytes.

Inhibitors of cell proliferation are known, e.g., chalones, lactoferrin, and prostaglandin, and may have colony inhibiting activity. Indeed, at least 20 inhibitors of hematopoietic growth factors have been described (8). The influences of cell-to-cell interactions also have considerable importance in stem cell activity. The monocyte-macrophages, in particular, are thought to have a

regulatory function because they not only produce colony stimulating activities (CSA) but also prostaglandin E_2, which has an effect opposite that of CSA.

The committed erythroid stem cells, BFU-E and CFU-E are measured by their ability to form colonies in methylcellulose or agar. Under the stimulus of Epo, they go on to form pronormoblasts, normoblasts, reticulocytes, and mature RBC. These production phases were looked at on SLS-1 by enumerating the number of reticulocytes. The type of cells (erythroid, myeloid, etc.) in the bone marrow and spleens of the rats were enumerated since, in the bone marrows and spleens of the rats, the circulating hematopoietic cells are formed. The RCM was measured by the isotope ^{51}Cr (E-141), as well as the RBC count. The plasma volume was measured by the use of ^{125}I-tagged albumin (E-141). As previously stated, the Hgb and Hct are measures of hemoglobin concentration. All of these factors come together to determine the total O_2-carrying capacity in the RBCM. The availability of O_2 is sensed by cells in the kidney, from which the glycoprotein hormone Epo originates. As mentioned before, this hormone stimulates the conversion of progenitor cells to differentiated cells of the erythroid series. The concentration in blood serum of the EPO hormone was determined in controls and in the rats exposed to the weightlessness of microgravity.

At the bottom of Figure 2 are listed other essential measurements performed on SLS-1. They include: body weights, food and water consumption, white blood cell counts and differentials, and platelet counts.

A summary of the hematological parameters measured on SLS-1 is given in Table 1.

The objective of the hematology studies was to gain an understanding of the regulatory parameters which modulate RBC production and destruction. The original questions addressed were:

OBJECTIVES

1. Determine if any changes in serum erythropoietin levels occurred in rats exposed to microgravity.
2. Determine if there were any changes in standard hematological parameters: hemoglobin, hematocrit, red blood cell count, platelet count, reticulocyte count, white blood cell count, and white blood cell differential count.
3. Enumerate lymphocyte subsets in peripheral blood.
4. Determine the effect of weightlessness on the responsiveness of erythropoietin-sensitive cells in *in vitro* cultures.
5. Determine if the rat is an appropriate model for hematological changes which occur in astronauts during space flight.

This report will be divided into the following chapters:

Chapter 1. Erythroid Parameters

Chapter 2. Spleen Studies

Chapter 3. Leukocytes and Platelets

Chapter 4. Lymphocyte Immunophenotyping

Chapter 5. Summary, Conclusions and Recommendations for Future Studies

Bibliography

Acknowledgments

Appendix: A. Materials and Methods

B. Tables and Figures not used in text

C. Raw Data on Individual Animals

Each chapter will contain: A. Significant Flight Results, and B. Significant Results of the Delayed Flight Profile Test (DFPT). All values presented are the means and standard error of the mean (SEM).

Table 1

Animal Parameters Measured in SLS1-012 Hematology Studies

A. General
   1. Body weight/mass
   2. Food consumption
   3. Fluid consumption
   4. Space craft environment
   5. RAHF & AEM environment

B. Peripheral Blood
   1. Hematocrit
   2. Complete blood count, including RBC indices, platelet count, and leukocyte differential and lymphocyte subsets
   3. Reticulocytes

C. Erythroid Regulatory Parameters
   1. Erythropoietin

D. Medullary Parameters
   1. Spleen weight
   2. Bone marrow and spleen differential counts; spleen histology
   3. Bone marrow BFU-E and CFU-E

Chapter 1

Regulation of Erythropoiesis in Rats During Space Flight

INTRODUCTION

Astronauts have been found to have a reduced red blood cell mass after exposure to microgravity (1-3). On the other hand, far fewer animal studies have been performed with varied results. Thus, the Soviets and their co-investigators have reported studies in rats flown on the following Cosmos flights: 605 (4-9), 690 (10), 782 (11, 12), 936 (11-15), 1129 (16), 1514 (8,17), 1667 (8,18), 1887 (8,19), and 2044 (20,21). Rats have also been studied on two NASA flights (22-26). Mice were flown and studied on Apollo XVII (27) and monkeys on two Cosmos flights (28,29).

In this chapter, the results of the erythroid parameters studied will be examined. These include:

Red blood count

Hemoglobin

Hematocrit

Mean corpuscular volume (MCV)

Mean corpuscular hemoglobin (MCH)

Mean corpuscular hemoglobin concentration (MCHC)

Reticulocytes (manual and flow cytometry)

Erythropoietin

Bone marrow differentials

Bone marrow response to EPO (CFU-E and BFU-E)

MATERIALS AND METHODS

See Appendix A.

RESULTS

The different housing groups were combined for the flight animals (Animal Enclosure Module [AEM] flight + Rodent Animal Holding Facility [RAHF] flight, AEM control + RAHF control).

This section will present the results for the erythroid parameters for both the flight and the DFPT. Those tables and charts not included in this chapter will be found in Appendix B and the results on individual animals will be found in Appendix C.

A. FLIGHT:

Erythroid Parameters of the Complete Blood Count:

Pre-Flight (L-4 - L-2): No significant differences in the erythroid parameters of the complete blood count (CBC) were seen between the control (AEM control and RAHF control) and flight (AEM flight and RAHF flight) animals.

Landing (R+0): Upon landing, statistical differences appeared for the MCV and MCHC between the flight and control groups. The flight animals' values were lower for the MCV and higher for the MCHC. Even though the computer determinations of these two groups are statistically, significantly different, examination of the means and standard deviations or the standard error of the mean showed very little difference.

Post-Flight (R+1 - R+9): The remainder of the rats were bled post-flight on R+1, R+3, R+4, R+8, and R+9. During this post-flight or "recovery" period, the flight animals showed significant increases on R+1 in the RBC, Hgb and Hct values. On R+3, the flight animals' values again were increased but only for the MCHC parameter. The remainder of the parameters on phlebotomy days showed no significant differences between control and flight rats.

Reticulocytes: The reticulocytes, either counted manually or in the flow cytometer, showed no significant differences between control and flight rats during preflight or at landing. Postflight, however, the flight animals showed significantly lower values than controls using the

flow cytometer method on R+1 and R+3. On R+4, both methods indicated that the flight animals had lower values compared to the controls. On R+8, the manual counts indicated that the flight animals had significantly higher values.

Erythropoietin: The serum Epo levels were tested on specimens obtained upon landing (R+0) and 9 days postlanding (R+9). No significant differences between the flight and control groups were found.

Bone Marrow: The responsiveness of the bone marrow to Epo was tested on R+0 for one group and its control and 9 days postlanding for the remaining group and its control. The early progenitor (BFU-E) showed the most differences on R+0, with flight animals having lower values than controls at the three doses of Epo (Fig. 1). The CFU-E, or later progenitors, were more variable, with flight animals having lower colony counts at the .25 and .50 U/ml dose levels on R+0 (Fig. 2). On R+9 with added Epo, there were no significant differences between the flight and control groups.

Bone Marrow Differentials: The only changes seen in the bone marrow differentials were on R+0. The flight animals were slightly but significantly higher in numbers of immature myeloid cells and eosinophils and in lower numbers of lymphocytes.

B. DFPT RESULTS:

1. R+0 Animals: The only major statistically significant differences were found in the clonal studies:
   a. CFU-E with 0.25 U Epo added. AEM control less than RAHF control.
   b. CFU-E with 1.0 U EPO added. The AEM control and RAHF flight had fewer colonies than the RAHF control group.
2. R+9 Animals - Major differences
   a. Erythroid parameters of the CBC
      1. RBC. The AEM control rats had lower values than the RAHF controls on R+0 and R+3 and than the AEM flight animals on L-4.
      2. Hgb. AEM control group was less than RAHF control on R+0.
      3. MCHC. On L-3, the AEM flight group had lower values than the RAHF flight animals.
   b. Reticulocytes: By the manual determinations on L-4, RAHF flight animals were decreased in comparison to RAHF controls.
   c. Bone marrow differentials. The mature erythroid cells of the AEM flight animals were increased in comparison to the RAHF flight group.
   d. Clonal studies - BFU-E. The AEM control group had a greater number of colonies than the RAHF controls in cultures with 1.0 U Epo added.

BODY WEIGHTS:

Body weights were taken on the animals at intervals, beginning at their arrival at Kennedy Space Center (KSC) and ending at the final phlebotomy at the Dryden Payload Receiving Facility (DPRF).

Flight: As seen in Fig. 3, all of the rats grew at a consistent rate up through R+0. Between R+0 and the remaining postflight period, however, the rats showed a distinct separation of growth patterns between the control and flight animals. Both groups had an unexplained loss in body weight from R+0 to R+1. The flight animals continued to lose weight through R+2 before regaining weight, starting at R+3. The control animals started regaining weight at R+2 but at a much slower rate than preflight.

DFPT: Fig. 4 depicts that all groups of the R+0 animals showed consistent and nominal growth patterns throughout the entire DFPT.

As shown in Fig. 5, the R+9 animals' growth pattern flattened out after R+0.

FOOD AND WATER CONSUMPTION:

Food and water also were measured from the rats' arrival to the end of postflight activities. During the flight, only the RAHF control and flight animals had food and water measured.

Flight: As seen in Figs. 6 & 7, the food and water consumption between control and flight animals are consistent with each other preflight. During the flight, distinct differences were noted. The flight animals consumed both food and water at a faster rate than the control animals, whose consumption decreased during this time. Upon landing through R+1, the decrease in food intake of both groups continued. The flight animals continued to have a decrease in water intake through R+1, while the controls resumed their normal intake of water from L+7 through the postflight period.

DFPT: R+0 and R+9 animals: As can be seen in Figs. 8-11, the DFPT food and water data does not present a concise pattern, as did the flight data. The reason for this lies with the fact that a lower number of samples in each group created a greater variability.

Age and RBC: As shown in Figs. 12 and 13, the RBC increased as the animals grew. This was true both during the flight determinations and the DFPT.

DISCUSSION

CBC Erythroid Parameters: In previous studies, variations have been found in the RBC parameters of laboratory animals flown in microgravity. Thus, Kalandarova has written that increased values, decreased values, and unchanged values have been reported.

The Spacelab 3 differences, depending on the age of the animals, were reported as shown in the following table (24-26):

	Small Rats	Large Rats
Hct	Increase*	Increase
RBC	Increase*	Increase
Hgb	Increase*	Increase
MCV	Decrease	Decrease
MCH	Decrease	NC
CHC	Decrease	Increase

* = Sig. <0.05

LeBlanc on Space Transport 8 (ST8) rats found a significant increase in RBC, Hgb, Hct, and MCV, but these animals were thought to be dehydrated (22). It is of interest that Gazenko (10) and Leon *et al.* (12) found evidence for a hemolytic component. However, in later studies, Leon *et al.* (14) found that when the animals were centrifuged in flight, to produce the effect of gravity, the hemolysis was prevented, indicating that the hemolysis probably resulted from the lack of gravity rather than other factors in the flight environment.

On SLS-1, we found no significant differences between flight and control animals on R+0 in their RBC, Hct and Hgb. The MCV of flight animals were lower than control animals, but their MCHC was increased. On R+1, the RBC, Hgb and Hct were increased and on R+3, the MCHC of flight animals increased. However, on R+9, there were no differences in the RBC parameters of flight and control animals.

Reticulocytes:

Previously, investigators have reported that the number of reticulocytes (young circulating red blood cells) in humans and animals exposed to microgravity are reduced in number immediately post-flight (3,9,10,19,30). This has been thought to be due to a slight depression in red blood cell formation due to exposure to microgravity. In the present studies, the reticulocyte values of the flight rats were not statistically different than the values of control rats on either R+0 or R+9. Lower values for flight animals were found on R+1, 3 and 4 when reticulocytes were counted by flow cytometry (Fig. 12). This was also true for manual counts on R+4, but on R+9 by manual counting, the reticulocytes of flight animals were increased compared to their controls.

Flow cytometry has been used to enumerate reticulocytes, allowing a large number of cells to be counted (31-33). This method eliminates the human element. Some authors have reported good agreement between the values obtained by flow cytometry and the standard manual method of counting reticulocyte numbers (33). We found that, although the trends were the same, the flow cytometric method gave distinctly higher values. Leach *et al.* (30,34,35), in human studies, also found that their flow cytometry values were higher than those obtained by manual methods. It is obvious that further studies using the two methods are needed and, perhaps, the preservation of cells will allow studies to be made in-flight.

Erythropoietin: There have been very few serum Epo level determinations carried out on flight rats. On SL3, we found the Epo levels measured by radioimmunoassay to be 15.4 ± 0.8 in flight animals and in the controls 19.0 ± 3.7. In 5 rats studied after the 2044 Cosmos (21),

flight animals had a level of 26.54 $\pm$ 0.77 and the synchronous controls 27.12 $\pm$ 1.78 (23). In the present studies, at R+0 there were no essential differences between flight and control animals.

DFPT: On R+9, the AEM flight animals had a statistically higher value than the AEM control animals and were also higher than either the RAHF control or flight animals. However, this increased level is of questionable physiological importance.

Studies in astronauts have shown the Epo levels to be decreased during flight and to sharply increase after landing, and to remain elevated through 14 days after landing (30,34-37). This postflight elevation was also found in Soviet Cosmonauts (37). Kalandarova (3) also reported that Cosmonauts in microgravity over two months had lower Epo levels than Cosmonauts in space for shorter periods of time.

Bone Marrow Colonies:

The earliest clonal studies on the effect of space flight measured the CFU-S. Vacek *et al.* found a decrease in CFU-S from spleen, liver and bone marrow cells of flight animals from Cosmos 1667 and 1514, 936, and 1887 (15-17,38). CFU-S are thought to be very early stem cells. On Cosmos 2044, Vacek *et al.* (38) found a decrease in the number of hematopoietic progenitor cells (Granulocyte-Monocyte-Colony Forming Cells [GM-CFC], BFU-E, CFU-E, Colony Forming Unit-Fibroblast [CFU-F]) in the tibial bone marrow cells in animals which had been injured prior to flight.

On SL3, we found significantly more erythroid colonies from bone marrow cells of flight animals in response to Epo at levels of 0.02 and 1.0U Epo/ml; but at the 0.20U Epo/ml level, the bone marrow cells from control animals formed more colonies (23-26). Results today may be more meaningful because the Epo being used is produced by recombinant technology, compared to the use of urinary Epo, which was less pure. In studies carried out post-Cosmos 2044, we found control marrow cells had increased BFU-E's both without added Epo and at the 0.25U Epo/ml level (21). There were no differences in the CFU-E colonies in the control and flight groups.

In the present SLS-1 studies, R+0 flight animals had a decreased number of BFU-E and in CFU-E at Epo dose levels at 0.25 and 0.50 U/ml, but not a 1.0 U/ml. There were no significant differences discerned on R+9. The results of clonal studies may indicate an inhibition of the earliest erythroid progenitor cells in the animals exposed to microgravity. By R+9, there were no differences observed between flight and control animals, which could be due to the readaption to 1-G.

Bone Marrow Differentials:

The results of bone marrow examinations showed, on the Cosmos flights, a decrease in the number of erythroid cells and lymphoid elements with a small increase in myeloblastic cells (5,7,8,13). The Soviet investigators also reported a decrease in the numbers of marrow cells. However, on Flight 936, the differences were not statistically reliable.

However, on SL3, we found no significant changes in the bone marrow differentials (23-26) and on Cosmos 2044, while some minor differences were found, there were no essential differences between flight and control bone marrow differential counts (21).

In mice on Apollo XII studies, Ellis *et al.* (27) found that the hyperoxic environment depressed erythropoiesis both in the flight and control animals. In monkeys, a decrease in bone marrow erythropoietic activity was found in flight animals (28,29). Six days after the Cosmos 2044 flight, the Soviet investigators reported there were marked signs of erythroid production activation.

In the present studies, on R+0, there were no differences between flight and control animals in their bone marrow differential counts. On R+9, the flight animals had a slight increase in the number of immature myeloid cells and eosinophils, but a slight decrease in the number of lymphocytes. The changes were probably not physiologically significant.

SUMMARY

Red blood cell parameters were examined in flight and control animals during the actual SLS-1 flight and again during DFPT. During the actual flight, there was no difference between flight and control animals for the following parameters:

A. Pre-Flight

Complete blood counts

Reticulocytes enumerated either manually or by flow cytometry. The manual counts gave lower values.

B. At R+0

Red blood cell counts

Hemoglobin

Hematocrit

Mean corpuscular hemoglobin

Reticulocytes, counted either manually or by flow cytometry. Manual counts were lower than flow.

Bone marrow differential

Erythropoietin

C. On R+9

Complete blood counts

Bone marrow clonal studies

Erythropoietin

The significant differences were found in the clonal studies. On R+0, the flight animals had significantly fewer BFU-E than the controls at all doses of added Epo. The CFU-E values were also lower for the flight animals at dose levels of 0.25 and 0.50 U Epo/ml. By R+9, there were no significant differences. These studies indicate that the earliest erythroid progenitor cells were probably decreased in number due to exposure to flight and would be compatible with studies showing a decrease in red blood cell mass in astronauts and rats exposed to microgravity.

The bone marrow cells, however, are able to respond to added erythropoietin and by R+9 have recovered in numbers.

At DFPT, inconsistent variations were found. While some differences were observed by severe statistical analysis, it is doubtful that they were of physiological significance and no consistent pattern seemed to be present.

It should be noted that there were some differences in caging at DFPT in comparison to the flight conditions. Further, the control animals were not flown to Dryden as were the flight controls.

Another caveat should be made. Due to the sheer number of tests performed, some statistical differences may appear without their being directly related to a difference due to exposure to microgravity.

Although no differences were found due to the manner of caging, the erythroid parameters change quite definitely with the growth of the animals.

Another difficulty arises when we attempt to compare the SLS-1 results with previous flights due to the different strains of rats used, the age of the animals, and the length of the flight.

Chapter 2

Effects of Space Flight on Rat Spleen Weight and Histology

INTRODUCTION

Previous studies of lymphatic tissues in space flight experiments with rats have shown changes. The Cosmos 605 space flight of 22 days demonstrated significant atrophy of spleen, thymus, and inguinal lymph nodes examined two days after the flight landed. At 27 days postflight, recovery had occurred, indicating reversible atrophy of lymphatic tissues (1). Leukopenia and lymphopenia were also reported with recovery to normal values in 60 and 30 days, respectively (2). These changes in lymphatic tissues were attributed to environmental stress associated with the longer flight. On the shorter, 7-day flight with SL3 there was a mild lymphopenia and spleen lymphocyte depletion (3).

The white pulp of the rat spleen is a typical example of lymphatic tissue. The red pulp contains some blood-forming elements like those in the bone marrow in these young animals. The purpose in examining the spleen (weight, histology and cytology) during the SLS-1 experiment was to look for effects of microgravity and other aspects of the flight experiment on this organ.

MATERIALS AND METHODS

(See Appendix A.)

RESULTS

The spleen to body weight ratio and hematoxylin and eosin (H&E) histology of a cross-section of the rat spleen showed no obvious differences for flight and control groups at either R+0 or R+9 (Table 1, Appendix B, chapter 2). An increase in body weight between R+0 and R+9 in flight animals was less than in the controls and was attributed to reduced food intake in flight animals compared with normal food intake in the ground-based controls that did not need to adjust to the landing and return to normal gravitational influence. In these same animals, brush preparations of spleen were made to evaluate cell differentials and no differences in flight and control groups were observed (Table 2, Appendix B, chapter 2).

Similarly, the DFPT studies on the same variables did not provide significant differences based on group or single animal caging (Tables 3 and 4, Appendix B, chapter 2).

DISCUSSION

While this flight with nine days of microgravity only caused a temporary reduction in food intake in the flight animals on landing and no discernible effect on spleen weight, histology or cell differentials, longer and shorter flights and duration of microgravity have produced significant changes, as shown in a review of the literature. Serova (4) discusses the relation of body weight changes to lymphatic tissue and comments on the post-flight functional hypokinesia or "physiological reduction of motor activity" in Cosmos biology experiments with rats. This behavior was present in the SLS-1 rats for about 8 hours postflight.

The most obvious basis and explanation for lymphatic tissue changes, where they have been noted, is possible stress of the experimental rats during the total preflight, flight, and postflight experiences. Inflight sampling of lymphatic tissues has the potential for separating out the portion (i.e., launch and flight vs. the total experiment) of the experience that contributes most to the stress reaction.

Even in SLS-1, the peripheral blood circulating lymphocytes showed some depletion when measured after landing (see Chapter 3). Probably the thymus, traditionally the most sensitive organ for measuring stress in young animals, should be examined in microgravity

studies. The classical work of Selye and his general adaptation syndrome is quite relevant to flight personnel and animals in the context of microgravity experiences. Selye recognized adrenal hypertrophy, lymphatic tissue atrophy and gastrointestinal ulcers as being the key events in chronic stress. These were central features in an adaptation syndrome (5,6).

SUMMARY:

Although there was some peripheral blood lymphocyte depression after landing in SLS-1 flight animals, when compared to controls, this was not detectable in the study of lymphatic tissue in the spleen. Other studies of lymphatic tissues in space flight experiments have shown significant atrophy of several lymphatic tissues. Fairly prompt recovery after landing has been reported.

The reported lymphatic tissue depression is most likely related to stress in the total experimental situation and future in-flight studies may show which portion of the experiment produces a stress reaction. All organ systems involved in the classical Selye stress adaptation syndrome could be monitored for relevance to humans and animals.

Chapter 3

Effects of Space Flight on Rat Peripheral Blood Leukocytes

INTRODUCTION

Several experiments on orbiting spacecraft during the past decade have shown significant changes in both the number and functional capacity of rat peripheral blood leukocytes. The basic mechanism for these changes during spaceflight has yet to be resolved. In rats flown on Cosmos 936 and 1129 flights, a leukocytosis with lymphopenia and neutrophilia occurred during the acute postflight period with values returning to control levels by 3 days (1-3). Ilyin *et al.* found a leukocytosis and eosinophilia in rats flown on Cosmos 605 (4). Rats flown on SL3 demonstrated no significant changes in the white blood cell counts but showed a mild lymphopenia and neutrophilia (5).

It is difficult to compare the results from the different flights since the duration of the flights differed, as did the strain of rats and the time the animals were sampled postflight. Studies conducted on the first 41 U.S. space shuttle astronauts demonstrated that the absolute number of lymphocytes and eosinophils in the peripheral circulation and lymphocyte blastogenesis were decreased after flight (6,7). Conversely, there was an almost universal doubling of the absolute neutrophil number and, often, an increase in the helper cluster destination (CD) T-lymphocyte population, as determined by flow cytometric analysis. Data from space shuttle flights 41B and 41D involving 11 crew members indicated a postflight decrease in circulating monocytes and B-lymphocytes (7). Prolonged spaceflight resulted in hypoplasia of lymphoid organs and alterations in mitogen-induced blastogenesis (7). This chapter includes the study of total and absolute leukocyte counts in both flight and control rats prior to and after exposure to microgravity.

MATERIALS AND METHODS

(See Appendix A)

RESULTS

Flight Animals:

R+0 Animals: The mean and standard error of the mean of total and absolute counts of peripheral blood leukocytes of control and flight animals are presented in Table 1. At preflight, there were no statistically significant differences in any of the parameters between the control and flight animals. At landing, there was a significant decrease in the number of total leukocytes and absolute counts of lymphocytes, monocytes and eosinophils of flight animals compared with controls. A slight decrease in the absolute neutrophil count of the flight animals compared with the controls was also observed.

R+9 Animals: On R+1 (1 day post landing) there was a doubling of the absolute neutrophil, monocyte and eosinophil numbers accompanied by a slight decrease in the total leukocyte and absolute lymphocyte counts of flight animals compared with the controls (Table 1). The platelet counts of flight animals were slightly higher than the controls. The values of all the parameters of flight animals returned to control levels by 9 days postlanding.

DFPT Animals:

R+0 Animals; The mean and standard error of the mean of total and absolute peripheral blood leukocyte counts are presented in Table 2. At L-3, the total white blood cell (WBC) counts of both AEM and RAHF flight animals were slightly to markedly higher compared with AEM and RAHF control animals, respectively. The absolute lymphocyte count of RAHF flight animals were markedly higher than the RAHF controls. At R+0, there was a marked increase in the total WBC count of RAHF flight animals compared with controls. There was a moderate increase in the number of absolute lymphocytes and monocytes of RAHF flight animals, compared with RAHF controls. Conversely, there was a marked increase in the absolute counts of both neutrophils and monocytes of RAHF flight animals compared with AEM flight animals.

R+9 Animals: At R+0, the white blood cell count (WBC) and absolute lymphocyte counts were markedly lower in RAHF flight animals, compared with controls as shown in table 3. Conversely, a moderate increase was observed in the number of neutrophils, monocytes, and eosinophils of AEM flight animals, compared with controls. No significant changes in the values of any of the parameters were observed at days 1,3,4,8, and 9 post flight. No significant changes were observed in the platelet counts of DFPT animals.

DISCUSSION

It was previously observed that, in rats exposed to microgravity, a leukocytosis with lymphopenia and neutrophilia occurred during the acute postflight period with values returning to preflight levels by 3 days (1-3). Several factors could contribute to these effects, including microgravity, stress, radiation, and interaction with other host biological systems. Also, it is difficult to compare the results from the different flights, since the duration of the flights differed as did the strain of rats and the time the animals were sampled postflight. In the present studies, there were statistically significant decreases in total white blood cell counts, and the absolute number of lymphocytes, monocytes and eosinophils, and a slight decrease in the neutrophil count at the time of landing. Twenty-four hours postflight, the neutrophil, monocyte and eosinophil counts of flight animals were higher than that of the control animals. The lymphocyte count returned to normal physiological levels but was still lower than that of the controls. The changes in the absolute number of lymphocytes, monocytes, and eosinophils in the current study indicated a similarity with the results reported by Taylor *et al*. (7). We also found a slight decrease in neutrophil counts rather than an increase, as indicated by some studies (5).

The results of peripheral blood leukocytes of DFPT animals indicated that they are a heterogeneous population. This heterogeneity is clearly demonstrated in the preflight period, which showed marked difference between control and flight animals in various peripheral blood leukocyte parameters, both among and within the groups, as presented in Tables 2 and 3. These changes and the variation in the growth rate that were shown by these animals made it difficult to study the effect of housing on these animals. Since the number of rats in the DFPT groups

(N=5) was much smaller compared to the actual flight number (N=19, N-20), more variability was observed between DFPT groups. The flight animals flown aboard the space shuttle Columbia during their preflight period showed no differences between or among the groups in any of the studied parameters. On the day of landing, these flight animals showed a significant decrease in total white blood cell counts and also in the absolute count of lymphocytes, monocytes, and eosinophils, compared with control animals. These leukocyte values returned to the level of control animals on day 9 postflight. This clearly demonstrated the effect of microgravity and/or other factor(s) (i.e. stress, radiation, etc.) on the blood values of the flight animals compared with DFPT animals.

The mechanism responsible for changes in peripheral blood leukocytes is not well understood. The number and functional activities of these cells are regulated by a complex network of cytokines and endocrine factors. Thus, spaceflight could affect the generation of these cells by affecting these regulatory mechanisms. Other studies also reported a decrease in the function of both human lymphocyte subsets and monocytes (7). Here again, it is possible that stress induced by spaceflight could change hormone levels, which could alter cytokine production by lymphocytes, monocytes, and other cytokine-generating cells. In addition, there could be an increased production of a soluble inhibitor which acts as negative regulators on the function and the production of these cells. These negative regulators, such as tumor necrosis factor a (TNF-a) and transforming growth factor b1 (TGF-b1), either have a direct effect on the response of these cells to other cytokines or affect the number of receptors on their surfaces. Also, it is well established that these negative regulators have either a direct or indirect effect on erythropoiesis, which leads to the inhibition of maturation of erythroid progenitor cells (9,10).

Our results indicated that the platelet counts of flight animals didn't differ from the ground controls either preflight or postflight. This is comparable to results reported in human studies (11).

SUMMARY

The effects of microgravity on peripheral blood leukocytes are as follows: On the day of landing (R+0), there was a significant decrease in the total white blood cell counts ($p<0.0001$) of flight animals in comparison to control animals. There was also a significant decrease in the absolute number of lymphocytes ($p<0.0001$), monocytes ($p<0.0001$), and eosinophils ($p<0.0001$) in the flight animals. A marked decrease in the number of neutrophils of flight animals was also observed. By day 3 post flight, the total and absolute leukocyte counts returned to control levels. These changes in peripheral blood leukocytes may be due to a stress reaction or physiological readaptation to microgravity.

To better understand the various factors affecting the leukocyte parameters of flight animals, inflight testing during the upcoming Space Life Sciences-2 (SLS-2) mission will be critical. We hypothesize that various factors are contributing to the changes in erythropoiesis of flight animals, either directly or indirectly. As the levels of serum Epo, Interleukin-3 (IL-3), and Interleukin-9 (IL-9), and the negative regulators possibly change due to microgravity, erythropoiesis will be directly affected. An indirect effect on erythropoiesis, such as stress, is very difficult to quantify. However, measuring serum corticosteroids and examining the weight and histology of the thymus aids in determining if the animals have been stressed.

Chapter 4

Effects of Microgravity on Rat Spleen and Peripheral Blood Lymphocytes

INTRODUCTION

There have been studies indicating that humans and laboratory animals exposed to the microgravity environment of space flight become immunodeficient (9,10). Immune cells, especially CD4 T-lymphocytes, have a major role in the regulation of hematopoiesis by interleukins and cytokines, which are elaborated when these cells are activated. Specifically, activated T-cells produce IL-3 and IL-9, together with Epo, promote the differentiation of progenitor hematopoietic cells along the erythrocytic lineage (3). In this study, we assessed the immunocompetence of the flight rats by immunophenotyping the peripheral blood and spleen lymphocytes to ascertain the immunodeficiency of the flight animals. The lymphocyte subsets were enumerated with monoclonal antibodies directed against T helper (CD4), T suppressor (CD8), and major histocompatibility complex (MHC) class II for B cells.

MATERIALS AND METHODS

(See Appendix A.)

RESULTS

Flight: The absolute number of lymphocytes (see Table 1) was compared for the flight and control animals in the groups tested on days 3 or 4 before launch (L-3/4), on recovery (R+0), and on number of mission days following recovery (R+9). A difference in the absolute lymphocyte counts was discerned in the rats in the flight group on R+0 when compared to the control group. Otherwise, there were no differences between the two animal groups.

The percentage and absolute number of the CD4, CD8, and B cells are also shown in Table 1. There were no differences detected between the animal groups prior to flight on L-3/4). There were significant decreases in the numbers of CD4, CD8, and B cells in the flight animals on the day of recovery, R+0, and even a decrease in the percent of B cells. The increased percentage

of CD4 and CD8 cells in the flight animals on R+9 did not have a corresponding increase in number of cells.

DFPT: No differences in the percent of CD4, CD8, or B cells were noted in the rats included in the DFPT studies (see Tables 2 and 3). However, some differences in the absolute counts were noted in the RAHF housed rats only in the groups where the lymphocyte counts were different.

Spleen Cell Immunophenotyping: The spleen cells from the flight and control animals were compared for percent reactivity on R+0 and R+9, where no differences were discerned. In the DFPT study, there was an increased level of B cells in the flight animal group.

DISCUSSION

Rats flown on SLS-1 returned to earth in a transient state of immunodeficiency because of a lowered lymphocyte count. The animals were all bled within 8 hours of the landing of the shuttle. The percent CD4, CD8 and B cells from the flight animals was not different from the control animals; whereas, the number of lymphocytes in each subset was significantly depressed. Inasmuch as an increase in the percent CD4 and CD8 cells was noted on R+9 in the flight animals, there was no corresponding increase in the absolute number of lymphocytes in these subsets. We speculate that there was an overproduction of these two T lymphocyte subsets to compensate for the lower numbers found on R+0. We have noted the overproduction of CD4 and CD8 lymphocytes in several kidney transplant patients made immunodeficient with the monoclonal antibody OKT3 or antithymocyte serum therapy following the completion of their treatment (Ichiki and Goldman, unpublished results). Further, we believe that the immunophenotyping values obtained from peripheral blood lymphocytes can better reflect transient immunodeficient states because it is possible to get the total white blood count and the percent lymphocytes, which cannot be obtained for bone marrow aspirates or spleen cells (8,9).

Some differences in the absolute number of CD4, CD8 or B cells were observed between the RAHF housed animals in groups where there were increased lymphocyte counts between the control and flight groups. It is possible that only differences discerned were found in the RAHF

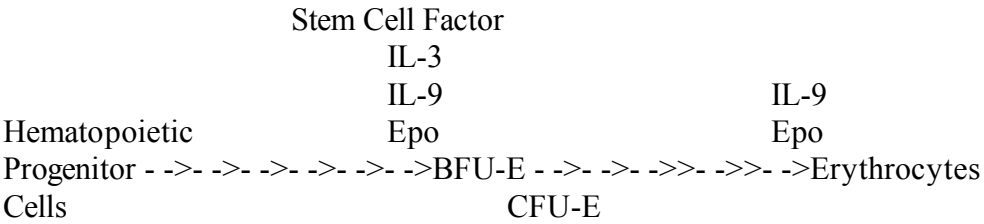
housed groups because of the larger number of animals (N=10) as compared to the AEM housed group (N=5). If housing affected these results, there would have been a consistent difference noted, which was not. Hence, the differences noted in the DFPT study only reflect the heterogeneity of the rats included in the study, which had been previously noted.

The depression of the number of lymphocytes evidently is due to factors such as microgravity, other physical stresses, physiological stresses, and other yet to be determined factors, which can impact the number of lymphocytes. The depressed white blood cell level on R+0 was in the normal range the next day, R+1 (see Chapter 3); hence, this transient state of low lymphocytes was very short lived. Blood specimens will be obtained from the rats on SLS-2 so we can compare the results from in-flight specimens to R+0 and R+9 samples. If there are no statistical differences between the results from the animals bled in space and the animals bled upon landing, we will be more confident in attributing the immunodeficient state to microgravity and not to the return to 1 g.

It was observed that cultures of human lymphocytes were not activated by the T cell mitogen, concanavalin A (Con A), in the cultures flown aboard Spacelab 1, as compared to the ground controls (2). This observation, in light of contemporary immunology, would suggest that the CD4 helper/inducer T-lymphocytes lacked the capacity to bind and endocytose the Con A, which would result in many events leading to the production of interleukin 2 (IL-2) and high affinity IL-2 receptors, and the subsequent production of many interleukins, cytokines, and growth factors. Consequently, there have been studies to determine whether the level of these immunological factors are affected by space flight in humans or laboratory animals (4,5,7), as well as cultured murine and human immune cells (1). Inasmuch as activated CD4 cells produce factors associated with immunological differentiation and proliferation, these cells also produce interleukins, cytokines and growth factors which are required for the differentiation and proliferation of other hematopoietic cells. Hence, immunodeficiency resulting from microgravity would impact hematopoiesis and, in our study, on erythropoiesis.

IL-9 and IL-3 were found to be abundant products of activated T cells and were suggested to be the most likely major factors contributing to the erythroid burst-promoting activity (BPA) (3). IL-11, which was originally isolated from a primate bone marrow stromal cell line, and the ligand for the c-kit encoded factor (stem cell factor or c-kit ligand) stimulate highly proliferative erythroid progenitors (7).

We consider the following a working model to study the role of interleukins, growth factors, and Epo for erythropoiesis:



It is our intention to assay for IL-3, IL-9, and stem cell factor, as well as for Epo, in the serum of the rats flown aboard SLS-2. By assaying for these factors in serum obtained from the rats in-flight and comparing the results postflight on R+0 as the immunophenotyping studies, it could be possible to demonstrate that the immunodeficiency caused by microgravity impacts erythropoiesis.

SUMMARY

Rats flown on SLS-1 returned to earth in an immunodeficient state because of their depressed level of peripheral blood lymphocytes. Although the percentage of CD4 helper/inducer T lymphocytes, CD8 suppressor/cytotoxic T lymphocytes, and B cells was not found to be depressed on R+0, the numbers of each of these T cell subsets and B cells were significantly lower. This state of immunodeficiency was observed to be transient because it was not observed on R+8.

Chapter 5

Summary, Conclusions and Recommendations for Future Studies

SUMMARY

The objective of these experiments was to aid in answering the question: What mechanism(s) is responsible for the anemia experienced by the astronauts? It is also to be determined if the rat is an appropriate animal model to study these mechanisms. The E012 experiment was carried out to make measurements of the various factors affecting erythropoiesis in a group of rats and their appropriate controls.

The results of these hematological studies indicated that on R+0 there was a significant decrease in the number of Epo-responsive erythroid progenitor cells as enumerated by the BFU-E progenitor cells. Also, the peripheral blood showed a significant decrease in the total WBC and in the absolute number of lymphocytes, monocytes, and eosinophils. Immunophenotyping studies of peripheral blood lymphocytes indicated a significant decrease in the absolute number of B-cells, T-helper cells, and T-suppressor cells. All values returned to the control levels by R+9. No significant differences between flight and control animals were observed in the red blood cells parameters (RBC, Hgb, Hct), serum erythropoietin level, and reticulocyte counts.

CONCLUSIONS

The exact mechanism(s) that lead to these observed changes during this flight is not completely defined. While the primary cause might be the influence of microgravity, the etiology is probably multifactorial. This might include altered hemodynamics, changes in oxygen demand or oxygen carrying capacity, and metabolic disturbances. In vertebrates, including man and rat, the overall control of red cell production is controlled by a complex network of hormones and cytokines which affect red blood cell production.

Changes we observed in the bone marrow's responsiveness to erythropoietin indicated that differentiation was either slowed or altered in some manner. With progenitor cells always present in the bone marrow, some mechanism(s) is preventing those cells from fully differentiating into mature red blood cells. It was previously thought that Epo was the major

determinant in erythropoiesis. However, as technology has developed, new cytokines are being identified as having an important part in the regulation of erythropoiesis, either positively or negatively. In addition to Epo, those that react positively include IL-3, IL-9, IL-11, and stem cell factor. Negative regulatory factors include TNF-a and TGF-b. Since we did not observe changes in the Epo levels post flight, it might be possible that these new cytokines may be involved, whether by increasing, suppressing, or altering the process of erythropoiesis in some form.

As the lymphocyte population decreases, the production of several cytokines could decrease, which could contribute to the reduction in red blood cell production. As new information becomes available, the enumeration of T-cell subsets, which was the initial step of our determining the role of lymphocytes in the complex network of hematopoiesis, may greatly contribute to the understanding of lymphocytes and erythrocytes.

In examining previously published data by other investigating teams, it is extremely difficult to make direct comparisons with their results and those presented in this report. The reasons for this are differences in length of flights, strains of rats, different post flight animal receiving times, housing differences (food and water included), and the general overall handling of the animals. However, it is not to say previously reported data is not useful. It provides a baseline and is used as a guide to the results other investigators are acquiring. With SLS-2 forthcoming and the basic scenarios being comparable to those of SLS-1, in addition to inflight experimentation, this data will be corroborated.

RECOMMENDATIONS FOR FUTURE STUDIES

1. Expanded inflight tissue procurement
2. Inflight Epo IP injections (E141)
3. Measure serum regulatory factors Epo, IL-3, IL-9, IL-11 and stem cell factor which have a direct and indirect effect on erythropoiesis.
4. Measure negative regulators (TNF-a and TGF-b) that suppress erythropoiesis.
5. Continue to gather basic hematological parameters
    a. Erythroid (RBC, Hct, Hgb, MCV, MCH, MCHC)

b. Reticulocytes

c. RBCM, plasma volume

d. Bone marrow differentials and cell cultures

e. Peripheral blood differentials

f. Peripheral blood lymphocyte subset enumeration

6. Examine thymus (weight, cellularity) and serum corticosteroids to determine stress levels

BIBLIOGRAPHY

Introduction

1. Johnson PC. The erythropoietic effects of weightlessness. In: Dunn CDR ed. Current concepts of erythropoiesis. New York: John Wiley, 1983: 279-300.

2. Tavassoli M. Anemia of spaceflight. Blood 1982; 60: 1059-1067.

3. Kimzey SL. Hematology and immunology studies. In: Johnston RS, Dietlein LF, eds. Biomedical Results from Skylab. Washington: NASA, 1977: 249-282.

4. Leon HA, Serova LV, Cummins J, Landaw SA. Alterations in erythrocyte survival parameters in rats after 19.5 days aboard Cosmos-782. Aviat Space Environ Med 1978, 49: 66-9.

5. Gazenko OG, Genin AM, Ilyin EA, Oganov VS, Serova LV. Adaptation to weightlessness and its physiological mechanisms (results of animal experiments aboard biosatellites). Physiologist 1980; 23(Suppl 6): S11-5.

6. Erslev AJ. Erythropoietin. Leuk Res 1990; 14: 683-8.

7. Quesenberry P, Levitt L. Hematopoietic stem cells. N Engl J Med 1979; 301: 755-60, 819-23, 868-72.

8. Dainiak N. Negative regulators of hematoietic stem cells and progenitors. Exp Hematol 1992; 20: 1154-5.

Chapter 1

1. Johnson PC. The erythropoietic effects of weightlessness. In: Dunn CDR, ed. Current concepts of erythropoiesis. New York: John Wiley, 1983: 279-300.

2. Tavassoli M. Anemia of spaceflight. Blood 1982; 60: 1059-1067.

3. Kalandarova MP. The effect of space flight factors on hemopoiesis. Kosm Biol Aviakosm Med 1986; 20: 7-17.

4. Rakova IA, Shvets VN. Morphologic study of the hematopoietic organs of rats during hypokinesia. Kosm Biol Aviakosm Med 1978; 12: 64-8.

5. Shvets VN, Krivenkova NP. Morphology of the bone marrow cells of rats on the Cosmos-690 biosatellite. Kosm Biol Aviakosm Med 1977; 11: 75-8.

6. Shvets VN, Portugalov, VV. Space flight effects on the hemopoietic function of bone marrow of the rat. Aviat Space Environ Med 1976; 47: 746-9.

7. Shvets VN, Vacek A, Kozinets GI, Britvan II, Korol'kov VI. Hemopoietic status of rats exposed to weightlessness. Kosm Biol Aviakosm Med 1984; 18: 12-6.

8. Serova LV, Chelnaya NA. Comparative analysis of hypergravity (2G) and weightlessness effect on the blood-forming system of male and female mammals. Physiologist 1990; 33(1 Suppl): S137-40.

9. Ilyin Ea, Serova LV, Portugalov VV, et al. Preliminary results of examinations of rats after a 22-day flight aboard the Cosmos-605 biosatellite. Aviat Space Environ Med 1975; 46: 319-21.

10. Gazenko OG, Adamovich BA, Grigoriev YG, Druzhinin YP, Ilyin EA, Popov V. Radiobiological experiment aboard the biosatellite Cosmos 690. Aviat Space Environ Med 1978; 49: 42-6.

11. Shvets VN, Portugalov V. Influence of factors involved in space flight on hematopoiesis. Biull Eksp Biol Med 1977; 83: 238-40.

12. Leon HA, Serova LV, Cummins J, Landaw SA. Alterations in erythrocyte survival parameters in rats after 19.5 days aboard Cosmos-782. Aviat Space Environ Med 1078; 49: 66-9.

13. Kozinets GI, Korol'kov VI, Britvan II, Bykova IA, Spitsyna NE. Morphofunctional properties of the peripheral blood and bone marrow cells of rats following a flight on board the Kosmos-936 biosatellite. Kosm Biol Aviakosm Med 1983; 17: 61-5.

14. Leon HA, Serova LV, Landaw SA. Effect of weightlessness and centrifugation on red blood cell survival in rats subjected to space flight. Aviat Space Environ Med 1980; 51: 1091-4.

15. Vacek A, Tkadlecek L, Shvets VN, et al. Space flight effects on haemopoietic stem cells of the bone marrow of rats. Cell Tissue Kinet 1982; 15: 643-9.

16. Vacek A, Bartonickova A, Rotkovska D, Michurina TV, Damaratskaya EI, Serova LV: The effects of weightlessness and increased gravity on hemopoietic stem cells of rats and mice. Physiologist 1983; 26 (Suppl): S131-2.

17. Vacek A, Serova LV, Rotkovska D, et al. Changes in the number of haemopoietic stem cells (CFU's) in the bone marrow and spleens of pregnant rats after a short space flight onboard the Kosmos-1514 biosatellite. Folia Biol (Praha) 1985; 31: 361-5.

18. Gazenko OG, Ilyin EA, Savina EA, Serova LV, Kaplanskii. Experiments on rats exposed in the Cosmos 1667 biosatellite (goals, protocols, results) Biol Aviakosm Med 1987; 212: 9-16.

19. Sidarenko AA, Chel'naya NA. The effect of a 13.5 day space flight on the blood profile and bone marrow of rats. In: Kosm Biol Aviakosm Med-Space Biology and Aerospace Medicine IXth All-Union Conference. Kaluga. June 19-21, 1990: 341-2.

20. Vacek A, Bartonickova A, Roskovska D, Bueverova EI, Michurina TI, Serova LV. Haemopoietic progenitor cells in the bone marrow of rats after a flight on the Cosmos 2044 Biosatellite. Proc of the XXIII International Symposium on Cosmic Biology and Medicine within the INTERCOSMOS Programme. Kosici, CSFR May 1990: 325-9.

21. Lange RD, Gibson LA, Driscoll TB, et al. Rat hematological parameters following flight on Soviet Biosatellite Cosmos 2044. Abs Int Symposium Biosatellite Cosmos. Leningrad Aug 12-15, 1991: 197-8.

22. LeBlanc. Report on blood studies from rats flown on STS-8. Unpublished data.

23. Lange RD, Jones JB, Johnson PC, Jr. Comparative aspects of hematological responses in animal and human models in simulations of weightlessness and space flight. Physiologist 1987; 30(1 Suppl): S113-6.

24. Lange RD, Andrews RB, Gibson LA, Congdon CC, Wright P, Dunn CDR, Jones JB. Hematological measurements in rats flown on spacelab shuttle SL-3. Am J Physiol 1987; 252: R216-21.

25. Lange RD, Andrews RB, Gibson LA, Wright P, Dunn CDR, Jones JB. Hematologic parameters of astrorats flown on SL-3. Physiologist 1985; 28(6 Suppl): S195-6.

26. Lange RD, Andrews RB, Gibson LA, Wright P, Dunn CDR, Jones JB. Hematological studies on rats flown on shuttle flight SL-3. In: Zanjani ED, Tavassoli M, Ascenseo JL, eds. Regulation of erythropoiesis. New York: PMA Pub. Corp., 1988: 455-66.

27. Ellis JT, Kraft LM, Lushbaugh CC, et al. Effects of cosmic particle radiation on pocket mice aboard Apollo XVII, appendix III: evaluation of viscera and other tissues. Aviat Space Environ Med 1975; 46: 639-54.

28. Ilyin EA, Korolkov VI, Kozlovskaya IB, Lobachik VI, Truzhennikov AN. The monkey in spaceflight. Physiologist 1991; 34(1 Suppl): S49-51.

29. Korolkov VI, Burkovskaya TE, Ivanova SM, Lobachik VI, Sidorenko LA, Britvan II. Influence of weightlessness on erythroid systems in monkeys. Physiologist 1992; 35(1 Suppl): S231-2.

30. Leach CS, Johnson PC: Influence of spaceflight on erythrokinetics in man. Science 1984; 225: 216-18.

31. Tanke HJ, Rothbarth PH, Vossen JM, Koper GJ, Ploem JS. Flow cytometry of reticulocytes applied to chemical hematology. Blood 1983; 61: 1091-7.

32. Tsuda I, Tatsumi N. Reticulocytes in human preserved blood as control material for automated reticulocyte counters. Am J Clin Pathol 1990; 93: 109-10.

33. Tichelli A, Gratwohl A, Driessen A, et al. Evaluation of the Sysmex R-1000, an automated reticulocyte analyzer. Am J Clin Pathol 1990; 93: 70-8.

34. Leach CS, Chen JP, Crosby W, et al. Hematology and biochemical findings of Spacelab 1 flight. In: Zanyani ED, Tavassoli M, Ascensao JL, eds. Regulation of erythropoiesis. New York: PMA Pub. Corp., 1988: 415-54.

35. Leach CS, Chen JP, Crosby W, et al. Spacelab 1 hematology experiment (INS103): influence of space flight on erythrokinetics in man. NASA Technical Memorandum 58268, Houston: Lyndon B. Johnson Space Center, 1985.

36. Leach CS, Johnson PC, Cintron NM. The endocrine system in space flight. Acta Astronautica 1988; 17: 161-6.

37. Legen'kov VI, Kiselev RK, Gudim VI, Moskaleva GP: Peripheral blood changes in the crew members of the Salyut-4 orbital space station. Kosm Biol Aviakosm Med 1977; 11: 3-12.

38. Vacek A, Rotkovska D, Serova LV, Michurina TV, Bartonickova A. the effects of space flight factors on hemopoietic stem cells of rats. Symposium Current Problems of Cosmic Biology and Medicine 1989: 271-6.

Chapter 2

1. Durnova GN, Kaplansky AS, Portugalov VV. Effect of a 22-day space flight on the lymphoid organs of rats. Aviat Space Environ Med 1976; 47: 588-91.

2. Rakova IA, Shvets VN. Morphologic study of hemopoietic organs of rats during hypokinesia. Kosm Biol Aviakosm Med 1978; 4: 64-8. (Russian), Space Biol Aerospace Med 1978; 12: 85-90. (English translation of above).

3. Lange RD, Andrews RB, Gibson LA, Congdon CC, Wright P, Dunn CDR, Jones JB. Hematological measurements in rats flown on spacelab shuttle SL-3. Am J Physiol 1987; 252: R216-21.

4. Serova LV. Weightlessness effects on resistance and reactivity of animals. Physiologist 1980; 23(Suppl 6): S22-6.

5. Anthony CP, Kolthoff NJ. Textbook of anatomy and physiology. St. Louis: C.V. Mosby, 1975: 534-44.

6. Selye H. Stress without distress. New York: Signet, 1975.

Chapter 3

1. Gazenko OG, Genin AM, Ilyin EA, Oganov VS, Serova LV. Adaptation to weightlessness and its physiological mechanisms (results of animal experiments aboard biosatellites). Physiologist 1980; 23(Suppl 6): S11-5.

2. Kozinets GI, Korol'kov VI, Britvan II, Bykova IA, Spitsyna NE, et al. Morphofunctional properties of the peripheral blood and bone marrow cells of rats following a flight on board the Kosmos-936 biosatellite. Kosm Biol Aviakosm Med 1983; 17: 61-5.

3. Shvets VN, Vacek A, Kozinets GI, Britvan II, Korol'kov VI. Hemopoietic status of rats exposed to weightlessness. Kosm Biol Aviakosm Med 1984; 18: 12-6.

4. Ilyin EA, Serova LV, Portugalov VV, et al. Preliminary results of examinations of rats after a 22-day flight aboard the Cosmos-605 biosatellite. Aviat Space Environ Med 1975; 45: 319-21.

5. Lange RD, Andrews RB, Gibson LA, Congdon CC, Wright P, Dunn CD, Jones JB. Hematological measurements in rats flown on Spacelab shuttle. Am J Physiol 1987; 252: R216-21.

6. Taylor GR, Dardano JR: Human cellular immune responsiveness following spaceflight. Aviat Space Environ Med 1983; 54: S55-9.

7. Taylor GR, Neale LS, Dardano JR. Immunological analyses of U.S. Space Shuttle crew members. Aviat Space Environ Med 1986; 57: 213-7.

8. Durnova GN, Kaplansky AS, Portugalov VV. Effect of a 22-day space flight on the lymphoid organs of rats. Aviat Space Environ Med 1976; 47: 588-91.

9. Khan KNM, Kociba GJ, Wellman ML, Reiter JA. Effects of tumor necrosis factor-alpha on normal feline hematipoietic progenitor cells. Exp Hematol 1992; 20: 900-3.

10. Miller KL, Carlino JA, Ogawa Y, Avis PD, Carroll KG. Alterations in erythropoiesis in TGF-B1-treated mice. Exp Hematol 1992; 20: 951-6.

11. Legen'kov VI, Kiselev RK, Gudim VI, Moskaleva GP: Peripheral blood changes in the crew members of the Salyut-4 orbital space station. Kosm Biol Aviakosm Med 1977; 11: 3-12.

Chapter 4

1. Chapes SK, Morrison DR, Guikema JA, Lewis ML, Spooner BS. Cytokine secretion by immune cells in space. J Leukoc Biol 1992; 52: 104-10.

2. Cogoli A, Tschopp A, Fuchs-Bislin P. Cell sensitivity to gravity. Science 1984; 225: 228-30.

3. Donahue RE, Yang YC, Clark SC. Human P40 T-cell growth factor (interleukin-9) supports erythroid colony formation. Blood 1990; 75: 2271-5.

4. Gould CL, Lyte M, Williams J, Mandel AD, Sonnenfeld G. Inhibited interferon-g but normal interleukin-3 production from rats flown on the space shuttle. Aviat Space Environ Med 1987; 58: 983-6.

5. Konstantinova IV, Sonnenfeld G, Lesnyak AT, ***et al***. Cellular immunity and lymphokine production during spaceflights. Physiologist 1991; 34(1 Suppl): S52-6.

6. Quesniaux VF, Clark SC, Turner K, Fagg B. Interleukin-11 stimulates multiple phases of erythropoiesis in vitro. Blood 1992; 89: 1218-23.

7. Sonnenfeld G, Mandel AD, Konstantinova IV, et al. Effect of space flight on immune responses: Bone marrow cell response to colony stimulating factor and leukocyte subsets. Physiologist 1992; 35(1 Suppl): S222-3.

8. Sonnenfeld G, Mandel AD, Konstantinova IV, et al. Effects of spaceflight on levels and activity of immune cells. Aviat Space Environ Med 61:648-653, 1990.

9. Sonnenfeld G, Mandel AD, Konstantinova IV, et al. Spaceflight alters immune cell function and distribution. J Appl Physiol 1992; 73(2 Suppl): 191S-195S.

10. Sonnenfeld G, Taylor GR. Effects of microgravity on the immune system. SAE Technical Paper Series, #911515, 1991.

APPENDIX A

1. Ogawa M: Methylcellulose clonal cell culture of murine and human erythropoiesis. In: Murphy MJ, Peschle C, Gordon AS, Mirand EA, eds. In vitro aspects of erythropoiesis. New York: Springer-Verlag, 1978: 248.

2. Burke WT, Brotherston G, Harris C. Improved technique for obtaining bone marrow smears from the rat. Am J Clin Pathol 1955; 25: 1226-8.

3. Harris C. The lymphocyte-like cell in the marrow of rats. Blood 1961; 18: 691-701.

ACKNOWLEDGMENTS

The authors wish to thank the following for their assistance and encouragement for which our experiments could not have been completed:

STS-40 Flight Crew

Ames Research Center Personnel

Kennedy Space Center Hangar L Personnel

Dryden Payload Receiving Facility Personnel

Nina Mushell - Experiment Support Scientist for E012

University of Tennessee Flow Cytometry Facility - Joseph E. Fuhr, Ph.D., Director

Hematology Laboratory, Department of Pathology, University of Tennessee Medical Center at Knoxville, Frances Jones, M.D., Chairman

Erika Amaba and Lisa Payne for their technical assistance

Paul Wright, M.S., for all statistical work

The original E012 experiment was designed by:

P.C. Johnson, M.D.*

C.D.R. Dunn, Ph.D.

Their many contributions are acknowledged.

*Deceased

APPENDIX A

MATERIALS AND METHODS

Experimental Design and Selection Process:

Animals: 163 Sprague-Dawley rats were obtained from the Taconic Farms (Indianapolis, IN). They were brought into the Kennedy Space Center at approximately five weeks of age. At that time, they were singly housed with their food and water given *ad libitum*. 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 any of those that did not show a consistent pattern were not chosen either for flight or as ground controls. At L-4, the rats were randomly divided into group or individually housed animals. The first injections and phlebotomies were performed on all of the animals on L-4 (R+9 animals) and L-3 (R+0 animals). Subsequent 24-hour blood samples were taken on L-3 (R+9 animals) and L-2 (R+0 animals). After the last blood sample was taken on L-2, the final flight selection was made. The only criteria available to the #E012 investigators in order to make this decision were the following:

Food and water consumption

Body weight

Complete blood cell count - focusing mainly on the red blood count and white blood count

Hematocrit

The flight selection was given to Ames personnel for random placement into the RAHF and AEM modules. Contingency plans were utilized to repeat this procedure. Thus, a second group of animals was prepared, selected, and placed into the modules.

Prior to launch, a malfunction of one of the RAHF water lixits prevented one of the RAHF rats from flying. This brought the number of RAHF animals flown to 19 from the original 20. The number of animals and their designated housing plan is seen in Table 1. There were 10 AEM flight animals and 19 RAHF animals flown aboard Columbia. On the ground were matched 10 AEM controls and 20 RAHF controls.

After the launch of Columbia, all supplies, equipment, and ground control animals were flown to the DPRF. This took place two days post launch. The AEM ground control animals were in a non-flight AEM while being transported to the Dryden facility. The RAHF animals were removed from their individual vivarium cages and placed in groups in regulation transportation boxes. Once at Dryden, the RAHF animals were then placed back into individual vivarium cages. The light and dark cycles for both the AEM and RAHF were kept the same as those flying on Columbia.

Figure 1 portrays the experimental plan. On R+0 or day of landing, five of the AEM control animals had their final 3 ml blood sample taken and were then killed to obtain other tissues. Upon arrival at the payload facility, five of the AEM flight and 10 of the RAHF flight animals followed the same protocol, followed by ten RAHF ground controls. The remainder of the animals, both flight and controls, were held 9 days post landing and then proceeded to follow the same protocol as on R+0.

Delayed Flight Post Test (DFPT):

The DFPT was performed at KSC. This test was performed to assure that results seen on SLS-1 were really the effect of microgravity and not housing, noise, etc. Environmental factors recorded aboard Columbia were simulated on the ground as much as was feasible. This included the light/dark cycle, temperature, humidity, noise, and housing. A non-flight RAHF and AEM were used for the simulation. Timing of all procedures were flight simulated. The only simulation not included was the transportation of the ground controls from KSC to Dryden 2 days post launch.

HEMATOLOGICAL TECHNIQUES UTILIZED:

Determination of hematological parameters were performed during the preflight period, on R+0, and during the postflight period (Table 2). The procedures were repeated during the DFPT. The total number of determinations on specimens collected for subsequent analysis are also presented in Table 2. Table 3 summarizes the methods used.

Blood Cell Counts: Automated blood cell counts were performed using the TOA Sysmex F-800^{TM} microcell counter. These measurements were made onsite shortly after the acquisition of the blood samples. Standard blood controls were analyzed prior to any test samples to ensure the performance of the machines. Standard procedures were used on 20 ml of blood, with the exception of the lysing solution used on the white blood cell count; three drops are standard, but seven drops were required in order to lyse all of the RBC's. All samples were analyzed twice and the average computed. Any obvious anomalies were discarded and the samples reanalyzed. The counts included: white blood cell count, platelet count, red blood cell count, hematocrit, hemoglobin, and red blood cell indices mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration.

Reticulocyte Counts:

Two methods were utilized in determining reticulocyte counts:

A. Flow Cytometry: Blood was collected via the rat tail vein as part of the regular blood sampling. Ten microliters were removed and placed into a prelabeled, heparinized microtube and held on ice until ready for shipment to Knoxville. The samples were shipped under refrigeration and, when received in the Knoxville laboratory, brought to room temperature. Each sample was thoroughly mixed. The blood sample, 5 ml, was pipetted into 1 ml of thiazole orange stain, mixed, and immediately placed in the dark at room temperature for a minimum of 30 minutes. The samples were remixed and placed on the Becton-Dickinson FACScan flow cytometer. A software program, ReticCount^{TM} by Becton Dickinson, San Jose, CA, analyzed the results of 10,000 events. The percentage and number of reticulocytes was then calculated.

B. Manual Reticulocyte Counts: The reticulocyte slides were prepared at the dissection site shortly after blood collection. Ten microliters of blood were added to 10 ml of New Methylene Blue-N. This mixture was allowed to stain for a minimum of 3 minutes, after which standard smears were prepared. The slides were returned to Knoxville, where they were counterstained using a Dif-Quik^{TM} staining procedure. A total of 2,000 red blood cells were

counted by 2 different technicians. The number of reticulocytes were noted and percentages recorded. The average between the two values was recorded.

Erythroid Colony Assay: The cultures were carried out according to the method described by Ogawa (1978), with some modifications. One femur was removed from each of the flight and control rats and was returned to the Knoxville laboratory under refrigeration, where the bone marrow was flushed into a 15 ml conical tube. The cells were washed 3 times using Iscove's Modified Dulbecco's Medium (IMDM) (Whittaker, Walkersville, MD) and suspended in a final concentration of 4×10^6 cells/ml. Four 15 ml conical tubes containing the culture mixture were prepared for each rat. One milliliter of the culture mixture contained 1.3×10^5 total bone marrow cells suspended in 0.8% methylcellulose (Terry Fox Laboratories, Vancouver British Columbia) in IMDM, 10% rat spleen-conditioned medium, L-glutamine, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4} beta-mercaptoethanol, 10,000 units/ml penicillin, 10,000 mg/ml streptomycin, and either one of 3 concentrations of Epo (0.25, 0.5, 1.0 U/ml) or none. One milliliter from each tube was aliquoted into one Petri dish, repeated 6 times, and incubated at 37^oC in a humidified atmosphere containing 7.5% CO_2. Any remaining cells were immediately frozen using a standard 90% fetal calf serum and 10% dimethyl sulfoxide (DMSO) mixture and placed under liquid nitrogen. On day 3 after culturing, three plates per each Epo dose were stained with 2,7 Diaminofluorene (DAF) and the CFU-E were enumerated. The other three plates per EPO dose were stained with DAF on day 7 and the BFU-E colonies were enumerated. All cultures were read using a Nikon inverted microscope.

Bone Marrow Differential: The bone marrow smears were prepared at the Knoxville laboratory from the head of the femur. The "paint brush" technique was used to make bone marrow preparations for differential counting (2,3). Bone marrow slides were fixed in absolute methanol for 10 minutes and then stained for 10 minutes with Wright's stain, after which tap water was added for 20 minutes to neutralize the stain. After rinsing, the slides were stained for 10 minutes with freshly prepared and filtered Giemsa stain. After rinsing and drying, 200 cell

differential counts were performed. The bone marrow cells were classified into the following cell types:

Small lymphocytes

Large lymphocytes

Plasma cells

Reticulum cells

Mature myeloid cells (metamyelocytes, bands, segmented)

Immature myeloid cells (myeloblasts, promyelocytes, myelocytes)

Eosinophils

Tissue Basophils

Mature erythroid cells (polychromatophilic and acidophilic normoblasts)

Immature erythroid cells (pronormoblasts and basophilic normoblasts)

The myeloid to erythroid ratio was calculated in order to determine which cell lineages were suppressed

Spleen Cell Differentials: Spleen slides were prepared at the DS using the same "paint brush" technique as the bone marrow. The slides were fixed and then transported back to Knoxville, where they underwent staining on a Miles Hema-Tek 1000^{TM}. Two hundred spleen cells were classified as small lymphocytes, large lymphocytes, myeloid cells, and erythroid cells.

Peripheral Blood Differentials: Peripheral blood smears were prepared at the DS using 10 ml whole blood plus 5 ml sterile saline. This method was utilized as a precaution in case the rats became dehydrated, causing the peripheral blood to increase in viscosity. The smears were fixed at the DS, transported to the home lab, and stained on the Miles Hema-Tek 1000^{TM}. Three personnel read separately 100 cells from each slide for a total of 300 cells and the results averaged.

Peripheral Blood and Spleen Lymphocyte Subsets Enumeration: A method for lymphocyte subsets, developed by Becton-Dickinson and printed in their monoclonal antibodies package, was modified to accommodate rat blood and spleen for the SLS-1 flight. Both the blood

and spleen cells were obtained at either KSC or Dryden and then shipped back to the Knoxville laboratory. The blood was held at room temperature and the spleen sections under refrigeration in RPMI-1640 medium supplemented with calf serum, L-glutamine, and beta-mercaptoethanol. The cells were then prepared by performing a series of washings using media and followed by incubation with specific amounts of the following monoclonal antibodies: (obtained from Bioproducts for Science, Indianapolis, IN)

43F - Leukocyte common antigen

48P - T-suppressor/cytotoxic

55F - T cells (helper) and macrophages

340 - B-lymphocytes

341 - Monocytes and macrophages

149A - Granulocytes (spleen only)

Both spleen and blood cells from each rat had a control determined simultaneously. The cells were fixed and enumerated on the Becton-Dickinson FACScan flow cytometer using the Consort 30 software. A total of 2,000 events were collected for each sample. All data were stored on discs and each rat's sample data individually analyzed at a later date.

Serum Erythropoietin Level: Prior to decapitation, blood (3 ml) was obtained from the tail vein using no anticoagulant. The blood was allowed to clot and then centrifuged to obtain the serum samples. The serum was immediately frozen to -70^{o}C. The samples were then transported to Knoxville on dry ice and stored at -70^{o}C. Epo was measured using a commercial radioimmunoassay kit manufactured by Diagnostic Systems Laboratories, Inc. (Houston, TX). The samples were counted and Epo levels calculated using the Cobra 5005 gamma counter and software by Packard (Downers Grove, IL).

Spleen Histology: Spleen samples were obtained at dissection. Weights were taken and blocks made. The histology sample (approximately 1/12 of the spleen) was placed in 50 ml of 10% neutral buffered formalin and kept at room temperature. The fixed samples were sent to

Knoxville for processing. Paraffin blocks were sectioned for Hematoxylin and Eosin staining of slides. The slides were examined by light microscope.

Statistical Analysis: Statistical analyses were performed by the biostatistician at the University of Tennessee Medical Center. The flight data from the AEM flight and RAHF flight animals were combined as were the AEM control and RAHF control data. However, for the DFPT, all groups were kept separate, include the rats killed on R+0, which were kept separate from those killed on R+9. The statistical test utilized varied depending on the variability of the data. The following tests were used throughout the data analysis:

Student t-test (equal variables)

Student t-test (unequal variables)

Wilcoxan (Z approximate)

Median (Z approximate)

The 012 experiment was carried out to make measurements of the various erythropoiesis factors in a group of rats and their appropriate controls. It was hoped to determine whether the rat was an appropriate model for the hematological changes which occur in astronauts during space flight.

1. There were no significant differences between flight and control animals for many parameters.
2. Differences were found for the following parameters
    a. The BFU-E in the bone marrow of flight animals were decreased in number an R+O
    b. the CFU-E of flight animals bone marrow were found to be decreased in response to EPO of the EPO dose levels of 0.25 and 0.50/u/ml.

       These differences had returned to normal by R+9.
    c. The white blood cell counts of flight animals were decreased.
    d. The absolute number of the following lymphocyte subsets in flight animals were decreased: B cells, T-helper and T-suppressor cells.
    e. Flight animals decreased their food intake post flight.
    f. The flight of control animals from Florida to California affected their food intake.
3. The red blood cell parameters (RBC, Hgb, Hct) of the rats increased as they grew.
4. The DFPT results were variable and indicated that the rat population differed from the flight animals and were perhaps heterogeneous.
5. Reticulocyte counts by the manual method were decreased in comparison to counts enumerated by flow cytometry.
6. It was pointed out that due to the number of tests performed that some differences could have been due to chance.
7. The literature was reviewed and the results indicate that further studies are needed.

CONCLUSIONS

The result of the present studies are at variance with previous studies. In part this may be due to the different lengths of exposure to microgravity, in part to the different strains of animals used and also due to the different sex of the animals flown. The isotope experiments of E141 indicate that when rats are exposed to microgravity that there is a reduction in RBCM. Since actual counts depend on the plasma volume the isotope studies are needed to confirm that the rat may serve as a model for erythropoiesis changes due to exposure to microgravity. The variable results from previous studies indicate that more studies need to be performed.

RECOMMENDATIONS FOR FUTURE STUDIES

1. A strategy for space biology and medical science for the 1980's and 1990's, the following recommendations were listed for the study of the anemia of space flight.
   a. There is a need for more detailed studies of the time course and magnitude of the anemia.
   b. Erythropoietin levels in the blood
   c. Red blood cell survival studies
   d. Bone marrow studies
   e. Clinical implications
2. An ad hoc committee of the Federation of American Societies of Experimental Biology has recommended that the following parameters be measured in ground based and in flight studies (pre, in, and post flight) red cell count*, hemoglobin*, hematocrit*, red cell mass*, blood volume*, plasma volume*, reticulocyte count*, erythropoietin*, plasma or serum haptoglobin, platelets*, red cell shape*, red cell size, blood P50, blood $PC0_2$, red cell 2,3 - DPG, red cell ATP, red cell sodium, bone marrow smear*.

   In flight animals the presence of skin petechiae* and subcutaneous and subserosal oozing of RBC*.
3. The items marked with an asterisk will be measured in SLS-2.

4. Since the above recommendations were made, many technical advances have been accomplished. These include growth factors, cytokines and receptors. It should now be possible to measure many of these including IL-3, IL-9, IL-11 an stem cell factor. In addition all the organs involved in the Selye adaptation syndrome could be examined. These include: Thymus, other lymphatic tissues, adrenal weights and other measures that relate to current concepts of acute and subacute stress.