Spaceflight affects bone formation in rhesus monkeys: a histological and cell culture study

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Received 14 June 2001; accepted in final form 26 April 2002

Zérath, Erik, Marc Grynpas, Xavier Holy, Michel Viso, Patricia Patterson-Buckendahl, and Pierre J. Marie. Spaceflight affects bone formation in rhesus monkeys: a histological and cell culture study. J Appl Physiol 93: 1047–1056, 2002. First published May 17, 2002; 10.1152/japplphysiol.00610.2001.—Using analyses of iliac crest cell and tissue, back-scattered electron imaging, and biochemical techniques, we characterized the effects of a 14-day spaceflight (Bion 11) on bone structure and bone formation in two 3- to 4-yr-old male rhesus monkeys compared with eight age-matched Earth-control monkeys. We found that postflight bone volume was 35% lower than preflight values in flight monkeys. This was associated with reduced osteoid (−40%) and mineralizing (−32%) surfaces and decreased bone formation rate (−53%). Moreover, flight monkeys exhibited trends to lower values of mineralization profile in iliac bone (back-scattered electron imaging) and to decreased osteocalcin serum levels (P = 0.08). The initial number of trabecular bone cells yielded in cultures did not differ in flight and control animals before or after the flight. However, osteoblastic cell proliferation was markedly lower in postflight vs. preflight at 9 and 14 days of culture in one flight monkey. This study suggests that a 14-day spaceflight reduces iliac bone formation, osteoblastic activity, and/or recruitment in young rhesus monkeys, resulting in decreased trabecular bone volume.

microgravity; primate; cancellous bone; osteoblasts; histomorphometry; back-scattered imaging; osteocalcin

SPACEFLIGHT HAS BEEN ASSOCIATED with decreased bone mass. This has been reported in humans by noninvasive techniques (6, 36, 44). However, the paucity of flight data in humans and human diversity in space experimentations has given considerable interest in the use of animals for space studies. Histological studies in growing rats have shown that bone loss during spaceflight is primarily due to diminished bone formation (13, 24, 46, 47). These results have mostly been obtained from the metaphyseal part of rat long bones, which is characterized by a very sustained growth activity. Moreover, rats’ loading pattern is different from that of humans, which makes it difficult to extrapolate spaceflight data from rats to humans. Even though no large-animal model is fully comparable to humans with regard to bone physiology, it is generally admitted that monkeys, and specifically rhesus monkeys, are a suitable model of human bone and calcium metabolism (14). However, few studies have been performed in nonhuman primates flown in space, because of stringent breeding requirements making it difficult to use these animals in space. The available data showed that bone mineralization parameters are markedly reduced during spaceflight (50). However, the time elapsed between reentry and surgery so far precluded early postflight bone cell investigations.

The present study was conducted under a joint French-American-Russian program aimed at defining microgravity-related changes in primate physiology and clarifying their underlying mechanisms. The purpose of bone studies conducted onboard the Bion 11 mission was to investigate by cell and tissue analyses, biochemistry, and back-scattered electron (BSE) imaging techniques the effects of spaceflight on bone metabolism in monkeys during a 14-day spaceflight. The primary rationale of this study was to collect bone biopsies as soon as possible after landing to avoid measurement of readaptation to normal gravity. Moreover, techniques of cell isolation and culture were specifically developed to investigate bone cell activities for the very first time in primates in this type of space experiment. Our results indicate that 14 days of spaceflight induce a measurable bone loss in iliac crest associated with altered bone formation activity, as evidenced by biochemical, osteoblastic, and mineralization parameters.

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METHODS

Animals

Two male rhesus monkeys (Macaca mulatta) were selected for flight on the basis of animals’ health and task proficiency at the Institute of Medical Primatology of Sochi (Russia). Monkeys were 3 to 4 yr old at the time of flight. The two flight monkeys were identified as monkey 357 and monkey 484. Eight monkeys of the same origin were used as ground controls. These control animals were assigned to two groups: vivarium animals (n = 5) and confinement animals (n = 3). At the time of the flight, the average weight of the animals was 4.78 ± 0.12 kg (Table 1). Vivarium animals (monkeys 448, 470, 474, 503, 513) remained in their individual cages during the entire experiment. Confinement animals (monkeys 447, 501, 534) were part of the flight pool candidates that had been successfully selected for flight during preliminary tests and had performed the same habituation and training as the two flight monkeys. Habituation consisted in wearing the flight jacket, being restrained in the flight chair, and being fed the special paste diet. Training consisted in performing selected cognitive tasks for psychomotor purposes. These animals had also been shipped to the launch base of Plesetzk (Russia) with flight monkeys on November 30, 1996, and were shipped back to Moscow (Russia) on the day after launch. Monkey 447 was placed into a flight “mock-up” capsule on the fourth day after launch of the flight animals. Monkeys 501 and 534 were placed into actual flight capsules as soon as they became available (i.e., 17 days after recovery). These last three monkeys were thus submitted to delayed simulation experiments that faithfully mimicked flight conditions (i.e., environmental confinement, restraint, and diet).

Bion 11 Flight: General Description

The experimental conditions of Bion 11 are described elsewhere (8, 11) and are quite similar to those of the previously published Bion 10 experiment (50). Briefly, the spacecraft, a modified Vostok capsule, was launched from the Plesetzk space base on December 24, 1996 at 1650 (Moscow time). After a 10-min 4-G acceleration period, the biosatellite was placed into orbit. The vehicle landed at 0802 on January 07, 1997 in Kazakhstan. The flight duration was thus 13 days and 15 h. The animals were recovered at 1030 in good health (i.e., they were alert, active, without signs of distress). Within 12 h after landing, they were flown back to the laboratory of the Institute of BioMedical Problems (Moscow), which was in charge of all pre- and post-flight operations on animals. During flight, the animals were maintained in a specifically designed chair. The atmosphere was maintained at 750 Torr ambient pressure, with 140–180 Torr PO2 and less than 1.6 Torr PCO2. Humidity was raised from 20 to 65% from the first to the last day of flight. The temperature in the capsule was maintained between 21 and 26.2°C depending on sunshine exposure during orbital flight. The monkeys were exposed to a 16.8-h light-dark cycle (lights on from 0800 to 2400 with a 60-lux light intensity). They were given 250 g of paste food through a feeder twice a day (at 1000 and 1800). In reward for successfully performed tasks, they were also given fruit juice with a maximum quantity of 50 ml four times a day (at 0800, 1300, 1600, and 2100). The paste diet contained 70% water to meet fluid requirements. Its protein-fat-carbohydrates ratio was 1:0.8:4 respectively, and calcium-phosphorus ratio was 0.8.

Bone Sampling

Iliac bone biopsies (weighing ~330 mg) were taken 24 h after landing by a veterinarian team according to the technique previously developed for that purpose (28). Briefly, the animals were anesthetized (ketamine 50 mg im and isoflurane 1–1.5% in the trinitrogen flow), and a 3-cm incision was made under sterile conditions on the anterior part of the iliac crest. After muscle dissection, a 0.5- to 0.6-cm-width sample was removed with a cutting pliers. Muscles, fascia, and skin were then successively closed. The time needed for this surgery was ~20 min. Bone sampling from the 10 monkeys was scheduled 5 mo preflight (preflight control biopsies, left iliac crest) and postflight (right iliac crest). Flight and confinement animals were all sampled on the same day after recovery from flight or confinement situations, respectively. All animals recovered quickly from surgery and resumed normal activity except for flight animal monkey 357. During recovery from anesthesia, this monkey exhibited an acute cardiorespiratory syndrome following extubation. It was unresponsive to active resuscitation procedures and died 2 h after the end of surgery. However, the bone biopsy taken from this animal was investigated in the same manner as the others. Consequently, 20 bone biopsies were analyzed in this experiment.

Immediately after sampling, bone biopsies were placed in a sterile buffered survival medium (PBS) and sent immediately to Paris, France, at +4°C. On arrival, bone biopsies were immediately cut under sterile conditions in two parts for histological and cell culture purposes. In these conditions, the osteoblast phenotype of isolated bone cells was preserved, as shown by alkaline phosphatase (ALP) activity, type I collagen synthesis, and osteocalcin synthesis (19, 48, 49; see also Bone Cell Growth and Phenotype).

Table 1. Body weight of flight, vivarium, and confinement monkeys, recorded preflight and postflight

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Group</th>
<th>Body Weight (preflight), kg</th>
<th>Body Weight (postflight), kg</th>
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<tbody>
<tr>
<td>357</td>
<td>F</td>
<td>4.90</td>
<td>4.30</td>
</tr>
<tr>
<td>484</td>
<td>F</td>
<td>5.10</td>
<td>4.50</td>
</tr>
<tr>
<td>448</td>
<td>V</td>
<td>5.20</td>
<td>4.90</td>
</tr>
<tr>
<td>470</td>
<td>V</td>
<td>4.20</td>
<td>4.40</td>
</tr>
<tr>
<td>474</td>
<td>V</td>
<td>4.15</td>
<td>4.10</td>
</tr>
<tr>
<td>503</td>
<td>V</td>
<td>4.80</td>
<td>5.00</td>
</tr>
<tr>
<td>513</td>
<td>V</td>
<td>4.85</td>
<td>5.30</td>
</tr>
<tr>
<td>447</td>
<td>C</td>
<td>4.80</td>
<td>4.60</td>
</tr>
<tr>
<td>501</td>
<td>C</td>
<td>5.30</td>
<td>4.80</td>
</tr>
<tr>
<td>534</td>
<td>C</td>
<td>4.50</td>
<td>4.30</td>
</tr>
</tbody>
</table>

F, flight; V, vivarium; C, confinement.
the first biopsy. Prior to the second bone biopsy, flight and confinement animals received a double calcein administration with a 7-day interval immediately preceding flight or confinement experiment and a third calcein administration when they were removed from flight or from confinement facilities (i.e., 1 day on/7 days off/1 day on/14 days off/1 day on/1 day off/sampling).

All parameters were measured by use of a semiautomatic system (Newtec, Nîmes, France) connected to a video camera. A Sony DXC-151AP color video camera was used for microscopy in transmitted light, and a Kappa CF 15/4 MCC video camera (Leica, Rueil-Malmaison, France) sensitive to dim-light exposure was used for microscopy under fluorescent lighting. Measurements were made in the cancellous area (overall surface area: 6 mm²). A ×2.5 objective lens was used for bone mass measurements, and a ×10 objective lens was used for the evaluation of bone mineralization activity.

The following parameters were measured.

**Bone structural parameters.** Bone volume (BV/TV, bone volume/tissue volume) was expressed as a percentage, being the part of the cancellous space filled with trabecular bone. Trabecular thickness (Tb.Th, in μm) was calculated by using four parallel lines from one edge to the other edge of each trabeculae. Number of trabeculae per millimeter (Tb.N) and trabecular separation (Tb.Sp, in μm) were estimated according to the method of Parfitt et al. (30). Wall thickness (W.Th, in μm) was calculated by use of polarized light by measuring four parallel lines from the cement line to the border facing the marrow of each bone packet (17).

**Static parameters of bone formation and resorption activity.** Osteoid surface (OS/BS, osteoid surface/bone surface) was expressed in percentage corresponding to the portion of bone surface covered with osteoid. Osteoid thickness (O.Th, in μm) was measured as the mean width of osteoid tissue. Osteoclast surface (Oc.S/BS, osteoclast surface/bone surface) was expressed as percentage corresponding to the portion of bone surface covered with osteoclasts.

**Dynamic parameters of bone formation activity.** Mineral apposition rate (MAR, in μm/day), calculated as the mean distance between double labels divided by interval labeling time; mineralizing surface (MS/BS, mineralizing surface/bone surface), expressed as a percentage, representing the part of the trabecular surface occupied by the fluorescent labeling of the corresponding step of the experimental schedule; bone formation rate (BFR, in μm²·μm⁻²·day⁻¹), formation period (FP, in days), activation frequency (Ac.f, in day⁻¹), and mineralization lag time (Mlt, in days) were calculated according to the method of Parfitt et al. (30). Parameters and abbreviations complied with the American Society for Bone Mineral Research nomenclature (30).

**BSE Techniques**

The biopsies embedded in block from which previous sections had been cut were polished with 1-μm diamond films. The biopsies were then washed in PBS, the marrow cells were flushed out using PBS, and the remaining bone was washed and placed in PBS before isolation of bone cells. In this study, no attempt was made to collect and culture marrow stroma cells. Trabecular bone cells were isolated from the cancellous area of iliac bone by collagenase digestion. To do this, the bone was dissected into small (1 mm³) fragments, which were placed in 0.25% collagenase (Sigma Chemical, St. Louis, MO) for 2 h at 37°C. The pieces of bone were gently shaken after 1 h of digestion. The bone fragments were then discarded, and the remaining cells were resuspended in PBS, centrifuged (1,300 rpm, 2 min), and washed in PBS. After centrifugation (1,300 rpm, 5 min), the cells were suspended in DMEM (Eurobio) supplemented with 10% FCS (Deutscher) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin, Gibco, Grand Island, NY) and counted by use of a hemocytometer (initial number of cells). The cells were then plated in 25-cm² culture flasks (Falcon, Becton Dickinson, Meylan, France) and cultured at confluence (2 wk) with the medium changed every 2 days. The cells were detached with 0.05% trypsin (Sigma Chemical) and plated as indicated below for evaluation of cell growth and osteoblast phenotype. Although it is possible that some osteocytes may be derived by the method used, this is certainly not the major bone cell population obtained because the collagenase digestion was not stringent.

**Bone Cell Growth and Phenotype**

Cell growth was evaluated as previously described (20). The cells were plated at a concentration of 10,000 cells/cm² in multiwell chambers (LabTek, Nunc, Naperville, IL) and cultured in DMEM with 10% FCS. One day after plating, the medium was replaced and the cells were cultured for 14 days. At days of culture 1, 3, 7, 9, and 14, the cells were labeled with 0.5 μCi/well of 6-[3H]thymidine (Amersham, Arlington Heights, IL). Four hours before the end of treatment, the cell layer was collected by trypsinization, DNA was precipitated with trichloroacetic acid, the trichloroacetic acid-insoluble fraction was dissolved in NaOH, and 6-[3H]thymidine incorporation into DNA was measured in aliquots by liquid scintillation. DNA synthesis was determined as the total (cumulative) 6-[3H]thymidine incorporated into DNA during the time-course period (20). In preflight monkeys, cell proliferation was assessed both by cell number and cumulative DNA synthesis. The latter was found to be directly proportional to cell number during a 14-day culture period (r = 0.69, P < 0.04, n = 9), indicating that DNA synthesis reflected osteo-
blastic cell proliferation. Therefore, cell proliferation after the flight was assessed only by DNA synthesis.

ALP activity and type I collagen synthesis, two parameters of osteoblast differentiation, were determined in confluent cells as described previously (19, 20, 48, 49). Briefly, bone cells were plated at 10,000 cells/cm² in multwell chambers and cultured in DMEM with 10% FCS. At confluence, the medium was removed and frozen for the determination of carboxy-terminal propeptide (P1CP) levels by a procollagen radioimmunoassay kit using a specific antibody (Orion Diagnostics, Espoo, Finland), and the results were expressed as nanograms of P1CP per milligram of protein. The intra- and interassay variability of the kit was 2.8 and 5.1%, respectively. The cells were rinsed with cold PBS, scraped in distilled water, and sonicated, and ALP activity in the cell lysate was determined by a colorimetric method (kit bio-Merieux, Lyon, France). Protein content of the cell lysates was determined colorimetrically (kit Bio-Rad, Ivry-sur-Seine, France), and the activity of the enzyme was expressed in nanomoles of p-nitrophenol released per minute per milligram protein. All cell culture analyses were performed without knowledge of monkey origin.

Biochemical Analyses

Serum osteocalcin was measured by a modification of the method of Patterson-Allen et al. (32). Highly purified monkey osteocalcin was used for radioiodinated tracer and standards, with rabbit antibody to bovine osteocalcin. Goat anti-rabbit immunoglobulin (Antibodies Incorporated, Davis, CA) was used to separate bound from free osteocalcin. Inter- and intra-assay variations were <6%.

Statistical Analyses

Data are expressed as means ± SE. Differences between pre- and postflight data for F, C, and V situations were tested by a nonparametric test (Kruskal-Wallis test) because of small group numbers, followed by Duncan's post hoc tests. Statistical significance was accepted at $P < 0.05$.

RESULTS

General

Although animals had been submitted to a 1-mo preflight habituation period, monkey 484 had no measurable food intake during the first 2 days of flight. Even though food intake was 300 g daily in this monkey thereafter, average food consumption was calculated to be 229 g per day throughout the flight (11). On the other hand, monkey 357 had a daily 350-g paste diet intake throughout the flight. At recovery, the monkeys were found to have lost ~600 g body wt (~12–13%) compared with preflight values (Table 1).

Histomorphometric Data

As shown in Fig. 1, the 14-day spaceflight induced a loss of bone mass in the iliac cancellous area as postflight BV/TV was significantly lower than preflight values in flight animals (~35%). It is noteworthy that these changes in bone mass parameters appeared to be markedly more pronounced in flight monkey 484 (~48%) than in flight monkey 357 (~23%). Decreased bone volume was associated with slightly lower Tb.Th and Tb.N and higher Tb.Sp, although differences did not reach statistical significance in these parameters (Fig. 1).

Most bone-formation parameters were decreased in flight animals compared with controls (Fig. 2, Table 2). The extent of osteoid surface (OS/BS) was 40% lower after flight than before flight (Fig. 3). Similarly, mineralizing parameters (MAR and MS/BS) were respectively 33 and 32% lower after flight. Consequently, the bone-formation rate at the tissue level (BFR) was significantly reduced in flight animals (~53%, Fig. 2). Again, the decrease in BFR was more pronounced in flight monkey 484 (~61%) than in monkey 357 (~46%). These changes in formation parameters were associated with a significant decrease (~27%) in the amount of bone formed (W.Th) in flight monkeys. Interestingly, we found that the changes in W.Th were positively correlated with changes in BFR ($r = 0.69, P = 0.027$).
and with changes in osteoid surface (OS/BS, \( r = 0.70, P = 0.036 \)). Finally, we found a lower, albeit nonsignificantly (\( P = 0.08 \)), activation frequency (Ac.f) in flight animals after spaceflight compared with controls, whereas formation period (FP) and mineralization lag time (Mlt) were found unchanged both in flight animals or in control groups (Table 2), as was Oc.S/BS (Fig. 2).

**BSE Analyses**

The mineralization profile (Fig. 4) shows the percent area of the biopsy in each gray-level bin from 2 (least mineralized) to 7 (most mineralized). After flight, flight monkeys exhibited lower values of area in bin 6 but higher values in bin 4, although differences did not reach statistical significance.

The comparisons between mineralization profiles were done by using the logit function \( \text{logit}(x) = \log\left(\frac{x}{1-x}\right) \), where \( x \) represents the average mineralization level. The decrease in \( \text{logit}(4) \) in flight monkeys exhibited in Fig. 5 together with changes in mineralization profile indicate a trend toward a lower mineralization profile in the iliac bone of these animals.

**Osteoblastic Cell Parameters**

The initial number of trabecular bone cells yielded in cultures was not significantly different in flight and control animals preflight (mean flight monkeys 2.89 ± 2.67 × 10⁶ cells vs. controls 2.97 ± 1.04 × 10⁶ cells) and postflight (mean flight monkeys 2.25 ± 1.79 × 10⁶ cells vs. controls 2.60 ± 1.02 × 10⁶ cells). Isolated bone cells showed osteoblast features in culture as assessed by alkaline phosphatase activity and type I collagen synthesis evaluated by P1CP levels (Table 3). ALP and P1CP levels could be measured in only one postflight monkey (monkey 484) and did not differ from levels measured in other postflight animals (Table 3). Figure 6 shows that osteoblastic cell proliferation, as measured by cumulative DNA synthesis, was markedly lower in one postflight vs. preflight bone samples at 9 and 14 days of culture (flight monkey 357). In this animal, bone cells were still viable, and DNA synthesis continued for up to day 14, although cell growth was lower than normal.

**Table 2. Bone formation parameters in flight, vivarium, and confinement monkeys**

<table>
<thead>
<tr>
<th></th>
<th>W.Th, ( \mu \text{m} )</th>
<th>O.Th, ( \mu \text{m} )</th>
<th>MS/BS, %</th>
<th>Ac.f, day⁻¹</th>
<th>FP, days</th>
<th>Mlt, days</th>
</tr>
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<tbody>
<tr>
<td><strong>Flight monkeys</strong></td>
<td></td>
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</tr>
<tr>
<td>Preflight</td>
<td>86.5 ± 5.5</td>
<td>19.0 ± 2.0</td>
<td>38.5 ± 5.5</td>
<td>1.11 ± 0.03</td>
<td>36.5 ± 5.1</td>
<td>8.05 ± 1.45</td>
</tr>
<tr>
<td>Postflight</td>
<td>63.5 ± 3.5*</td>
<td>15.5 ± 3.5</td>
<td>26.0 ± 2.0*</td>
<td>0.70 ± 0.10</td>
<td>35.4 ± 2.7</td>
<td>8.45 ± 0.83</td>
</tr>
<tr>
<td><strong>Vivarium monkeys</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Preflight</td>
<td>81.4 ± 5.1</td>
<td>18.6 ± 2.4</td>
<td>35.6 ± 4.2</td>
<td>1.21 ± 0.21</td>
<td>34.4 ± 1.9</td>
<td>7.89 ± 0.97</td>
</tr>
<tr>
<td>Postflight</td>
<td>83.8 ± 3.5</td>
<td>22.0 ± 2.2</td>
<td>40.6 ± 6.3</td>
<td>1.20 ± 0.32</td>
<td>32.4 ± 3.3</td>
<td>8.56 ± 1.29</td>
</tr>
<tr>
<td><strong>Confinement monkeys</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preflight</td>
<td>82.7 ± 4.6</td>
<td>19.0 ± 0.5</td>
<td>39.3 ± 2.5</td>
<td>1.32 ± 0.06</td>
<td>35.1 ± 1.4</td>
<td>8.44 ± 0.54</td>
</tr>
<tr>
<td>Postflight</td>
<td>86.0 ± 8.8</td>
<td>22.0 ± 2.0</td>
<td>42.7 ± 2.8</td>
<td>1.52 ± 0.28</td>
<td>36.1 ± 6.7</td>
<td>9.40 ± 1.82</td>
</tr>
</tbody>
</table>

Values are means ± SE. W.Th, mean wall thickness; O.Th, osteoid thickness; MS/BS, mineralizing surface; Ac.f, activation frequency; FP, formation period; Mlt, mineralization lag time. *Difference between pre- and postflight values is significant compared with data from control monkeys.
Biochemical Analyses

Even though the difference did not reach statistical significance, serum osteocalcin levels were found to be 26% lower in flight monkeys after flight compared with preflight values (78.0 ± 4.0 vs. 105.5 ± 7.5 ng/ml, postflight vs. preflight respectively, P = 0.08) (Fig. 7). In contrast, vivarium and confinement monkeys did not exhibit any significant change in serum osteocalcin levels (P = 0.40 and P = 0.50, respectively).

DISCUSSION

The results of this study suggest that a 14-day spaceflight affected iliac bone formation in young growing rhesus monkeys, resulting in decreased iliac bone volume. The effects of spaceflight on bone formation were previously reported in rats (13, 24, 43, 46–48). The present results in monkeys are of importance considering the absence of bone biopsy data from humans in spaceflight. This is also a new finding in nonhuman primates. Indeed, previously obtained biopsy samples have been collected too late after recovery from space to permit valid postflight cell activity assessment in monkeys (50). In the present study, bone biopsies were harvested within hours after landing, which avoided measurements of readaptation to normal gravity. We found that spaceflight inhibited cancellous osteoblast activity as evidenced by the decreased extent of osteoid formed. These changes were confirmed by the alterations in both the extent of mineralizing surface and mineral apposition rate resulting in decreased bone formation rate. Interestingly, wall thickness, which is an index of the amount of matrix synthetized by a team of osteoblasts over a total cycle period and further mineralized (29), was also found to be decreased in flight monkeys. The determinants of wall thickness are matrix capacity of osteoblasts, together with osteoblast density (31). However, indirect evidence has been shown to support a major contribution of impaired osteoblast recruitment to the pathogenesis of decreased wall thickness in osteopenia (31). The correlations found between wall thickness and bone formation rate on one hand, and between wall thickness and osteoid surfaces on the other hand, strongly suggest that the reduction in the amount of bone formed during spaceflight was caused by a fall in osteoblastic cell density (29). This hypothesis may be supported by the fact that Ac.f, which is the probability that a new cycle of remodeling is initiated at any point on the bone surface, tended to be decreased in flight animals. This is also supported by ultrastructural data from the same experiment showing a lower number of actively forming osteoblasts in iliac bone samples of flight animals compared with controls (37). This result is also consistent with our previous findings in unloaded rats, reporting that decreased mechanical loading on bone is

Fig. 3. Iliac trabecular bone from one flight monkey preflight (A) and postflight (B). Sections were stained by use of the Goldner trichrome technique. Note the markedly shorter and thinner extent of osteoid (dark staining, arrows) in the postflight compared with the preflight biopsy. Ca-b, calcified bone; Bo-m, bone marrow.

Fig. 4. Mineralization profile in preflight biopsies and in flight, confinement, and vivarium monkeys. Data show the %area of bone at various mineralization levels (gray level bin from 2, least mineralized, to 7, most mineralized).

Fig. 5. Results of the logit function on data from back-scattered electron analyses in iliac bone from monkeys of the Bion 11 mission. Each bar is the mean (±SE) of data obtained in each group of monkeys, preflight (open bars) and postflight (filled bars). logit(4) = log(proportion > 4/proportion ≤ 4), where 4 represents the average value mineralization levels.
Table 3. Osteoblast characteristics in bone cells isolated from iliac crest in preflight and postflight monkeys

<table>
<thead>
<tr>
<th></th>
<th>Alkaline Phosphatase</th>
<th>Type I Collagen</th>
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<tbody>
<tr>
<td></td>
<td>Activity,</td>
<td>Synthesis,</td>
</tr>
<tr>
<td></td>
<td>nmol PNP·min⁻¹·mg⁻¹·protein⁻¹</td>
<td>µg·P1CP·µg⁻¹·protein⁻¹</td>
</tr>
<tr>
<td>Preflight (8)</td>
<td>4.6 ± 0.5</td>
<td>27.1 ± 5.2</td>
</tr>
<tr>
<td>Postflight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flight (1)</td>
<td>3.7</td>
<td>17.4</td>
</tr>
<tr>
<td>Vivarium (3)</td>
<td>3.8 ± 1.2</td>
<td>17.0 ± 1.3</td>
</tr>
<tr>
<td>Confinement (3)</td>
<td>4.8 ± 1.0</td>
<td>25.2 ± 6.2</td>
</tr>
</tbody>
</table>

Data are the means ± SE. The number of animals is given in parentheses. PNP, p-nitrophenol; P1CP, carboxy-terminal propeptide.

associated with lower bone formation activity and lower recruitment of osteoblastic cells (19). Therefore, we can hypothesize that decreased bone formation during spaceflight could be due to both lower recruitment of osteoblastic cells and matrix-forming activity.

We isolated cells from trabecular bone with the aim of identifying changes in osteoblast proliferation and/or differentiation induced by spaceflight in monkeys. We found that these cells exhibit characteristics of the osteoblast phenotype such as ALP activity and type I collagen synthesis. We also found that osteoblastic cell proliferation was markedly decreased in one monkey (monkey 357) but not in the other (monkey 484). We consistently found that osteoid surface was more decreased in the former than in the latter monkey. This suggests that spaceflight reduced osteoblastic cell proliferation in parallel with osteoblast activity. The difference in bone cell growth rate between the two flight monkeys could also be due to the fact that the capacity of osteoblasts to recover from spaceflight-induced effects was higher in one monkey than in the other. This hypothesis is consistent with our previous data showing discrepancy in recovery trends in iliac bone structure parameters between the two monkeys of the Bion 10 experiment (50). In this previous work, we found that mineralizing surface was markedly higher (+40%) in one monkey than in the other when evaluated 53 days after returning to Earth. The reason for this discrepancy is not clear. Sources of variability of histomorphometric data can be due to the measuring technique (intraxindividual variations) or to variations due to biological differences (interindividual variations). Intraxindividual variations of primary measurements of histomorphometric parameters have been evaluated to be 10–14% (10), which is far lower than the difference observed between the two present flight monkeys. On the other hand, it has been shown that, for osteoid surfaces, 80% of the total variance was due to true variation between individuals (42). It could be argued that the lower proliferation capacity of osteoblastic cells of monkey 357 could be related to the postsurgery death of this animal. However, no argument may be raised in favor of this hypothesis, because this animal had undergone a thorough medical examination before surgery, performed by a multinational (American-French-Russian) veterinarian team, and all physiological parameters had been monitored during and after anesthesia. Furthermore, the postmortem examination indicated that the animal’s death was caused by acute aspiration asphyxia during recovery from anesthesia (11). This rules out the possible occurrence of changes before or during surgery.

The concept of a regulated pattern of differential gene expression during osteoblastic and bone maturation has been evidenced in vitro with the pattern of expression of c-fos and c-jun (proliferation), type 1 collagen and alkaline phosphatase (differentiation), and finally osteocalcin and osteopontin (mineralization) (40). It has been shown that spaceflight alters the pattern of gene expression for cell differentiation in osteoblasts (3), with lower mRNA levels of osteocalcin and type I procollagen (2). These results obtained in rats are consistent with the hypothesis that inhibition of bone formation during spaceflight could be mediated by downregulation in osteoblasts of gene expression for bone matrix proteins. This is supported by our data reporting a lower amount of osteoid matrix on bone surfaces in flight monkeys than in control animals. However, mineralization lag time, a timing index of

Fig. 6. Proliferation rate of trabecular bone cells isolated from iliac bone, as determined by cumulative DNA synthesis. Bone cells were plated at the same density (2,000 cells/cm²) and cultured for 1, 3, 7, 9, and 14 days. Each point is the mean (±SE) of cultures obtained from data in each group of monkeys (vivarium, confinement) and in each of the 2 flight monkeys.

Fig. 7. Serum osteocalcin levels evaluated in flight, confinement and vivarium monkeys of the Bion 11 mission. Each bar is the mean (±SE) of data obtained in each group of monkeys, preflight (open bars) and postflight (filled bars).

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bone matrix mineralization, was found unchanged, which supports the hypothesis that the mean time interval between deposition and mineralization of matrix was not affected by spaceflight. Although Mlt remained unchanged, it seems that mineralization quality was affected by spaceflight, as exhibited by the decrease in mineralization profile in BSE analyses of bone, which agrees with data reported in the previous spaceflight with monkeys (50).

The hereby-reported decrease in static and dynamic parameters of bone formation in flight animals, associated with unchanged resorption parameters, resulted in decreased BV/TV, which confirms previously reported data (50). However, the structural determinatives of this change are not the same, as Tb.Sp tended to increase in this flight, whereas it did not in the previous Bion experiment. The reason for this difference may be related to the duration of the flight, which was longer in the present study (14 days) than in the previous one (11.5 days) and consequently may have had more impact on bone structure.

The etiology of spaceflight-induced bone changes is poorly known. Mechanical unloading may be responsible for the alterations in bone formation associated with spaceflight. Characteristic losses of appendicular and axial bone (4) and changes in collagen composition of bone (23) have been demonstrated in a primate model of immobilization. Strains applied to bone have been suggested to stimulate directly or indirectly the production of biochemical mediators such as growth factors or prostaglandins (9, 15, 21). Among these factors, transforming growth factor (TGF)-β is one candidate known to increase the number and activity of osteoblasts by autocrine and paracrine actions (22). A decrease in mRNA levels for TGF-β has been reported in long bones of rats flown for 11 days (45), and continuous infusion of TGF-β2 has been proven to prevent bone loss in unloaded rats (18). Apart from local factors, systemic skeletal response to spaceflight cannot be ruled out. Bed-rest studies in humans have evidenced decreased levels of parathyroid hormone and 1,25(OH)2D (1), which could account for the decreased rate of bone cell activity observed. However, although plasma levels of vitamin D or parathyroid hormone may be transiently altered in humans during spaceflight, this cannot account for the changes observed in bone remodeling in space (25, 39).

The role of altered nutrition in the genesis of the observed bone changes must also be discussed. Eating disorders may be associated with reduced bone mineral content in humans (5, 7), and a 30% food restriction has been shown to alter skeletal development in rhesus monkeys (16). Several hypotheses have been proposed to explain this relationship, including reduced mechanical loading, altered hormone levels, and dietary factors such as reduced calcium and energy intake. It indeed appears that bone remodeling changes are measurable after prolonged and marked energy (~40%) and/or calcium (~80%) restrictions (5, 7, 41). However, the precise mechanisms that explain the bone effects of starvation are still unclear, even though it has been reported that acute fasting may influence the circadian rhythm of bone remodeling (38). In the present investigation, even though starvation effects on bone metabolism could not be excluded, the overall result on bone of this short duration (2 days) food restriction in the early stage of spaceflight is likely to be negligible with regard to the overall effects of the 2-wk sojourn in microgravity. Furthermore, calcium or energy restriction (or both) have been shown to induce an increase in bone resorption (41), whereas we observed unchanged bone resorption and decreased bone formation in the present study.

Finally, we cannot exclude the effect of stress in these events. Stressful situations are known to enhance glucocorticoid levels (26), thereby affecting osteoblastic cells (12). We also found in the present work that serum osteocalcin levels tended to decrease in flight animals. Serum osteocalcin may be influenced by stressful situations (27, 34, 35), and microgravity conditions may be considered to be mentally as well as physically stressful. Osteocalcin synthesis and secretion have been reported to be decreased in rats in response to simulated or real microgravity (2, 33). Moreover, we recently showed in rats that the effects of spaceflight on bone formation are independent of the glucocorticoid status of animals (49). In the present study, except for weightlessness, confinement was intended to simulate the environmental conditions of flight, and, interestingly, we found that biochemical, cellular, and histological indexes of bone metabolism were unaffected by confinement. Histomorphometric measurements indicating decreased bone formation in flight monkeys after flight are consistent with the trend for lower serum osteocalcin levels in the same monkeys postflight, suggesting that the observed bone changes can be mainly attributed to spaceflight. This contrasts with stress-related results obtained in peripheral long bones in monkeys of the same age on the ground after a 2-wk restraint (4, 23). The difference may be explained by the fact that, in the latter studies, monkeys were fully immobilized by application of total body cast, whereas in our studies arm and leg movements remained possible in flight and confined monkeys.

In conclusion, despite experimental difficulties regarding the low number of experimental subjects, bone tissue analyses from the Bion 11 spaceflight provide new biochemical, tissue, and cellular data suggesting that spaceflight affects static and dynamic parameters of bone formation in iliac bone of young rhesus monkeys. These alterations suggest that osteoblastic activity and/or recruitment was altered, resulting in decreased bone structure and mineralization parameters. These results are in good agreement with previous works in monkeys and demonstrate that, without countermeasures, weightlessness induces significant bone loss. Further studies are required to investigate the degree to which these alterations could occur with longer flights, as well as the mechanisms of these changes and the measures that could be used to limit or abrogate them.
We express deep gratitude to the Russian (Institute for Medical and Biological Problems, particularly Eugene Ilyin, Inessa Kozlovskaya, and Vyacheslav Korolkov), and American (National Aeronautics and Space Administration, particularly Mike Skidmore, Richard Grindeland, Joe Bielitski, and Shawn Bengston) support teams whose extraordinary efforts made this work possible. We also thank Antoine Bernardé, Pradeep Danturi, Sylvie Renault, Cathérine André, Benoït Noël, Philippe Delamony, and Monique Hott for technical assistance. Finally, we thank Martine Chassagne for type-script and Danielle Freund for English translation.

This work was supported by the Centre National d'Études Spatiales (French space agency).

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