Effects of spaceflight and PEG-IL-2 on rat physiological and immunological responses

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Chapes, Stephen K., Steven J. Simske, Gerald Sonnenfeld, Edwin S. Miller and Robert J. Zimmerman. Effects of spaceflight and PEG-IL-2 on rat physiological and immunological responses. J. Appl. Physiol. 86(6): 2065–2076, 1999.—Sprague-Dawley rats were subjected to two 8-day spaceflights on the space shuttle. Rats housed in the National Aeronautics and Space Administration's animal enclosure were injected (iv or sc) with pegylated interleukin-2 (PEG-IL-2) or a placebo. We tested the hypothesis that PEG-IL-2 would ameliorate some of the effects of spaceflight. We measured body and organ weights; blood cell differentials; plasma corticosterone; colony-forming units (macrophage and granulocyte macrophage); lymphocyte mitogenic, superantigenic, and interferon-γ responses; bone marrow cell and peritoneal macrophage cytokine secretion; and bone strength and mass. Few immunological parameters were affected by spaceflight. However, some spaceflight effects were observed in each flight. Specifically, peritoneal macrophage spontaneous secretion of tumor necrosis factor-α occurred in the first but not in the second flight. A significant monocytopenia and lymphocytopenia were detected in the second but not in the first flight. The second mission produced bone changes more consistent with past spaceflight investigations. PEG-IL-2 did not appear to be beneficial; however, this was mostly due to the lack of spaceflight effects. These studies reflect the difficulty in reproducing experimental models by using current space shuttle conditions.

pegylated interleukin-2; animal enclosure module; space shuttle; bone; lymphocyte; macrophage

In animals and humans, spaceflight has dramatic effects on several physiological and immunological parameters, including bone homeostasis (37, 61, 62), muscle mass (38, 51), immune cell number and function (53), hematopoiesis (27, 47–49, 60), and fluid shifts and cardiovascular changes (41, 58). Serum glucocorticoid levels (34) and lymphocyte blastogenic responses (31, 54, 55) have also been reported to change; however, these responses are not consistently altered by spaceflight (24, 31, 33). This variability may be related to parameters such as flight duration and flight platforms (e.g., shuttle vs. biosatellites) as well as to individual subjects' adaptation to the stresses of spaceflight (launch, microgravity, motion sickness, and landing acceleration). Recently, more frequent shuttle flights have allowed measurements of immunological and physiological parameters. Even with regular spaceflight, exact flight replications remain difficult and will probably remain so in the foreseeable future. Therefore, a consensus understanding of the effects of spaceflight on immunological and physiological systems is still being developed.

Because of the physiological consequences of spaceflight, there is considerable interest in development of effective countermeasures to the consequences of weightlessness. The immunosuppression and altered bone and muscle metabolism are of particular concern as longer manned missions are planned. To address these issues, we treated rats immediately before spaceflight with pegylated interleukin-2 (PEG-IL-2), a longer acting preparation of recombinant human aldesleukin interleukin-2 (IL-2) (15, 16, 57, 65). Some of the reported consequences of spaceflight on the human immune system include depressions in activity and numbers of natural killer cells, T cells, and monocytes. IL-2 and PEG-IL-2 have been shown to increase both the absolute numbers and activities of these cell types in both humans and animals (2, 32, 52, 63). Therefore we hypothesized that PEG-IL-2 treatment of rats would influence these and, perhaps indirectly, other parameters during spaceflight. To test this hypothesis, data were collected during two 8-day shuttle flights (Immune 1, STS-60; and Immune 2, STS-63) from 12 flight rats (6 controls and 6 PEG-IL-2-treated animals), as well as from four ground-based studies designed to control for spaceflight environmental factors.

MATERIALS AND METHODS

Animals. The animal handling protocols used for these studies were reviewed by the Kennedy Space Center (KSC) veterinary staff and approved by the appropriate National Aeronautics and Space Administration (NASA) animal care and use committee. Specific pathogen-free, male, 5- to 6-wk-old rats were obtained from Charles River Laboratories (Sprague-Dawley-derived CD rats, Wilmington, MA; Immune 1) or Taconic Laboratories (Sprague-Dawley rats, Germantown, NY; Immune 2). Rats were monitored for food and water intake and for health status for 1 wk by the veterinary staff at KSC before launch or use in control experiments. Rats weighed ~185–200 g at the time of treatment.
On their return from spaceflight, the rats were examined by the KSC veterinarian. After recovery of the animals in the Immune 1 study, two of the six rats injected iv with PEG-IL-2 and five of the six control rats injected with diluent were found to exhibit various degrees of damage to their tails, including tissue necrosis, loss of tissue, and gangrenous tissues. There were no other abnormal clinical observations. Examination of tail tissues by a veterinary pathologist confirmed the hypothesis that the tail conditions were consistent with thermal damage, possibly due to the use of heated water to enlarge the lateral tail veins before iv injection. None of the rats selected for flight showed any symptoms on inspection by the veterinarian before the launch, and none of the ground-based animals exhibited any abnormalities during the course of these studies. The data obtained from the rats with damaged tails were not statistically different from the data obtained from the other rats with respect to any of the analyses that were done. Because the number of individuals in each treatment group was small (n = 6) and there were no statistically valid reasons for excluding rats with tail damage, we followed our original experimental protocol for the Immune 1 flight.

To kill the rats, we first anesthetized them by injection of 75 mg/kg ketamine hydrochloride ip (Ketaset; Fort Dodge Labs, Fort Dodge, IA) and 5 mg/kg xylazine (Rompun; Miles Labs, Shawnee Mission, KS); then they were exsanguinated by cardiac puncture. Flight conditions. The experiments were conducted on shuttleflights STS-60 (Immune 1, February 1994) and STS-63 (Immune 2, February 1995). Both Immune 1 and 2 were 8-day flights that lasted 193 and 198 h, respectively. Animals were housed in NASA’s animal enclosure module (AEM) (8, 46), with six rats per AEM, two AEMs per flight, in the shuttle middeck. During Immune 1, animals were exposed to a mean temperature of 25.0 ± 0.1(±1)°C (range, 21.9–26.3°C) and an average of 26.3 ± 0.1°C (range, 24.7–29.4°C) during Immune 2. Animals had access to solid food bars and water ad libitum, and they were exposed to a 12:12-h light-dark cycle.

Two ground-based studies were designed to evaluate the potential impact of some of the environmental aspects of the flight conditions. Twelve (Immune 1) or 18 (Immune 2) rats were maintained in AEMS (2 in Immune 1, 3 in Immune 2) kept in a light-, temperature-, humidity-, and CO2-controlled chamber. In each study, one-half the number of animals were treated with PEG-IL-2, and one-half the number of animals were placebo controls. These animals, the flight controls, were treated on a 24-h delay compared with the flight animals to allow for the computer downlinking of the shuttle AEMS’s data on temperature, humidity, CO2, and exact light-dark cycles. The vivarium controls, a second group of 12 rats (6 treated, 6 controls), were maintained under normal vivarium conditions and were treated on a 48-h time delay relative to the shuttle animals.

Dissections commenced ~3 h after shuttle landing and were completed in <6 h for Immune 1 and in ~4 h for Immune 2. Regression analysis showed no correlation between the time animals were killed and the responses measured in these studies, either in the flight studies or in the multiple preflight verification tests that were performed before the Immune 1 and 2 flights. Samples were shipped by express courier to the laboratories where assays were conducted. Preflight verification tests were done to show that cell and tissue responses would not be affected by the time delay between tissue recovery and assay. Unless otherwise stated, samples were kept cold at ~4°C from the time of dissection until assay.

PEG-IL-2 treatment. Rats for Immune 1 were treated with PEG-IL-2 (0.5 mg/kg iv; Chiron, Emeryville, CA), whereas rats in the Immune 2 study were injected with PEG-IL-2 (1.0 mg/kg sc, in 200–300 ml pyrogen-free saline) 2–3 h before transfer to either the AEMs or vivarium housing. The two treatment routes and dose levels resulted in similar areas under the curve on the basis of the known pharmacokinetic behavior of PEG-IL-2 in rats (65), while, at the same time, the pharmacodynamics of these two routes could be compared (sc-to-iv comparisons; Sharon A. Chen, Chiron Corporation, personal communication). These treatments resulted in no evidence of toxicities attributable to PEG-IL-2 (22, 56). The treatments were selected on the basis of preliminary ground-based dose-finding experiments.

Body and organ weights. Body weights were taken at the time of dosing and when the animals were killed. Brain, heart, kidneys, liver, lungs, trachea, spleen, and thymus weights were also obtained when the animals were killed.

Hematology and corticosterone analysis. Whole blood was collected, by cardiac puncture, into 10-ml heparinized syringes. A portion of this blood sample was shipped overnight on wet ice to Consolidated Veterinary Diagnostics (Sacramento, CA) for determination of complete cell counts and differentials.

The remaining portion of this blood sample was sent under the same conditions to Kansas State University for corticosterone analysis. The heparinized blood was centrifuged to separate plasma ~24 h after collection and was stored at −20°C until the corticosterone concentration was quantitated by competitive radioimmunoassay. Plasma and stock corticosterone solutions were extracted with ethyl acetate before the initiation of the radioimmunoassay, as described previously (21). The lower limit of sensitivity for rat plasma samples was determined to be ~10 ng/ml.

Bone marrow macrophage (M) and granulocyte-macrophage (GM) colony-forming unit (CFU) assay. Macrophage and granulocyte-macrophage colony-stimulating factor (M-CSF and GM-CSF, respectively)-dependent macrophage colony formation from bone marrow cells was assayed by using a modification of procedures described previously (49). Briefly, bone marrow cells were obtained from the femora by removing the bone end with the use of Dulbecco’s modified Eagle’s medium (DMEM) and a 21-gauge needle. The cells were passed through the needle three times to break up clumps, resuspended in 15 ml of DMEM that contained 10% fetal bovine serum (FBS), and then placed on ice and shipped overnight to Kansas State University (CFU-M) or to the Carolinas Medical Center (CFU-GM) for assay. The cells were pelleted, treated with 0.17 M NH4Cl to hypotonically lyse the red blood cells, centrifuged, and resuspended at a concentration of 1.67 × 106 cells per 1.5 ml of DMEM containing 0.3% agar, 10% FBS, and 150 ng/ml of recombinant human M-CSF (Chiron) or 100 U/ml recombinant GM-CSF (Genzyme, Cambridge, MA). After 7–8 days of culture, several microscope fields were scored for colonies (25–50 cells).

Lymph node and spleen cell proliferation assays. Lymphocytes were obtained by expression of the cells from the spleen or the axillary and inguinal lymph nodes through a tissue sieve (Fallon, no. 2350). The cells were resuspended in 15 ml of DMEM that contained 10% FBS and were placed on ice and shipped overnight to Kansas State University or the Carolinas Medical Center for assay. Before assay, splenic lymphocytes were centrifuged, resuspended in 0.17 M NH4Cl, centrifuged, resuspended, and washed twice with DMEM that contained 2% FBS and 50 µg/ml of gentamycin sulfate. Lymph node lymphocytes were treated similarly, except the
hypotonic lysis was omitted because few red blood cells contaminated the lymph node samples. Lymphocytes (5 × 10^6 in 100 µl) were added per well in 96-well, flat-bottom microtiter plates (Costar, Cambridge, MA). Wells received an additional 100 µl of DMEM supplemented with 1 × 10^{-3} M 2-mercaptoethanol, 2% FBS, and 50 µg/ml of gentamycin sulfate, with or without either 1) phytohemagglutinin (PHA; 5 µg/ml final concentration; Wellcome Biotechnology, Research Triangle Park, NC), 2) concanavalin A, (ConA; 5 µg/ml final concentration; Sigma Chemical, St. Louis, MO), 3) dextran-liopolysaccharide (dextran at 10 µg/ml final concentration; Sigma Chemical); liopolysaccharide (LPS; final concentration 10 µg/ml; E. coli O55:B5; Difco, Detroit, MI), or 4) staphylococcal enterotoxins A or B (SEA or SEB, respectively; 10 µg/ml final concentration; Toxin Technologies, Sarasota, FL). The cells were incubated for 48–72 h and were then pulsed with 0.5 mCi per well of [3H]thymidine for 6–8 h before harvest. The cells from each well were harvested with a Cambridge PHD cell harvester on glass-fiber filters, placed in scintillation-counting fluid, and counted on a Packard-500 scintillation counter. Stimulation index (SI) was calculated by

\[ SI = \frac{cpm \text{ stimulated sample}}{cpm \text{ sample in medium alone}} \times 100 \]

where cpm is counts per min.

Cytokine assays. Bone marrow cell cytokine secretion was assayed by incubating 5 × 10^4 isolated cells in 3 ml of RPMI supplemented with 10% FBS for 18–20 h, as previously described (4, 5). Clarified supernatants were stored at −70°C for cytokine assay. Bone marrow cells were assayed for secretion of M-CSF, IL-6, and transforming growth factor-β (TGF-β).

Resident peritoneal macrophages were obtained by washing the rat peritoneal cavity with 100-ml of ice-cold phosphate-buffered saline (PBS). The cells were pelleted, resuspended in medium, and shipped to Kansas State University by using the above-described procedures. Macrophages were centrifuged, resuspended in 0.17 M NH₄Cl, centrifuged, and washed twice with DMEM containing 2% FBS and 50 µg/ml of gentamycin sulfate. After cells were counted, 1 × 10^5 cells per well were added to 16-mm tissue culture wells (24-well clusters, Costar). Macrophages were incubated for 2 h, washed, and then refed with either 1 ml of medium alone or medium containing LPS (12.5 µg/ml) or SEA (10 µg/ml). Cells were incubated for 18 h, and clarified supernatants were stored at −70°C until cytokine assays were performed.

IL-6 concentrations in the supernatants were determined by an MTT bioassay by using the IL-6-dependent cell line B9 (26) and a recombinant human IL-6 standard curve with a sensitivity of 0.1 pg/ml (R&D, Minneapolis, MN). TGF-β content was assessed by an MTT bioassay by using the TGF-β-sensitive cell line CCL64 (17) and a recombinant TGF-β standard curve with a sensitivity of 0.1 pg/ml (R&D). M-CSF concentrations in the supernatants were determined by an MTT bioassay by using the M-CSF-dependent cell line B6MP102 (12) and a recombinant human M-CSF standard curve with a sensitivity to 5 ng/ml. Tumor necrosis factor-α (TNF-α) was determined by an MTT bioassay by using the TNF-sensitive cell line LM-929, as described previously (20). Sensitivity of the assay was 0.5 U/ml (specific activity: 200 pg/unit).

Interferon-γ (IFN-γ). Levels of IFN-γ were determined in supernatant fluids of spleen cells placed in 96-well culture dishes (3 × 10^6 cells/ml) and challenged with 5 µg/ml ConA (23). Cultures were incubated for 48 h at 37°C in 5% CO₂. After the supernatant fluids were harvested, IFN-γ levels were determined for Immune 1 by using the virus-inhibition test previously described by Gould et al. (23). For Immune 2, IFN-γ concentration was quantified by using a rat IFN-γ ELISA kit (Biosource International, Cupertino, CA) following the manufacturer’s recommendations. The plates were read at 450 nm by using an ELISA plate reader (Dynatech, Chantilly, VA). IFN-γ titers, expressed in picograms per milliliter, were derived by comparison with standard curves determined along with the samples.

Bone processing. The left humerus (Immune 2 only), femur, and tibia (both flights) were collected from each rat, cleaned of nonosseous tissue, and frozen (−70°C) for storage. After they were shipped to the University of Colorado, the bones were thawed slowly (4°C, 18 h) before they were rewetted for 1.5 h in PBS (10) before mechanical tests were performed.

Mechanical tests were performed in flexure by using an Instron 1331 servohydraulic testing system (44). For Immune 1, the femora and tibiae were tested to failure at a deflection rate of 10 mm/min sampled at 33 Hz. The span lengths for testing were 15 mm for the femora and 20 mm for the tibiae. Mechanical testing on the bones from Immune 2 were performed on the femora (10-mm span length), humeri (10 mm), and tibiae (15 mm) and sampled at 20 Hz. The spans were shortened for Immune 2 to prevent oblique fractures that occurred among 10–15% of the bones in Immune 1. For all tested bones, force-deflection parameters of strength (P; in Newtons), deflection (d; in millimeters) and energy (in milli-Joules), were calculated by using custom software at the elastic (ε) limit (Pₑ, dₑ, and Aₑ), maximum force (m: Pₓ, dₓ, and Aₓ), and failure (f: Pₓ, dₓ, and Aₓ × lₓ × Aₓ), where Aₓ is energy absorbed by the bone during the elastic part of the curve force-deflection curve, Aₓ is the energy absorbed by the bone up to the maximum force, and Aₓ is energy absorbed by bone up to failure. Stiffness (S) was calculated from Pₓdₓ. These properties have been described in more detail elsewhere (44, 45).

After mechanical testing, the bones were allowed to air dry (25°C, 48 h, Bone-M), then dried at 105°C for 24 h (Dry-M), and ashed at 800°C for 24 h (Min-M), where M is the mass determined by the procedure. The percent mineral (%Min) was calculated from (Dry-M/Min-M) × 100.

Statistical analysis. The bone data were analyzed by analysis of variance (ANOVA) followed by Scheffe’s test to compare different groups. All other data were assessed for normality by using the Shapiro-Wilk’s normality test. Normally distributed data (all presented data: Shapiro-Wilk’s W ≥ 0.85) were analyzed by a two-way ANOVA. Sets with F-scores ≤ 0.05 were analyzed for intergroup differences by using Student t-tests. Sets were considered statistically significantly different by t-test analysis with P < 0.05. However, for some data, P values < 0.1 are presented to show trends toward significance. Analyses were done with the Statmost for Windows Statistical Package (Data Most, Salt Lake City, UT).

RESULTS

Physiological parameters. We assessed the overall health and immunological status of the animals by measuring body weight, thymus weight, spleen weight, and plasma corticosterone levels (Tables 1 and 2). The young rats used in these experiments were actively growing. PEG-IL-2-treated rats did not gain as much weight as the control animals did, measured as either the absolute number of grams gained or as a percentage of their baseline weight (Table 1). This difference was statistically significant (P < 0.05) in only four of the six comparisons, however, probably because of the small
number of animals in each group. Similarly, it also appeared that, in general, animals housed in AEMs gained more weight than did the respective vivarium-housed animals. Again, statistical significance was found in only two comparisons. PEG-IL-2 treatment caused an increase of from 8 to 21% in the spleen-to-brain weight ratio across all the groups, independent of the animals’ flight or housing status (Table 2). There did not seem to be any differences between the routes of treatment or the dose levels. Splenomegaly is an expected consequence of IL-2 treatment (2, 32).

To assess animals’ responses to the stresses of spaceflight, plasma corticosterone concentrations and thymic weights were measured. Elevated glucocorticoid levels in animals have been shown to cause lymphoid organ atrophy (9), and thymocytes, in particular, are known to be unusually susceptible to corticosteroids (7). In Immune 1, there was a progressive increase in the mean plasma corticosterone concentrations from vivarium animals to AEM-housed ground controls to flight rats that was not affected by PEG-IL-2 treatment (Table 1). However, because of the limited number of animals per group and interanimal variation, these differences were not statistically different (P < 0.05), except between the flight and vivarium animals when the PEG-IL-2 and control groups were combined.

Perhaps as a reflection of the influence of these corticosterone levels, a similar trend was found for the thymus weights as a percentage of brain weight in Immune 1. The thymus weights of the flight and the AEM-ground control groups were both larger than thymus weights in the vivarium ground controls, but these results were not statistically significant. In the vivarium group, PEG-IL-2 treatment appeared to result in a decrease of ~10% in the thymus-to-brain weight ratio, whereas there was a trend toward an increase in this ratio in the other two groups.

In Immune 2, the flight group also had the highest plasma corticosterone concentration. In contrast to Immune 1, however, the vivarium animals had higher corticosterone levels than the AEM-housed ground control rats had. Once again, PEG-IL-2 treatment did not appear to significantly influence the corticosterone levels measured at recovery.

Again, in contrast to the results obtained in Immune 1, in Immune 2 there were no differences in the thymus-to-brain weight ratios between any of the groups. PEG-IL-2 treatment resulted in thymic atrophy in the vivarium group, as observed in Immune 1; however, this effect was also extended into the other two groups as well.

Hematology. Complete blood cell counts and differentials were obtained at recovery to assess the influence of spaceflight and PEG-IL-2 treatment. In Immune 1, there were no differences in the number of total white cells or the absolute number or percentage of monocytes or lymphocytes between any of the groups (Table 3). However, the absolute number of neutrophils was significantly higher in the PEG-IL-2-treated flight and AEM-ground control animals, compared with the vivarium group. In these same groups, there tended to be the same increase in neutrophils in the placebo-treated animals as well, although this was only a trend compared with the vivarium-placebo group.

In the Immune 2 experiment (Table 3), a somewhat different profile of peripheral blood cells was obtained. The number of monocytes decreased in the flight animals compared with the other two groups, independent of treatment; in some cases, this was statistically significant (Table 3). PEG-IL-2 treatment had no effect on the flight animals, although there was a trend which was not significant toward a decrease in the number of monocytes in the two ground-based studies.

The absolute number of lymphocytes was decreased in the Immune 2 flight animals, again independent of treatment status, which was statistically significant, as indicated. PEG-IL-2 treatment tended to increase the
number of lymphocytes, although this was statistically significant only in the AEM animals, which may reflect the larger number of animals in that group. There were no differences in the absolute number of neutrophils between the various groups, although on a percentage basis, the flight animals had more neutrophils than did either of the ground-based groups.

There were no consistent changes observed in any of the red blood cell or platelet parameters that could be attributed to either flight or treatment status (data not shown).

Lymphocyte proliferation and INF-γ secretion. Rat lymphocyte blastogenesis was depressed after some spaceflights when examined after landing (40). Moreover, spaceflight affects lymphocytes dramatically at the cellular level (11, 13). Therefore, we examined the postflight blastogenic responses of splenic and lymph node cells. There were no consistent spaceflight effects on either spleen or lymph node cell proliferation after stimulation with various T and B cell agonists (Table 4). There was animal-to-animal variation that limited the number of sample comparisons that were statistically significant. The spleen cell response to PHA of flight rats injected with saline was significantly less (P < 0.05) than that of ground control rat spleen cell responses to PHA (Table 4). However, the spleen cell response to PHA was the poorest of all the agonists. Interestingly, ex vivo spleen cell proliferation of cells from rats injected with PEG-IL-2 tended to be greater than cells recovered from saline-injected rats (P < 0.1,

Table 4. Effects of spaceflight and PEG-IL-2 on lymphocyte proliferation by source

<table>
<thead>
<tr>
<th>Group</th>
<th>PEG-IL-2</th>
<th>Medium Control, counts/min</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PHA</td>
<td>Con-A</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flight</td>
<td>−</td>
<td>2.698</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>Flight</td>
<td>+</td>
<td>2.203</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>AEM</td>
<td>−</td>
<td>2.249</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>AEM</td>
<td>+</td>
<td>2.234</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Vivarium</td>
<td>−</td>
<td>1.648</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>Vivarium</td>
<td>+</td>
<td>2.457</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flight</td>
<td>−</td>
<td>1.305</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Flight</td>
<td>+</td>
<td>1.633</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>AEM</td>
<td>−</td>
<td>1.332</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>AEM</td>
<td>+</td>
<td>1.158</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Vivarium</td>
<td>−</td>
<td>1.730</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Vivarium</td>
<td>+</td>
<td>1.117</td>
<td>1.6 ± 0.1</td>
</tr>
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</table>

Values are means ± SE; n = 6 rats per treatment group. Sprague-Dawley rats were injected iv with 0.5 mg/kg PEG-IL-2 (+) or saline (−). Lymphocytes were stimulated ex vivo with phytohemagglutinin (PHA), concanavalin A (Con A), dextran-lipopolysaccharide (Dextran/LPS), staphyloccocal enterotoxin A or B (SEA or SEB, respectively) for 48 h (lymph node) or 72 h (spleen). Statistical difference between treatment groups, as determined by Student's t-test: *different from similarly housed, saline-treated group, P < 0.05.
A more limited data set was obtained from the Immune 2 experiment. There were no significant (P > 0.1) spaceflight or housing effects on the ConA, SEA, SEB, or dextran-LPS-induced proliferative responses of spleen or lymph node lymphocytes (data not shown). However, unlike the Immune 1 spleen cell response, PEG-IL-2 did not enhance the Immune 2 spleen cell proliferative responses.

ConA-stimulated spleen cells were assessed for secretion of IFN-γ 48 h after stimulation. There were no significant effects of spaceflight on Immune 1 or Immune 2 (Fig. 1). In contrast to the significant increase in ConA-induced splenic T cell proliferation by PEG-IL-2 in animals housed in AEMs (flight and ground control), there was no similar effect on IFN-γ secretion. Although we used different assays to assess IFN-γ for samples analyzed for Immune 1 and 2, there were no statistically significant differences between any of the treatment groups. This was consistent with the results of our proliferation studies.

Macrophage and granulocyte-macrophage development from bone marrow cells and cytokine secretion. Experiments with rats flown in space on Russian biosatellites (48, 49) or on the space shuttle (27) have indicated depressed bone marrow macrophage progenitors when colony formation was assessed postflight. In an attempt to confirm and extend those data, we also assessed CFU-M and CFU-GM in the bone marrow and marrow cell secretion of various cytokines. CFU-M and CFU-GM, formed from bone marrow from rats in the Immune 1 (Fig. 2) and Immune 2 experiments; however, the overall numbers were much lower in Immune 2 (Immune 2 data not shown). It was not clear whether the lower colony numbers were due to sample preparation at KSC or whether the CFU-M and CFU-GM from this group of animals was just lower. But given that the CFU-M and CFU-GM colony assays were set up in different laboratories, the lower CFU was not attributable to individual assay setup. None of the CFU-M colonies grew from the vivarium rats in Immune 1, and those data could not be presented. There were no flight-dependent effects on either CFU-M or CFU-GM in either Immune 1 or 2. The CFU-M formed from marrow of flight rats injected with PEG-IL-2 was statistically lower than were the CFUs of the other Immune 2 treatment groups, but the low CFU numbers (e.g., 3 vs. 6–8 colonies) are difficult to assess.

When freshly harvested bone marrow cells were assayed for their secretion of various cytokines, there was notable animal-to-animal variation. However, there were no significant differences between flight rats and ground control rats when we quantitated IL-6 in Immune 1 and 2 (Fig. 3), although the lower IL-6 secretion
of bone marrow cells of rats from Immune 2 paralleled the number of CFU-M and CFU-GM formed. Similarly, there were no statistically significant differences between treatment groups when we assayed for CSF-1 (values ranging from 3 to 80 pg/ml) in Immune 1 or TGF-β in Immune 2 (values ranging from 321 to 570 ng/ml; data not shown).

Peritoneal macrophage cytokine secretion. In addition to quantitating CFU development and bone marrow cell cytokine secretion, we assessed the basal and inducible concentrations of TNF-α and IL-6 that were secreted by resident peritoneal macrophages. Resident macrophages from both Immune 1 control groups (AEM or vivarium) secreted low basal concentrations of TNF-α that were not augmented by PEG-IL-2 injection (Table 5). The macrophages from the saline-injected flight group had a higher basal TNF-α secretion (P < 0.1). All macrophages secreted much higher concentrations of TNF-α when stimulated with LPS or SEA (P < 0.05; Table 5). The Immune 1 macrophages secreted high concentrations of IL-6 (~100–300 ng/ml), which were not significantly augmented by stimulation by either LPS or SEA (data not shown). The macrophages from rats in the Immune 2 experiment secreted TNF-α in a pattern opposite to what was seen in the Immune 1 experiment. Macrophages from both the control groups secreted higher concentrations of TNF-α, and LPS and SEA minimally enhanced the response (Table 5). The macrophages from flight rats secreted significantly lower (P < 0.05) basal concentrations of TNF-α than did macrophages from control groups, and the response was enhanced by SEA and LPS, although there was some variation between agonists. The secreted IL-6 concentrations were much lower for Immune 2 (1–3 ng/ml). Moreover, the amount of IL-6 secreted was not consistently enhanced by incubating the macrophages with either LPS or SEA. In general, neither flight nor PEG-IL-2 injection appeared to affect peritoneal macrophage IL-6 secretion in the Immune 1 or Immune 2 experiments.

Bone physiology. Significant bone changes were not observed in the Immune 1 flight (Table 6). Stiffness (S), strength (Pm), deflection, and energy properties were not significantly affected by flight or PEG-IL-2 treatment with the exception of tibia Pm, which was significantly (10–15%) lower for the pooled flight groups than the Pm recorded for the pooled AEM or vivarium groups. Mass (Bone-M, Dry-M, and Min-M; data not shown) properties were not significantly altered. For example, the Dry-M of femora of flight rats was 379 ± 32 mg for the flight rats, 393 ± 31 mg for the AEM rats, and 379 ± 22 mg for the vivarium rats on Immune 1. No statistically relevant effects on the material property of %Min were observed for Immune 1.

The bone-related results for Immune 2 were quite different from those of Immune 1 (Table 7). In the femur, deflection and energy parameters (data not shown) were similar among all groups. However, S (18%) and Pm (19%) were considerably less for the flight groups than for the AEM controls. These flight-induced changes were also manifested in mass measurements.

<table>
<thead>
<tr>
<th>Table 5. Effects of spaceflight and PEG-IL-2 on resident peritoneal macrophage TNF-α secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment Group</td>
</tr>
<tr>
<td>Immune 1</td>
</tr>
<tr>
<td>Medium</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>LPS</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>SEA</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Immune 2</td>
</tr>
<tr>
<td>Medium</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>LPS</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>SEA</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Values are means ± SE of 6 rats per treatment group except Immune 2 AEM control (n = 9). TNF-α, tumor necrosis factor-α; &lt;, Mean was less than the sensitivity of the assay. Statistical difference between treatment groups as determined by Student t-test: *significantly different from medium control, P &lt; 0.05; †significantly different from similarly treated (PEG-IL-2) AEM control, P &lt; 0.1; ‡significantly different from similarly treated (PEG-IL-2) AEM control, P &lt; 0.05.</td>
</tr>
</tbody>
</table>
which for the femora were (in Dry-M) 362 ± 16 mg for
the flight rats, a significantly (P < 0.05) higher 420 ±
30 mg for the AEM rats, and a significantly higher 398
± 46 mg for the vivarium rats. Min-M was also
significantly (12%) lower in pooled-flight groups com-
pared with pooled-AEM controls or vivarium controls.

Similar relative differences in stiffness, strength, and
mass values were observed in the tibiae in flight groups
compared with AEM groups. In addition to these flight-
induced trends, the following trends were observed for
Immune 2 bone data: 1) the material property, %Min,
was not affected by flight, 2) the vivarium controls had
femoral properties similar to the AEM controls, but
tibial properties similar to the flight groups, and 3) no
PEG-IL-2-induced bone effects were observed.

**DISCUSSION**

At present, at least 18 shuttle missions have carried
rats housed in AEMs. This unprecedented flight fre-
cuency will allow for the compilation of a significant
amount of data on the effects of spaceflight on various
physiological systems. The problem with these data is
that there will be significant differences in the flight
profiles endured by the animals. The Immune 1 and 2
flights provided our research team with two 8-day
shuttle flights to investigate changes in rat physiology.
Despite the similar flight durations and the same
research team performing the postflight analyses, there
were still notable differences in some of the data
obtained from each flight. In particular, macrophage
TNF-α secretion, blood cell distribution, and bone
structural and mass properties showed inconsistent
changes. These differences may be attributable to sig-
nificant variations in the flight profiles. Although Im-
mune 1 and 2 were both 8-day flights, the times of
launch and landing were very different. The animals in
Immune 1 were dissected in the evening, and the
animals in Immune 2 were dissected midmorning.
These times are close to the antithetical peaks for daily
circadian rhythms (42). Although the plasma corticoste-
rone concentrations of the vivarium rats in both flights
were very similar (127 ± 22 vs. 90 ± 16 ng/ml),
circadian variations cannot be discounted. The change
in supplier of Sprague-Dawley rats between flights also
could have contributed to some of the differences, al-
though preflight verification tests indicated that the
animals were comparable. Housing differences could
also have played a role. The average temperatures of
the AEMs of Immune 2 were higher than in Immune 1
(P < 0.001), possibly leading to temperature stress.

Table 6. Effects of Immune 1 spaceflight on bone properties

<table>
<thead>
<tr>
<th>PEG-IL-2</th>
<th>Femora</th>
<th>Tibia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S, N/mm</td>
<td>Pm, N</td>
</tr>
<tr>
<td>+</td>
<td>94.3 ± 14.2 +</td>
<td>73.8 ± 12.1</td>
</tr>
<tr>
<td></td>
<td>97.0 ± 6.8</td>
<td>73.4 ± 13.2</td>
</tr>
<tr>
<td>AEM</td>
<td>+</td>
<td>96.0 ± 15.3</td>
</tr>
<tr>
<td></td>
<td>98.2 ± 16.3</td>
<td>89.0 ± 14.5</td>
</tr>
<tr>
<td>Vivarium</td>
<td>+</td>
<td>107.7 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>103.4 ± 10.4</td>
<td>76.2 ± 2.3</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 for Flight and Vivarium groups; n = 9 for AEM groups. Data are representative bone data from STS-63 (Immune 1) and STS-63 (Immune 2). *Significantly different from pooled AEM groups or pooled Vivarium groups, P < 0.05.

Table 7. Effects of Immune 2 spaceflight on bone properties

<table>
<thead>
<tr>
<th>PEG-IL-2</th>
<th>Femora</th>
<th>Tibia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S, N/mm</td>
<td>Pm, N</td>
</tr>
<tr>
<td>+</td>
<td>182 ± 13</td>
<td>138 ± 10</td>
</tr>
<tr>
<td></td>
<td>181 ± 12</td>
<td>144 ± 13</td>
</tr>
<tr>
<td>AEM</td>
<td>+</td>
<td>217 ± 14</td>
</tr>
<tr>
<td></td>
<td>220 ± 19</td>
<td>182 ± 15</td>
</tr>
<tr>
<td>Vivarium</td>
<td>+</td>
<td>209 ± 10</td>
</tr>
<tr>
<td></td>
<td>211 ± 13</td>
<td>157 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6 for Flight and Vivarium groups; n = 9 for AEM groups. Data are representative bone data from STS-63 (Immune 2). *Significantly different from pooled AEM groups or pooled Vivarium groups for all S and Pm measurements, P < 0.05. †Pooled Vivarium group values of S and Pm in tibiae are significantly different from pooled AEM values, P < 0.05.
Temperature has been attributed as a stress factor for AEM-housed rats in previous flights (25). Long-term temperature stress on the rats would have been reflected as a reduction in thymus weight as a percentage of brain weight. There were no thymus weight differences between vivarium, AEM, or flight-treatment groups in either Immune 1 or 2 in the saline-injected animals. This suggests that long-term stress may not have been significant and would contrast with previous studies that showed spaceflight-induced thymic atrophy (18, 31). However, the thymic atrophy that is normally induced by PEG-IL-2 administration was clearly absent in AEM and flight rats in Immune 1. Therefore, the impact of housing stress is not unequivocal. It is possible that each variable alone may not have changed the animals' response from normal significantly. However, several variables combined may have compounded the effects on the animals. Indeed, this may have occurred with the animals that suffered tail pathology during the Immune 1 flight. When animals were prepared for intravenous injection, their tails were dipped in warm water to dilate the tail vein. Although there was no apparent injury to the rats at the beginning of the flight, subclinial scaling may have been exacerbated by changes in fluid distribution and/or circulation during flight. This should warrant future investigation.

The AEMs had some effects on the rats that were distinguishable from the effects of spaceflight. For example, AEM ground control rats gained more weight than did vivarium ground controls. We attribute this to the fact that there was more room in the vivarium cages. Six rats were housed in ~1,613 in.³ in the vivarium compared with 571 in.³ in an AEM. The rats had 64% less volume in the AEM in which to move, and the restricted AEM environment probably lowered the amount of energy used by the rats and allowed them to gain weight. It is not clear why Immune 1 flight rats gained less weight than AEM controls and why Immune 2 flight rats gained more weight than AEM controls. Weight gain was not related to stress, because flight rats in both experiments had similar plasma glucocorticoid concentrations.

There was a stepwise increase in plasma corticosterone concentrations in Immune 1 from vivarium rats to AEM rats to flight rats that correlated with blood neutrophilia. The flight rats in Immune 2 had neutrophilia comparable to that of flight rats in Immune 1. Because glucocorticoid concentrations affect cell extravasation (9), these data are not unexpected and are consistent with other spaceflights. Neutrophilia is seen in astronauts after their return from flight (54, 55). Sonnenfeld et al. (49) found an increase in the number of myeloid cells staining for antileukocyte antibody compared with vivarium controls after rats were recovered from Cosmos 1887. Allebboom et al. (1) found that a significant neutrophilia developed postflight in rats flown in the Space Life Science (SLS)-1 mission. Moreover, rats bled in space during SLS-2 and analyzed by that same group also exhibited neutrophilia (28). Therefore, blood neutrophilia appears to be a problem that can develop in space, may return to normal values on adaptation, and then can be induced again during landing.

The diminution in the number of blood monocytes in Immune 2 flight rats is similar to the monocytopenia seen in humans (55) and rats (1). The lack of a similar depression in monocyte numbers in Immune 1 rats is consistent with another more recent data set that showed that numbers of monocytes are not affected by spaceflight in 124 shuttle astronauts who were tested (33). The differences between Immune 1 and 2 probably reflect similar variability between individuals and studies. Analysis of rat blood during and after SLS-2 indicated that monocytopenia was a response induced by the shuttle landing (28). Therefore, monocyte differences between Immune 1 and 2 could be due to variations in landing conditions.

The failure of spaceflight to inhibit lymphocyte proliferation and IFN-γ secretion contrasts human responses measured after both short- and long-term spaceflights (19, 29) with proliferative (40) and spleen cell IFN-γ secretion responses (23) of rats subjected to spaceflight. However, some data indicate that lymphocyte responses are organ, mitogen, and flight dependent (31, 39, 40). Therefore, our results are not unprecedented. The fact that spleen cells from PEG-IL-2-injected Immune 1 flight rats responded, as well as did spleen cells from similarly injected ground controls, suggests that the IL-2 receptor was functional. This would be consistent with data showing that IL-2 receptor expression on lymphocytes was normal or higher than in ground controls when assayed immediately after flight or after subsequent in vitro stimulation (25, 40, 48, 49).

The failure of PEG-IL-2 to enhance spleen cell responses of Immune 2 rats was expected on the basis of preflight verification tests. The PEG-IL-2 was delivered to the spleen after subcutaneous injection, because splenomegaly still occurred in treated rats. Therefore, splenic hypertrophy is not predictive of splenocyte proliferative activity in this system. Interestingly, the splenic atrophy associated with spaceflight by other investigators (18, 25) was not seen in our saline-treated flight animals of Immune 1 or 2. This supports the hypothesis that our flight animals did not suffer long-term flight stress and that there was some consistency in our animal handling.

This study included the first postspaceflight analysis of secreted peritoneal macrophage cytokines. Although spaceflight did not affect IL-6 secretion, macrophages were spontaneously activated, and they secreted TNF-α ex vivo after the Immune 1 flight. Although this was not confirmed after the Immune 2 flight, peritoneal rat macrophages recovered from rats flown on STS-77 also made significantly more TNF-α in response to LPS compared with macrophages from ground controls (12a). Spleen cells from rats flown on the SLS-2 mission also secreted more TNF-α than did ground controls (30). Given that macrophages make more TNF-α when flown in space in vitro (11, 14), the possibility that macrophages may become hyperresponsive is a serious con-
cern. The cachectic activity of TNF-α could have severe consequences on astronauts' tissues (59). Additional flight experiments will be necessary to resolve how frequently this is a problem. We do not have a good explanation for the spontaneous secretion of TNF-α by control rats in the Immune 2 experiment. The macrophages from Immune 2 flight rats exhibited the classic activation pattern (i.e., medium controls, low; agonist-treated, high). Therefore, we do not believe the spontaneous TNF-α secretion by the control macrophages was caused by contaminating pyrogens. If pyrogen contamination occurred during handling, the flight rat macrophages would also have been activated in medium alone.

The failure of spaceflight to inhibit CFU-M or CFU-GM colony formation in Immune 1 and Immune 2 contrasts with data obtained in several previous spaceflights (27, 47–49). The fact that the assays for CFU-M and CFU-GM were consistent, yet were done in different laboratories for the Immune 1 or 2 flights, indicates that the results were not due to technical differences from past mission analyses. The flights in which CFU-Ms or CFU-GMs were lower than in ground controls were all relatively long flights (≥12 days) compared with the 8-day flights of Immune 1 and 2. Perhaps bone marrow progenitors are affected slowly by spaceflight, and it takes a long time to detect diminution in precursor numbers. Bone marrow cell production of cytokines was not affected by spaceflight; this would be consistent with the fact that the stromal cells that nurture various stem cell populations appeared to be functioning normally in Immune 1 and 2. It would also be compatible with data showing that secretion of another CSF, rat IL-3, was not affected by a 7-day space shuttle flight (23). However, that was a spleen cell measurement. Moreover, later studies found that spleen cells secreted higher concentrations of IL-3 after postflight stimulation of cells taken from rats flown on STS-54 for 7 days (35). Because M-CSF, TGF-β, and IL-6 had not been measured in earlier flights, it is hard to put our results into the context of the diminished colony formation that was seen previously. This issue warrants further attention, given that depression of M-CSF secretion by bone marrow cells correlated with reduced CFU-M formation in antithrombinically suspended mice (3).

The modest effects on bone of the Immune 1 flight are not unprecedented (64). It is unlikely that the results are a consequence of the use of different suppliers for the rats on the two missions, because spaceflight-induced bone alterations have been found in several distinct rat strains (37). Alternatively, when rapidly growing rats failed to show changes in bone mass and formation after a 17-day flight, a subsequent analysis revealed that group-housed rats may be less susceptible to bone alterations than are individually housed rats (64). Therefore, housing stress and activity may be critical factors that affect bone. Perhaps warmer AEMs in Immune 2 than in Immune 1 could have contributed a kind of "housing stress."

In Immune 2, both bone structural and bone mass properties were significantly lower in flight groups than in AEM groups. This indicates a change in overall bone size rather than a change in bone material properties. This latter conclusion is supported by the lack of significant changes in %Min. Based on the high rate of bone formation in rats at this age, the flight effects are almost certainly due to reduced bone formation caused by flight (36).

The difference between vivarium and AEM controls for tibial mechanical properties on Immune 2 is not readily explainable. These differences are not supported by differences in mass, which were similar for the Immune 2 AEM (386 ± 37 mg Dry-M) and vivarium groups (369 ± 43 mg Dry-M). Regardless of these unanswered questions, it is clear that the Immune 2 mission produced bone changes more consistent with past spaceflight investigations (43, 50) than did the Immune 1 mission. In neither flight were PEG-IL-2–induced bone effects observed.

In conclusion, the results of Immune 1 and 2 show that the physiological responses of rats to spaceflight are highly variable. Many flight conditions that are beyond the control of the investigative team may contribute to host physiological variability. Animal heterogeneity and experimental protocols may also be factors. For these reasons, we found it difficult to test the experimental hypothesis that PEG-IL-2 would ameliorate some of the effects of spaceflight. With the exception of attenuating the neutrophilia of flight rats in Immune 1, we found that PEG-IL-2 was not generally therapeutic. More importantly, it has become clear that we have yet to detail an all-encompassing paradigm for the effects of spaceflight on bone and immune systems.

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