Effects of a long-term spaceflight on immunoglobulin heavy chains of the urodele amphibian *Pleurodeles waltl*

Rachel Boxio, Christian Dournon, and Jean-Pol Frippiat

*Laboratoire de Biologie Expérimentale et Immunologie, Université Henri Poincaré-Nancy 1, Vandœuvre-lès-Nancy cedex, France*

Submitted 1 September 2004; accepted in final form 2 November 2004

**Boxio, Rachel, Christian Dournon, and Jean-Pol Frippiat. Effects of a long-term spaceflight on immunoglobulin heavy chains of the urodele amphibian *Pleurodeles waltl*. J Appl Physiol 98: 905–910, 2005. First published November 5, 2004; doi:10.1152/japplphysiol.00957.2004.—A variety of immune parameters are modified during and after a spaceflight. The effects of spaceflights on cellular immunity are well documented; however, little is known about the effects of these flights on humoral immunity. During the Genesis space experiment, two adult *Pleurodeles waltl* (urodele amphibian) stayed 5 mo onboard Mir and were subjected to oral immunization. Animals were killed 10 days after their return to earth. IgM and IgY heavy-chain transcripts in their spleens were quantified by Northern blotting. The use of the different VH families (coding for antibody heavy-chain variable domains) in IgM heavy chain transcripts was also analyzed. Results were compared with those obtained with ground control animals and animals reared in classical conditions in our animal facilities. We observed that, 10 days after the return on earth, the level of IgM heavy-chain transcription was normal but the level of IgY heavy-chain transcription was at least three times higher than in control animals. We also observed that the use of the different VH families in IgM heavy-chain transcripts was modified by the flight. These data suggest that the spaceflight affected the antibody response against the antigens contained in the food.

**During spaceflights, many factors, such as microgravity, stress, radiation, and disruption of circadian rhythm, combine to influence a variety of physiological functions. Spaceflight has been shown to affect a variety of immune parameters in humans and animals (3, 27). Alterations in leukocyte blastogenesis (10, 21), increase in polymorphonuclear leukocytes (9, 30, 32), and modification of the production of some cytokines, such as interferon-γ, were reported after spaceflight (13). Exposures to spaceflight also modified the distribution of leukocyte subpopulations (26). This could be mediated in part by changes in adhesion molecules (15, 30). Natural killer cell activity was severely inhibited in humans and rats subjected to spaceflight (19, 20, 22, 25). Reactivation of latent viruses, such as the Epstein-Barr virus, was reported after a flight (28, 29). Delayed hypersensitivity skin test responses to common recall antigens were determined during spaceflight and were found to be inhibited during short- and long-term missions (8, 12, 31). T-lymphocyte responsiveness to mitogens was also severely depressed during and after spaceflights (7, 8) because the expression of both IL-2 and IL-2Ra genes was significantly inhibited (37).

By comparison, humoral immunity has only rarely been studied in cosmonauts and astronauts. No significant changes in plasma levels of immunoglobulin were observed after short-term spaceflights (30, 36), but increases in the level of serum immunoglobulin, particularly total IgA and IgM, were reported after long-term flights (18). Moreover, studies on the effects of spaceflight on the antibody response to specific antigens have not been reported to date.

In a previous unpublished work, we showed that the urodele amphibian *Pleurodeles waltl* produced two types of antibodies, IgM and IgY, with urodele IgY being the physiological counterpart of mammalian IgA molecules. We have also determined that this animal possesses at least 10 families of VH genes (coding for antibody heavy-chain variable domains), 8 JH and 6 D segments (R. Boxio, C. Dournon, and J. P. Frippiat, unpublished observations). Recently, we have demonstrated that *P. waltl* uses the same genetic mechanism as higher vertebrates for rearranging its VH genes (JH and D segments) during B lymphocyte differentiation (Ref. 11; R. Boxio, C. Dournon, and J. P. Frippiat, unpublished observations).

Using *P. waltl* as a model system, we examined the effects of spaceflight on immunoglobulin heavy-chain repertoires.

**MATERIALS AND METHODS**

**Animals.** Adult *P. waltl* (3-yr-old females) reared in three different experimental conditions were analyzed in this work. The first group was composed of two *P. waltl* that were reared in classical conditions, i.e., reared in running tap water at a constant temperature of 18-20°C and fed twice a week with ground meat and *Chironomus plumosus* larvae. The second group was composed of two *P. waltl* that stayed for 5 mo onboard the Mir space station for the Genesis experiment performed during the Perseus French-Russian mission that occurred in 1999. These animals were designated V3R and V4R. The V stands for “vol.” which indicates flown animals; 3R and 4R refer to the number of small red pearls attached to the animals’ tails to identify each animal (e.g., 3R means 3 red pearls). These animals were reared in classical conditions in our animal facilities before the flight, but the feeding and living conditions were significantly modified onboard the space station. Animals were kept in the dark at 18 ± 2°C on towels dampened with Steinberg buffer ([in mM] 5.8 NaCl, 0.067 KCl, 0.034 Ca(NO₃)₂·4H₂O, 0.008 MgSO₄·7H₂O, 0.46 Tris·HCl, pH 7.4). They were fed three times a week with a dietetic food with a high energetic value, a high level in essential nutrients, and highly digestible ingredients (Prescription Diet Canine/Feline a/d, Hill’s Pet Nutrition, Sophia-Antipolis, France). This food, originally designed to feed cats and dogs, was diluted in water and administered using a syringe with a catheter, which was introduced into the stomach of the animals. This force-feed method was developed because *P. waltl* cannot take food.
IMMUNOGLOBULIN CHANGES AFTER SPACEFLIGHT

by themselves under microgravity conditions (35). More details about the Genesis experiment can be found in Ref. 35. After their return to earth, animals were reared in classical conditions until their death 10 days later. This delay was justified by the fact that, for another research program, we needed freshly laid eggs from the flown animals. Originally, four males and four females of *P. waltl* were launched in space, but only two females survived. The other six animals died on the 17th and 18th day after launch, probably by anoxia due to a breakdown in the ventilation system of the space station. After this incident, it was decided to keep the two surviving females in separate boxes, which were ventilated twice a day until the end of the mission. The third group was composed of two *P. waltl* reared in our laboratory in the same conditions as onboard Mir, i.e., force fed three times a week with the same dietetic food administered using a syringe and a catheter. These animals were designated S1R and S4R. S stands for “sol,” which indicates animals reared on earth; 1R and 4R refer to the number of small red pearls used to identify each animal (see above). Note that red pearls were on the left side of the tail of flown animals and on the right side of the tails of ground controls. Animals were treated in accordance with National Legislation and The Council Directive of the European Communities on the Protection of Animals Used for Experimental and Other Scientific Purposes 86/609/EEC.

**RNA extraction and Northern blotting.** Spleens from the two animals reared in classical conditions were mixed and RNA was extracted from this mixture, using the acid guanidine thiocyanate-phenol-chloroform method (5), to determine the average IgM and IgY heavy-chain transcription level. Total RNA was also isolated from the spleens of S1R, S4R, V3R, and V4R. RNA concentrations were determined by ultraviolet spectroscopy. These quantifications were approximate because pigments remained in our RNA preparations. Moreover, the amount of pigments were variable from one RNA extraction to another. Therefore, RNA concentrations determined by ultraviolet spectroscopy were approximate, and the same amount of RNA could not be loaded in each well of the agarose gels used to perform the Northern blotting experiments. We did not further purify our RNA samples because spleens of *P. waltl* are small and, therefore, produce limited amounts of RNA (~25–40 μg of total RNA). RNA quality was checked by running an aliquot of RNA on a 0.8% agarose gel. Two Northern blotting experiments were performed. Ten micrograms of total RNA, amounts calculated using the concentrations determined by ultraviolet spectroscopy, were loaded on 1% agarose, 1.5% formaldehyde gels in MOPS buffer (20 mM 3-[N Morpholino]-propanesulfonic acid, pH 7.0, 5 mM sodium acetate, 1 mM EDTA). After electrophoresis, gels were stained in 300 ml of diethyl pyrocarbonate-treated water that contained 0.5 μg/ml ethidium bromide. RNA was transferred to Hybond-N+ nylon membranes (Amscherm Pharmacia Biotech, Orsay, France) in ×10 SSC and baked at 80°C for 2 h. Filters were prehybridized in 50% formamide, ×5 SSPE, 0.02 M EDTA, 0.3% SDS, 5% dextran sulphate, 600 μg/ml salmon sperm DNA, and ×5 Denhardt’s solution at 42°C for 4 h. One membrane was hybridized with a mixture of GAPDH- and Cμ-specific probes; the other membrane was hybridized with a mixture of GAPDH- and Cυ-specific probes. The Cμ and Cυ probes were obtained by PCR using primers CU1 + CU2 (CU1: 5′-GGGTGGCGGGCATCTCTTGAGCA-3′; CU2: 5′-GGGGCAGTGGTAAAGGCTCC-3′) or CY1 + CY2 (CY1: 5′-GATCAACCATCAAGGCTCC-3′; CY2: 5′-ATGGCACTCAGAGGTCTACAC-3′), and plasmid DNA containing the constant regions of IgM or IgY heavy chains as template. The 381-bp Cμ probe contained 270 bp from CU1, the first constant domain of IgM heavy chains, and 111 bp from CU2. The 381-bp Cυ probe contained 300 bp from CY1, the first constant domain of IgY heavy chains, and 81 bp from CY2. Probes were labeled with [α-32P]dCTP using the Random Primed DNA labeling kit (Roche, Meylan, France). Filters were washed twice in ×2 SSC, 0.1% SDS for 30 min at room temperature, twice in ×0.5 SSC, 0.1% SDS for 30 min at 42°C, and finally once in ×0.1 SSC, 0.1% SDS at 55°C. After being washed, the numbers of counts per minute (cpm) in the GAPDH, IgM, and IgY bands were counted directly on the membranes using an Instant imager (Packard). The number of cpm is proportional to the abundance of the transcript. Then, we calculated the ratio between the number of cpm in the IgM or IgY band and the number of cpm in the GAPDH band within each sample. These ratios were independent of the amount of RNA loaded on the gel, as we compared the number of cpm for IgM or IgY and the number of cpm for GAPDH within the same RNA sample, which was hybridized simultaneously with the Cμ- and GAPDH-specific probes or with the Cυ- and GAPDH-specific probes. These ratios indicate the IgM or IgY heavy-chain transcription levels determined by comparison with a control (GAPDH) revealed simultaneously in each sample. Finally, these levels of transcription were expressed as a percentage of the IgM or IgY transcription levels determined with the RNA extracted from the mixture of the spleens of the two *P. waltl* reared in classical conditions in our animal facilities. For visualization, filters were exposed on β-max films (Amersham Pharmacia Biotech, Orsay, France).

**Construction and analysis of the VH Cμ libraries.** We have amplified by 5′-RACE the V(D)J rearrangements contained in IgM heavy-chain mRNA from the spleens of the two ground control animals (S1R and S4R) and the two *P. waltl* that stayed onboard Mir for 5 mo (V3R and V4R), and the mixture of the spleens of the two *P. waltl* reared in classical conditions. For this purpose, we used the Smart Race cDNA kit (Clontech, Palo Alto, CA) and the CMGSPI (5′-CCACGTTAGGTGTTAGTCCGAGG-3′/primer, which anneals to the *P. waltl* Cμ1 domain. For each RNA sample, a PCR product of 0.6 kb containing the V(D)J rearrangements associated with Cμ was obtained, gel purified, and cloned in the pGEM-T Easy vector (Promega, Lyon, France) to produce five VH Cμ libraries. There was one library per animal, except for animals reared in classical conditions, because their spleens were mixed. Probes specific for the first nine *P. waltl* VH families were labeled with [α-32P]dCTP and used to screen by colony hybridization 250 clones from each VH Cμ library.

**Statistics.** Frequency data were analyzed by χ2 analysis. Differences were considered significant at P ≤ 0.05.

**RESULTS**

**Quantification of IgM and IgY heavy chain transcripts.** We quantified, by Northern blotting, IgM and IgY heavy chain transcript in the spleens of the animals reared in the three experimental conditions described in the first part of MATERIALS AND METHODS. Two membranes were prepared with the same RNA samples. One membrane was hybridized with Cμ- and GAPDH-specific probes (Fig. 1A), the other one was hybridized with Cυ- and GAPDH-specific probes (Fig. 2A). Membranes were washed at high stringency, and the radioactivity in each band was counted using an Instant imager. The ratio between the number of cpm in the IgM or IgY band and the number of cpm in the GAPDH band of each sample indicated the IgM or IgY heavy-chain transcription level. Quantification results are shown in Figs. 1B and 2B. Figure 1B indicates that the transcription level of IgM heavy chains was not affected by the feeding and living conditions. Indeed, similar results are observed in ground controls (S1R and S4R), in V3R and V4R that stayed onboard Mir for 5 mo, and in the control (animals reared in classical conditions in our animal facilities). However, when the same experiment was performed to quantify the IgY heavy-chains transcript (Fig. 2B), we observed a clear difference between animals that stayed for 5 mo onboard Mir (V3R and V4R) and those reared on earth (S1R, S4R, and control). The IgY heavy-chain transcription level is at least three times higher after the 5-mo stay in space.
Use of the different VH families. Five VH Cμ libraries were built to assess the contribution of the different VH families to the repertoire of IgM heavy chains. There was one library for animal S1R, one library for S4R, another one for V3R, one for V4R, and finally one library constructed from RNA extracted from the mixture of the spleens of two P. waltl reared in classical conditions in our animal facilities. Two hundred fifty clones per library were randomly chosen and analyzed by colony hybridization, with probes specific for the first nine VH families (Fig. 3). Family X was not analyzed because we had previously determined that its contribution to the expressed repertoire was negligible (data not shown). VH family use was found to be very similar in S1R and S4R animals and in V3R and V4R animals. Families I, II, and VI are found, respectively, in 5, 28, and 58% of the S1R and S4R IgM heavy chains, whereas in animals reared in classical conditions (control), these families are found in 29, 43.5, and 9%, respectively, of the IgM heavy chains. These profound changes are likely due to the immunization of the animals with the antigens contained in the food. Indeed, the food provided to ground control animals (S1R and S4R) has a composition that is completely different from the food P. waltl got in classical conditions (control). Thus heavy chains of specific IgM are mainly made with VH genes from the VH II and VH VI families.

**DISCUSSION**

Humoral response of the urodele amphibian P. waltl. Figure 3 demonstrates an important change of VH family use in IgM heavy chains between unimmunized (control) and immunized (S1R, S4R, V3R, and V4R) animals. Immunization is associated with an important decrease in the use of the VH I family and an increase in the use of the VH VI family, which has its own combination of canonical structures for its H1 and H2 loops (R. Boxio, C. Dournon, and J. P. Frippiat, unpublished observations), thereby indicating a change in the functionality of the antibodies. This observation is in accordance with previous publications indicating that immunization of P. waltl induces the production of specific antibodies of the IgM class (4, 33, 34).

Our results (Fig. 3) also showed that a restricted number of VH families was used to produce IgM heavy chains: families I and II in unimmunized animals, families II and VI in immunized animals. A similar bias was also observed in...
human immunoglobulin lambda light chains (16). Out of 11 Vκ families, 3 are found in ~90% of the lambda light chains. This restriction is, therefore, a general and normal feature of antibody production.

**Effects of long-term spaceflight.** After their 5-mo stay in space, V3R and V4R animals had a 16% lower body mass compared with control animals, whereas S1R and S4R animals had only a 3% lower body mass at the end of the experiment. The V3R and V4R loss of weight is mainly explained by the fact that these amphibians laid during their stay onboard Mir. Eggs represent ~8–9% of the body weight. Gridley et al. (14) reported a 10–12% decrease in the body mass of mice after a 12-day space shuttle mission. Our *P. waltl*, which are among the vertebrates that stayed for the longest time onboard a space station, did not lose so much weight because they were force fed during the mission (35).

We previously showed that IgY molecules represent 6% of the antibodies found in the spleen and 43% of the antibodies found in the intestinal mucosa of *P. waltl* reared in classical conditions (R. Boxio, C. Dournon, and J. P. Frippiat, unpublished observations). *P. waltl* IgY molecules are therefore the physiological counterpart of mammalian IgA molecules. Northern blotting experiments showed that, 10 days after the return to earth, the transcription level of IgY heavy chains in the spleens of V3R and V4R is at least three times higher (Fig. 2). Interestingly, it was reported that spaceflights can modify the leukocyte distribution in the organism (24, 26). An increase in mouse splenic lymphocyte percentages was reported after a short-term spaceflight (24). The increase of IgY heavy-chain transcription in the spleens of V3R and V4R could therefore result from a change in the distribution of IgY-producing cells in *P. waltl* bodies.
As explained above, S1R, S4R, V3R, and V4R were fed with a food that induced an immune response. We can clearly see in Fig. 3 that these amphibians use mainly the VH II and VH VI families, whereas unimmunized P. waltl (control) use mainly the VH I and VH II families in their IgM heavy chains. However, this figure also indicates that families II and VI are found, respectively, in 28 and 58% of the S1R and S4R IgM heavy chains and in 61 and 24% of the V3R and V4R IgM heavy chains. These changes in the use of the VH II and VI families are unlikely due to an infection of the animals in the space station as they were kept in separate boxes from day 19 of the experiment up to the end of the mission. These changes, rather, reflect the impact of the flight on the immune response against the antigens contained in the food of the two P. waltl reared onboard Mir. During spaceflight, animals are subjected to microgravity, radiation, and stress. It is impossible to know which parameter affected the immune response of our animals. A direct effect of microgravity on the expression of the IL-2 and IL-2Rx genes was recently demonstrated (37). Microgravity could therefore also modify the expression of other genes, such as those involved in the V(D)J recombination machinery, and, consequently, modify the use of the VH families. It has been known for some time that radiation exposure can lead to changes in gene expression pattern. Recently, Nelson et al. (23) conducted full genome microarray studies of the nematode C. elegans and showed that 599 of 17,871 genes analyzed (3.4%) showed differential expression after exposure to gamma rays, protons, or high-energy iron particles. Amundson et al. (1) also performed radiation-induced genes in human peripheral blood cells. Using microarray analysis, they detected a large number of radiation-induced genes and found that DNA repair genes, including the DNA-dependent protein kinase gene, were among the set whose transcriptional profiles were altered. The list of significantly induced genes is available at http://rex.nci.nih.gov/RESEARCH/basic/lbc/forname.htm. Interestingly, it was previously demonstrated that DNA-dependent protein kinase is involved in V(D)J recombination (2, 17). There is a functional overlap between DNA repair and V(D)J recombination. Therefore, a change in DNA-dependent protein kinase expression could perhaps affect the recombination process. Finally, stress should also be considered because it can alter the cellular and humoral immune responses.

In conclusion, our work suggests that a long-term spaceflight could modify the distribution of some B lymphocytes and the antibody response of P. waltl. Despite the fact that the immune system of amphibians is different from that of mammals, the changes in the use of the VH families observed after spaceflight could perhaps be applicable to mammals since we previously demonstrated that P. waltl uses the same recombination machinery as higher vertebrates (Ref. 11; R. Boixio, C. Douron, and J. P. Frippiat, unpublished observations). Further studies are required to confirm this hypothesis.

Acknowledgments

We thank the French cosmonaut Jean-Pierre Haigneré for efficient practical expertise, all of our Russian colleagues for the preparation and realization of the space mission, the Centre National d’Etudes Spatiales board for management, and Christiane Tankosic for technical support in our laboratory.

Grants

This work was supported by the Ministère de la Jeunesse, de l’Éducation Nationale et de la Recherche, and by the Centre National d’Etudes Spatiales through grants DAR4800000024 and 8626.

References


J Appl Physiol • VOL. 98 • MARCH 2005 • www.jap.org