Effects of confinement (110 and 240 days) on neuroendocrine stress response and changes of immune cells in men

A. CHOULKÈR,1 L. SMITH,1 F. CHRIST,1 I. LARINA, I. NICHIPORUK,2 V. BARANOVA,2 E. BOBROVNIK,2 L. PASTUSHKOVA2, K. MESSMER,3 K. PETER,1 AND M. THIEL1
1Clinic of Anaesthesiology and 2Institute for Surgical Research, University of Munich, 81366 Munich, Germany; and 3Institute of Biomedical Problems, 123007 Moscow, Russia

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Choukèr, A., L. Smith, F. Christ, I. Larina, I. Nichiporuk, V. Baranov, E. Bobrovnik, L. Pastushkova, K. Messmer, K. Peter, and M. Thiel. Effects of confinement (110 and 240 days) on neuroendocrine stress response and changes of immune cells in men. J Appl Physiol 92: 1619–1627, 2002; 10.1152/japplphysiol.00732.2001.—The aim of the study was to evaluate the effects of long-term confinement on stress-permissive neuroendocrine and immune responses in humans. Two groups of four male subjects were confined 240 days (group 240) or 110 days (group 110) in two space modules of 100 or 200 m³, respectively. During confinement, none of the volunteers developed psychic stress as could be examined and verified by a current stress test. However, in group 240 but not in group 110, the diurnal rhythm of cortisol secretion was slightly depressed and the urine excretion of norepinephrine significantly increased. The innate part of the immune system became activated as seen by a rise in the number of circulating granulocytes and the enhanced expression of β2-integrins. In contrast, the ratio of T-helper to T-suppressor cells decreased. All these effects, observed during confinement, were even more pronounced in both groups when values of endocrinological and immunological parameters were compared between before and 1 wk after the end of the confinement period. Hence, return to normal life exerts pronounced effects to a much higher degree, irrespective of how long or under which conditions individuals were confined. Because the delayed-type hypersensitivity skin reaction against recall antigens remained unaffected, it is to be presumed that confinement appears to induce distinct sympathoadrenergic activation and immunological changes but no clinically relevant immunosuppression.

Spaceflight can affect every organ system, and it causes a number of physiological changes (immune changes, muscle atrophy, demineralization of bones). Alterations in immune responses during and after spaceflight have been reported in humans (15). Such effects (29, 31) could be a consequence of microgravity and radiation (18, 25). Besides physical forces, environmental stress factors could also play an important role in inflight as well as in postflight changes of the immune response. However, the quantification of environmental stress factors seems to be difficult because isolation, confinement, artificial circadian rhythm, as well as altered social interactions may altogether chronically affect the neuroendocrine stress and immune responses (20).

Because Russian, European, and American space agencies plan to establish long-term presence of people in space [International Space Station (ISS); mission to Mars], ground-based simulation studies are frequently preferred to investigate possible unwanted changes of health under standardized conditions. Accordingly, ground-based studies have been carried out to mimic the effects of low gravitation [e.g., by long-term hypokinesia in head-down tilt (HDT) at 6° (7, 9, 17, 40) or by water immersion (8)]. Confinement can be induced operationally in submarines, polar stations, and deep-sea laboratories as well as during expeditions in the Antarctica. Even deep-sea diving can, to a certain extent, reflect in-space conditions (a return to Earth or surface being impossible for a number of days even in the case of emergency), although hyperbaric conditions (100% oxygen) do not reflect life in space (27). Studies in a slightly pressurized diving chamber, where the conditions of daily life and work resemble those in space, mimic conditions in space better, but they have not yet been studied beyond a duration of 60 days (12).

In the present study, we investigated the isolated effects of group confinement for 110 and 240 days on stress-permissive neuroendocrine and immune responses in humans. To this end, the same variables were determined that were shown in a previous ground-based study to be sensitive enough for the detection of psychic stress and neuroendocrine-related changes of the immune system in healthy volunteers who had been subjected to HDT (5). Because the number and volume of blood samples allowed to be drawn were strictly limited, overall reactivity and cooperation of the nonspecific and specific parts of the immune response were additionally assessed by the delayed-type hypersensitivity (DTH) reaction of the skin against a panel of standardized recall antigens.

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MATERIALS AND METHODS

SFINCSS99, Study Groups, and General Conditions

In the future, ISS astronauts and cosmonauts will form one international crew living in different national space modules for longer periods of time. To simulate these conditions, the Simulation of Flight of the International Crew on the Space Station (SFINCSS99) project was carried out in the Institute of Biomedical Problems in Moscow in 1999 and 2000. All the subjects investigated were selected in accordance to medical criteria of cosmonaut selection and already had experience in extreme environmental conditions (e.g., member of astronaut or cosmonaut team). The general conditions were set to mimic the environmental conditions (gas composition, pressure, humidity and temperature, noise) as well as the living conditions on board the ISS. Regarding the latter, main inflight operations were simulated (e.g., docking, visiting groups), communication with mission control was performed (each 90 min for 30 min), and nutrition and sanitary concerns were mimicked. Daily life was subjected to a 24-h schedule: 8 h of daily work, 8 h of sleep, 3 h of spare time, 2 h of physical training, 2 h for meal preparation and eating, and 1 h for personal hygiene. Day and night cycles were kept by external timers (bright light in the module from 7 to 23 h).

After approval from the local ethical committee and receipt of informed consent, the first group of four healthy Russian male subjects (aged 41.8 ± 5.5 yr) was confined for 240 days in a container resembling a Russian space module (100 m²) where working zone and sleeping area were combined in one room (further designated as group 240). The second group of four subjects (3 Russian and 1 German volunteer, aged 38.2 ± 9.0 yr) spent 110 days (further designated as group 110) in the second chamber that was larger (200 m²) and contained a separate sleeping area for each subject. Both modules were connected to each other. The number of individuals who could be investigated was limited by the volume of the space modules studied.

According to the study protocol, saliva samples and tests of psychic well-being were taken before confinement (Pre 110 and Pre 240), monthly during confinement [group 110: months 1–3 (M1–M3); group 240: months 1–7 (M1–M7)], and 1 wk after the end of the confinement period (Post 110 and Post 240). Peripheral blood samples were withdrawn from the cubital vein before and 1 wk after confinement (Pre and Post 110 and Pre and Post 240) and additionally monthly (M1–M7) during confinement in group 240 to evaluate possible changes of white blood cells. In addition, DTH skin reaction against seven recall antigens was determined in both groups before and after confinement. Blood samples were analyzed in part immediately after acquisition or prepared and stored for further analyses.

No true control group (a matched group of individuals subjected to daily life outside of the chamber for the same period of time) could be studied because of the long-term approach of the present investigation and because of financial reasons. Hence, in agreement with the design of previous studies (5, 24), changes were compared to the Pre values within each group.

Current Stress Test

The current stress test (CST) was developed and validated by German psychologists (21) and was already applied during a study on the effects of 120 days of 6° HDT (5). The test is composed of six pairs of contradictory feelings of increasing intensities between which the subject has to decide. From the sum of values obtained, the final CST score is calculated, which may range from 1 (no stress) to 6 (maximal stress). The CST is a self-estimating, one-page paper test that is performed within 1 min and whose answers cannot be recalled by the test person because of the composition of the questionnaire.

Cortisol

Because glucocorticoids are known to be periodically secreted in response to a variety of environmental and hormonal stimuli (e.g., psychic stress and physical exercise), which alone and/or together with cortisol might affect the immune system, free cortisol was determined repeatedly in saliva samples collected in the morning (8:00 AM) and in the evening (8:00 PM). Saliva was collected by chewing on a cotton swab for 30–45 s, which was stored in a SALIVETTE device tube (Sarstedt, Nürnbrecht, Germany). Samples were frozen, and free-cortisol concentrations were quantified by RIA according to the instructions of the manufacturer (Cort-CT2 kit, CIS Bio International, Gif-sur-Yvette, France). To monitor the circadian rhythm of cortisol secretion, the ratio between morning and evening (m/e) cortisol values was calculated.

Prolactin

EDTA-containing plasma was aliquoted from blood specimens after centrifugation (600 g for 5 min at 2°C) and stored at −80°C until determination of prolactin by using a commercially available assay (ELECSYS, Roche Diagnostics, Mannheim, Germany).

Catecholamines

Dopamine, norepinephrine, and epinephrine were determined in 24-h urine samples collected monthly in each group at the same time points when saliva was collected and blood samples were withdrawn. After collection of urine in a container prefilled with hydrochloric acid (10%), urine samples were kept frozen until analysis by HPLC (Chromsystems, Martinsried, Germany). The amounts of catecholamines secreted were calculated on the basis of total catecholamine excretion in urine pooled during 24 h (μg/24 h).

Cell Counts

The concentration of lymphocytes, polymorphonuclear leucocytes (PMNL), and monocytes was determined from EDTA-anticoagulated whole blood specimen (Coulter multisizer, Coulter Electronics, Luton, UK).

β₂-Integrins

After incubation of aliquots of cell suspensions with FITC-labeled monoclonal antibody IB4, which specifically binds to β₂-integrins, fluorescence intensities were analyzed by using a FACSScan (Becton Dickinson, San Jose, CA). The expression of adhesion molecules was thereafter calculated as previously described (32) and expressed as relative fluorescence units. Because determination of lymphocyte surface antigens and β₂-integrins could not be performed in Moscow, blood samples were separated and transported to Munich, Germany at 20°C (for Simultest IMK) and at 0°C (β₂-integrins) and analyzed within 12 h after blood sampling. Additional control experiments were carried out to detect possible alterations due to transportation and delayed determination.
**Superoxide Anion Production by Phagocytes in Diluted Whole Blood**

The production of the superoxide anions (O$_2^-$) was determined in whole blood samples by photometric measurement of the reduction of cytochrome c, occurring spontaneously or after stimulation of PMNL with the chemotactic tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP; final concentration 10$^{-6}$ M, Sigma Chemical, Deisenhofen, Germany). For this purpose, a total of 1 ml of heparinized whole blood was aliquoted in four plastic tubes each prefilled with prewarmed (37°C) reaction mixture of 1.4 ml of Hanks’ buffered salt solution, cytochrome c (0.625 mg/ml), and cytochalasin B (2.5 μg/ml). Tube 1 had no further additives, whereas tubes 2 and 3 contained fMLP in the absence and presence of superoxide dismutase (50 U/ml). Tube 4 contained superoxide dismutase (50 U/ml). All concentrations mentioned above are final concentrations. After incubation of the reaction mixtures at 37°C for 15 min, cellular components were pelleted by centrifugation (5 min, 600 g). Thereafter, the supernatants were transferred to a microtiter and the absorbances were determined in triplicates on a multichannel automated photometer (550 nm). Thereafter, absorbances were determined in triplicates on a multichannel automated photometer (550 nm). From the difference between the absorbances determined in samples 2–4 and samples 1–3, the stimulated and the spontaneous production of O$_2^-$ was calculated as previously described (19). The results are expressed as calculated O$_2^-$ production in nanomoles per 1 × 10$^6$ phagocytes per 15 min.

Because of technical problems at the time points M2 to M5, O$_2^-$ production could not be determined in two of four subjects, and therefore data obtained from the remaining two subjects were excluded from further statistical analysis.

**T and B Cells**

To demonstrate possible changes of the specific part of the immune system, the percentages of functionally different subpopulations of lymphocytes were determined. To this end, we measured the expression of cell surface differentiation [cluster of differentiation (CD)] antigens of blood lymphocytes: CD3 on all T lymphocytes; CD4 and CD8 on T-helper, T-suppressor, and T- cytotoxic lymphocytes; and CD19 only on B-lymphocytes. CD56 and CD16 are coexpressed predominantly on a specific subgroup of lymphocytes, the natural killer (NK) cells. All the cell surface markers described here were determined by incubation of cells with specific fluorochrome-labeled monoclonal antibodies and subsequent analyses by flow cytometry (FACSScan, Becton Dickinson) according to the instruction of the assay manufacturer (Simultest IMK-Lymphocyte, Becton Dickinson). Further details of FACS-based immunophenotyping are published elsewhere (5).

**Cytokines and C-reactive Protein**

EDTA-anticoagulated blood specimens were centrifuged at 600 g for 5 min in a cooled centrifuge (2°C) and stored at −80°C until determination of plasma cytokines and C-reactive protein (CRP). Quantitative analysis of interleukin-6 (IL-6), interferon-γ (INF-γ), and CRP was performed by commercially available assay (TINA-Quant, Roche Diagnostics).

**DTH Reaction**

To monitor cellular immunoreactivity, the DTH reaction was tested. The standardized recall antigen multitest skin test (Immignost, Biosyn, Fellbach, Germany) contains seven different antigens that are injected by a test stamp intracutaneously by a slight push on the stamp toward the skin of the inner arm. To determine the DTH, the erythemas formed 48 h later were determined in their smallest and largest diameters, and the mean diameter was calculated. The test can be applied repeatedly, without sensibilization and with high reproducibility (16). It was performed in both groups before confinement (Pre) and 7 days after confinement (Post).

**Statistics**

The present study is a part of a large study coordinated by the Institute of Biomedical Problems in Moscow. Although one would have wished to investigate more than four subjects per confinement group, logistic, personnel, and financial resources set clear limits. Blood, saliva, and DTH analyses were performed in both groups before and after confinement, whereas in group 240 blood samples could be additionally taken during the confinement period. Because living conditions were different between the groups (e.g., volume of chamber), each group was analyzed separately despite the fact that the modules of both groups were connected to each other and social interaction between the groups was possible at any time. Statistical analyses of each group were performed with respect to the limitations of the small number of subjects. Data were tested for normal distribution by one-sample Kolmogorov-Smirnov test and compared with the Pre values of the same subjects by repeated-measures ANOVA with adjustment for multiple comparisons by Bonferroni. Two-tailed level of significance was set at $P < 0.05$. Data are presented as means ± SE. All statistical analyses were performed by SPSS program 10.0 (SPSS, Chicago, IL).

**RESULTS**

**Group 110: Psychoneuroendocrine Changes**

**CST score.** The averaged CST scores in group 110 showed only small variation during confinement (M1–M3: CST < 2.3) in the range of values usually observed in persons having no stress (normal range 2.4–2.8). After confinement, the mean CST score increased slightly (Post 110: 2.67; Table 1).

**Cortisol.** Morning and evening concentrations of free cortisol in saliva were elevated in Pre confinement. This was also the case during confinement, but morning and evening cortisol values always showed a circadian rhythm with higher levels in the morning followed by a decline in the evening. The calculated m/e was in the normal range (3–5) before and during confinement (Pre 110, M1–M3). Interestingly, a drop in m/e was observed after the end of confinement (Post 110: 1.4; Table 1).

**Prolactin.** The plasma concentrations of prolactin were almost doubled after confinement (Pre: 7.63 ng/ml; Post: 13.65 ng/ml), but they still remained in the physiological range (3–23 ng/ml; Table 1).

**Catecholamines.** Total urine secretion (μg/24 h) of dopamine and epinephrine remained unchanged during the entire observation period. This was also the case for the urine concentrations of norepinephrine, with the exception of a significant increase after the end of confinement ($P < 0.05$, Post 110 vs. Pre 110 or M3; Table 1).

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Table 1. Current stress test score, saliva cortisol, plasma prolactin, and urine catecholamine secretion for group 110

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference Values</th>
<th>Pre</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST</td>
<td>2.4–2.8</td>
<td>2.75 ± 0.31</td>
<td>2.25 ± 0.49</td>
<td>2.12 ± 0.41</td>
<td>2.24 ± 0.41</td>
<td>2.67 ± 0.53</td>
</tr>
<tr>
<td>Cortisol (morning 8 AM), nmol/l</td>
<td>13–17</td>
<td>32.38 ± 7.24</td>
<td>40.45 ± 6.19</td>
<td>21.68 ± 5.14</td>
<td>17.85 ± 1.53</td>
<td>28.7 ± 10</td>
</tr>
<tr>
<td>Cortisol (evening 8 PM), nmol/l</td>
<td>3.5–5</td>
<td>17.30 ± 7.91</td>
<td>18.68 ± 4.1</td>
<td>19.83 ± 7.64</td>
<td>16.95 ± 7.26</td>
<td>25.93 ± 8.48</td>
</tr>
<tr>
<td>Cortisol (m/e)</td>
<td>3.0–5</td>
<td>2.87 ± 0.82</td>
<td>2.49 ± 0.59</td>
<td>3.28 ± 2.2</td>
<td>2.84 ± 1.8</td>
<td>1.41 ± 0.43</td>
</tr>
<tr>
<td>Prolactin, ng/ml</td>
<td>3.0–23.3</td>
<td>7.63 ± 2.66</td>
<td>NA</td>
<td>NA</td>
<td>13.65 ± 8.94*</td>
<td>110</td>
</tr>
<tr>
<td>Dopamine, μg/24 h</td>
<td>190–450</td>
<td>256.63 ± 60.4</td>
<td>262.08 ± 14.92</td>
<td>186.40 ± 31.26</td>
<td>287.94 ± 29.96</td>
<td>278.10 ± 38.28</td>
</tr>
<tr>
<td>Norepinephrine, μg/24 h</td>
<td>22–105</td>
<td>31.54 ± 6.17</td>
<td>29.94 ± 6.71</td>
<td>34.38 ± 7.96</td>
<td>33.84 ± 2.96</td>
<td>60.56 ± 3.91*</td>
</tr>
<tr>
<td>Epinephrine, μg/24 h</td>
<td>4–20</td>
<td>11.32 ± 4.42</td>
<td>4.26 ± 0.1</td>
<td>5.02 ± 1.37</td>
<td>7.85 ± 1.43</td>
<td>9.85 ± 3.25</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 subjects. Pre; 1 wk before confinement started; M1, first month of confinement; M2; second month of confinement; M3; third month of confinement; Post, 1 wk after confinement; CST, current stress test; m/e, ratio of morning to evening; NA, not applicable. Reference values were obtained from the literature or from the manufacturer of the assay kits (CST (21), salivary cortisol (23, 37), prolactin (34), urine catecholamine secretion (hospital’s clinical laboratory)). *P < 0.05 vs. Pre; †P < 0.05 vs. Pre or M3. For explanations of statistical analyses, see MATERIALS AND METHODS.

Group 110: Immunological Changes

**Cell counts.** Number of blood leukocytes increased after confinement (Pre 110: 3.83 × 10⁹/μl; Post 110: 4.49 × 10⁹/μl). This rise in white blood cells was mainly due to increased numbers of circulating neutrophils (PMNL +68%) and lymphocytes (+44%), whereas monocyte counts remained unchanged (Table 2).

**Adhesion molecules.** Numeric expression of β₂-integrins (CD18/CD11b) on PMNL was elevated after the end of the confinement period (Pre 110: 45.4 relative fluorescence units; Post 110: 53.4 relative fluorescence units; Table 2).

**O₂ production.** The spontaneous as well as the chemotactic tripeptide (FMLP; -6 M)-stimulated production of O₂⁻ remained unchanged (Table 2).

**Lymphocyte populations.** The total number of circulating lymphocytes increased slightly after confinement but demonstrated no changes in the relative percentages of T and B cells and CD16/CD56-positive NK cells. After confinement, the percentages of the T-helper (CD3⁺CD4⁺) lymphocytes decreased, whereas suppressor (CD3⁺CD8⁺) cells increased, respectively. As a result, the calculated CD4⁺/CD8⁺ ratio decreased slightly but not significantly (Pre 110: ~1.8, Post 110: ~1.5; Table 2).

**Cytokines and CRP.** The plasma concentrations of IL-6 and INF-γ did not show any changes and remained in the lower physiological range at all time points (IL-6) or below the detection threshold (<0.1 pg/ml for INF-γ). The same was true for CRP plasma concentrations (<0.01 mg/l; Table 2).

**DTH.** The DTH reaction to seven recall antigens injected intracutaneously was in the normal physiological range (>10 mm) for all subjects studied. Compared with Pre 110, a slight but not significant increase was observed Post 110 (see Table 5).

Group 240: Psychoneuroendocrine Changes

**CST.** During confinement in group 240, the averaged CST scores varied but remained in the normal physiological range, indicating no stress. However, CST

Table 2. Cell counts, expression of β₂-integrins, superoxide anion production, lymphocyte populations, and cytokine plasma concentrations for group 110

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference Values</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes, ×10⁶ cells/μl</td>
<td>4.0–9.5</td>
<td>3.83 ± 0.49</td>
<td>4.49 ± 0.57</td>
</tr>
<tr>
<td>PMNL, ×10⁹ cells/μl</td>
<td>1.6–6.6</td>
<td>1.58 ± 0.17</td>
<td>3.16 ± 0.42*</td>
</tr>
<tr>
<td>Monocytes, ×10⁹ cells/μl</td>
<td>0.2–1.0</td>
<td>0.15 ± 0.03</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>Lymphocytes, ×10⁹ cells/μl</td>
<td>1.5–4.0</td>
<td>0.83 ± 0.08</td>
<td>1.20 ± 0.11</td>
</tr>
<tr>
<td>IB4, relative fluorescence units</td>
<td>35–45</td>
<td>45.40 ± 12.00</td>
<td>53.4 ± 5.4</td>
</tr>
<tr>
<td>O₂⁻ (stimulated), nmol·1 × 10⁶ PMNL·15 min⁻¹</td>
<td>5.5–15</td>
<td>11.94 ± 5.62</td>
<td>6.91 ± 2.4</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>&lt;12.5</td>
<td>1.23 ± 0.27</td>
<td>1.31 ± 0.36</td>
</tr>
<tr>
<td>INF-γ, pg/ml</td>
<td>&lt;15.6</td>
<td>&lt;0.01 ± 0</td>
<td>&lt;0.01 ± 0</td>
</tr>
<tr>
<td>CRP, mg/dl</td>
<td>0.07–8.2</td>
<td>0.07 ± 0.07</td>
<td>0.07 ± 0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 subjects. IB4, β₂-integrins; O₂⁻¹ superoxide anion; IL-6, interleukin-6; INF-γ, interferon-γ; CRP, C-reactive protein. Reference values were obtained from the literature or from the manufacturer of the assay kits [cell counts (34), β₂ integrins (33), O₂⁻ (38), lymphocytes (BD IMK-lymphocyte assay kit, n = 63); cytokines and CRP plasma concentrations (34)]. *P < 0.05 vs. Pre. For explanations of statistical analyses see MATERIALS AND METHODS.

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scores slightly increased again when confinement was finished (M6: 2.62 vs. Post 240: 2.96; Table 3).

Cortisol. Physiological concentrations of morning and evening salivary cortisol were obtained before the confinement period (morning: ~16 nmol/l, evening: ~6 nmol/l), giving calculated m/e in the normal range (~3.5). This ratio did not change during the first months of confinement (M1–M3). Thereafter, the circadian rhythm of cortisol secretion was altered as verified by the reduction of m/e (M4: 1.4; M5: 2.0) but normalized during confinement (M6: 2.7, M7: 4.2). However, after confinement was finished the ratio visibly decreased again (Post 1.98; Table 3).

Prolactin. The concentration of prolactin was in the lower physiological range before confinement and increased after the onset of confinement and remained elevated throughout the entire observation period (Table 3).

Catecholamines. The basal (Pre 240) urine catecholamine excretion of dopamine, norepinephrine, and epinephrine (μg/24 h in pooled urine collected within 24 h) showed normal values in the lower physiological range. During confinement the urine excretion of norepinephrine first increased significantly (M1 vs. Pre 240, P < 0.05) but returned to basal norepinephrine values (M6, M7). After confinement was finished, norepinephrine concentration significantly increased, again (Post 240 vs. Pre, M2, M3, M4, M6, and M7; P < 0.05). The changes of dopamine exhibited the same kinetics but did not reach the level of significance. The low excretion of epinephrine did not change during the entire observation period (Table 3).

Group 240: Immunological Changes

Cell counts. Determination of cell counts revealed an increase in the number of circulating blood leukocytes from the second month onward (M2) until the end of the confinement period (Post 240). The increase in the total leukocyte count was mainly due to the significantly increased concentrations of PMNL (M2, M3, and M4 vs. Pre 240; P < 0.05) and lymphocytes, whereas monocyte counts always remained low (Table 4).

Adhesion molecules. Expression of β2-integrins showed slightly elevated, but still physiological, values at Pre 240. β2-Integrins further increased after the second month of confinement (M2) and remained elevated throughout the confinement period (M6 vs. Pre, P < 0.05; Table 4).

O2 production. The spontaneous and fMLP-stimulated O2 production determined before confinement was slightly elevated and returned to normal physiological values (1, 38) during confinement. No significant differences were observed for the O2 production between Post 240 and Pre 240 (Table 4).

Lymphocyte populations. The total number of lymphocytes increased after confinement but still remained in the physiological range. No changes occurred in the relative percentages of T and B cells, CD16/CD56-positive NK cells, CD3+CD4+ T-helper cells, and CD3+CD8+ T-suppressor cells throughout the confinement period.

However, at Post 240, the percentage of T lymphocytes decreased and the concentration of circulating CD3+CD4+ T-helper cells was reduced. Because CD3+CD8+ T-suppressor cells increased in all subjects, the ratio calculated between T-helper and T-suppressor cells was significantly reduced (Post 240 vs. M2, M3, M4, M6, and M7; P < 0.05; Table 4).

Cytokines and CRP. The plasma concentrations of the proinflammatory cytokines IL-6 and INF-γ remained unchanged in the lower physiological range.

Table 3. Current stress test score, saliva cortisol, plasma prolactin, and urine catecholamine secretion for group 240

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference Values</th>
<th>Time of Confinement</th>
</tr>
</thead>
<tbody>
<tr>
<td>CST, score</td>
<td>2.4–2.8</td>
<td>Pre M1 M2 M3 M4 M5 M6 M7 Post</td>
</tr>
<tr>
<td></td>
<td>2.37 ± 0.14</td>
<td>2.58 ± 0.16 ± 0.38 ± 0.08 ± 0.2 ± 0.41 ± 0.14 ± 0.31 ± 0.32</td>
</tr>
<tr>
<td>Cortisol (morning 8 AM), nmol/l</td>
<td>13–17</td>
<td>16.39 ± 1.88 ± 1.65 ± 7.84 ± 8.89 ± 1.28 ± 4.39 ± 4.98 ± 5.88 ± 5.85</td>
</tr>
<tr>
<td>Cortisol (evening 8 PM), nmol/l</td>
<td>3.5–5</td>
<td>6.54 ± 2.56 ± 0.99 ± 0.79 ± 0.29 ± 2.21 ± 2.59 ± 1.62 ± 0.89 ± 0.14</td>
</tr>
<tr>
<td>Cortisol (m/e)</td>
<td>3.5–5</td>
<td>5.31 ± 0.98 ± 3.63 ± 2.08 ± 1.66 ± 0.54 ± 1.16 ± 0.23 ± 1.89 ± 4.25</td>
</tr>
<tr>
<td>Prolactin, ng/ml</td>
<td>3.0–23.3</td>
<td>6.86 ± 1.99 ± 4.57 ± 2.96 ± 3.19 ± 4.6 ± 1.54 ± 2.89 ± 6.8 ± 2.1</td>
</tr>
<tr>
<td>Dopamine, μg/24 h</td>
<td>190–450</td>
<td>198.13 ± 50.13 ± 39.03 ± 40.31 ± 16.55 ± 14.08 ± 44.63 ± 12.57 ± 27.74 ± 96.3</td>
</tr>
<tr>
<td>Norepinephrine, μg/24 h</td>
<td>23–105</td>
<td>30.72 ± 7.64 ± 5.58* ± 5.27 ± 3.58 ± 2.93 ± 4.18 ± 4.19* ± 2.85* ± 43.47</td>
</tr>
<tr>
<td>Epinephrine, μg/24 h</td>
<td>4–20</td>
<td>10.51 ± 3.5 ± 0.53 ± 2.07 ± 1.52 ± 0.36 ± 2.47 ± 0.67 ± 1.56 ± 7.7</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 subjects. M4, fourth month; M5, fifth month; M6, sixth month; M7, seventh month. Reference values were obtained from the literature or from the manufacturer of the assay kits [CST (21), saliva cortisol (23, 37), prolactin (34); urine catecholamine secretion (hospital’s clinical laboratory)]. * P < 0.05 vs. Pre. † P < 0.05 vs. Pre, M2, M3, M4, M6, and M7; ‡ P < 0.05 vs. M4. For explanations of statistical analyses, see MATERIALS AND METHODS.

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Table 4. Cell counts, expression of β2-integrins, superoxide anion production, lymphocyte populations, and cytokine plasma concentrations for group 240

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference Values</th>
<th>Time of Confinement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes, ×10^3 cells/μl</td>
<td>3.5–9.5</td>
<td></td>
</tr>
<tr>
<td>PMNL, ×10^3 cells/μl</td>
<td>1.6–6.6</td>
<td></td>
</tr>
<tr>
<td>Monocytes, ×10^6 cells/μl</td>
<td>0.2–1.0</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes, ×10^6 cells/μl</td>
<td>1.5–4.0</td>
<td></td>
</tr>
<tr>
<td>PMN, relative fluorescence units</td>
<td>35–45</td>
<td></td>
</tr>
<tr>
<td>O₂ (stimulated), nmol·1 × 10^6</td>
<td>5.5–15</td>
<td></td>
</tr>
<tr>
<td>O₂ (spontaneous), nmol·1 × 10^6</td>
<td>20–50</td>
<td></td>
</tr>
<tr>
<td>CD3, % gated lymphocytes</td>
<td>59–85</td>
<td></td>
</tr>
<tr>
<td>CD19, % gated lymphocytes</td>
<td>6.4–23</td>
<td></td>
</tr>
<tr>
<td>CD4, % gated T lymphocytes</td>
<td>29–75</td>
<td></td>
</tr>
<tr>
<td>CD8, % gated T lymphocytes</td>
<td>11–38</td>
<td></td>
</tr>
<tr>
<td>CD56, % gated lymphocytes</td>
<td>5.6–31</td>
<td></td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>&lt;12.5</td>
<td></td>
</tr>
<tr>
<td>INF-γ, pg/ml</td>
<td>&lt;15.6</td>
<td></td>
</tr>
<tr>
<td>CRP, mg/dl</td>
<td>0.07–8.2</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 subjects. Reference values were obtained from the literature or from the manufacturer of assay kits (cell counts (34); β2 integrins (33); O₂ (38); lymphocytes (BD IMK-lymphocyte assay kit, n = 63), cytokines, and CRP plasma concentrations (34)). *P < 0.05 vs. Pre. †P < 0.05 vs. M2, M3, M4, M6, and M7. For explanations of statistical analyses, see MATERIALS AND METHODS.

(II-L6) or below the detection threshold (<0.1 pg/ml for INF-γ). The same was observed for CRP (Table 4).

**Discussion**

Previous studies in mice, sheep, and monkeys, as reviewed by Schmitt and Schaffar (28), already demonstrated a moderate role of isolation on neuroendocrine and immunological functions. Because of the various and highly complex social interactions of humans, experimental conditions seem not to be fulfilled by animal models. The evaluation of the effects of confinement, especially during spaceflights, remains very important, because confinement in space may lead to psychic stress and to a compromised immune response in humans (15). The compromise of immune functions parallel to an increased probability of inflight infections may confirm the hypothesis that negative events (stress factors) can lead to negative emotional states (distress) that are related to changes in the innate part of the human immune system (11).

To systematically analyze the role of space-related factors with influence on the psychoneuroendocrine regulation and immunity, ground-based studies have already been carried out to simulate effects of long-term microgravity (24, 30) or confinement (10, 26). In a previous ground-based study mimicking reduced gravitation for 120 days by permanent bed rest (6° HDT), our laboratory observed substantial psychic stress alterations of the circadian rhythm of cortisol secretion, an elevation of excretion of stress-permissive catecholamines (e.g., norepinephrine), and changes in cell counts and functions of immune cells in six subjects (5).

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These changes most likely reflect stimulation of the
unspecific and the depression of the specific immune
system by prolonged HDT (5).

On the basis of the results of the HDT study (5) and
of the findings made by other groups in confinement
studies (10, 26), we investigated in part the same
variables in response to long-term confinement for 110
and 240 days in a space module, reflecting the condi-
tions in a multimodule orbital station. True control
groups (e.g., the same or a matched group of individu-
als subjected to daily life outside of the chamber for the
same period of time), however, could not be studied.
Because of the long-term approach of the present in-
vestigation, the same group of volunteers did not agree
to undergo an additional experimental period of 110 or
240 days. This might also have had a “bias” effect on
the subsequent experimental period that can only be
overcome by a crossover design, which was not possible
primarily because of financial reasons. To compensate
the lack of a true control group, we compared the values
obtained in the same subjects before confine-
ment was started, representing an experimental de-
sign previously described (5, 24).

Confinement and Neuroendocrine Changes

Exposure to stress factors might influence the im-
une system due to the activation of the hypothalamo-
pituitary-adrenal axis and the sympathoadrenergic
response by elevated levels of stress-permissive hor-
mones such as cortisol and catecholamines (6). The
reliability of the determination of psychic stress by
the CST was validated (21) and already applied in
space research (30). Quantitative determination of
salivary cortisol, serum prolactin, and urinary cate-
cholamine excretion has also been shown to be a
reliable method to assess stress-sensitive hormonal
changes (2, 6, 13, 36).

Pre vs. Post. In a comparison of the aforementioned
parameters within each group (group 110 and group
240) before and after 110 and 240 days of confinement,
respectively, it is obvious that psychic stress, as deter-
mained by the CST, did not develop to any significant
extent, whereas confinement seems to affect the neu-
roendocrine response as suggested by the alterations in
the circadian rhythm of cortisol secretion. Under phys-
iological conditions, salivary cortisol levels show a cir-
cadian rhythm in three- to fivefold higher concentra-
tions in the morning than in the evening (m/e), which
was below 2 after confinement in both groups. Prolac-
tin concentrations increased clearly after confinement.
The excreted concentrations of dopamine and norepi-
nephrine also increased significantly (Tables 1 and 3).
Thus, although psychic stress was not detected by the
CST, changes of secretion of cortisol, prolactin, and
catecholamines demonstrated a neuroendocrine re-
sponse at least after confinement in both groups.

During confinement. To determine whether these
changes already occurred during the confinement pe-
riod, additional saliva and urine samples were taken
monthly during confinement in both groups.

In contrast to group 110, group 240 exhibited a slight
increase of CST scores and a significantly elevated
excretion of norepinephrine already in the first months
of confinement. These changes showed a tendency to
normal values in the sixth (M6) and seventh (M7)
month. This was also observed for the diurnal secretion
of cortisol, which was altered in the fourth and fifth
months (m/e 1.4 or 2) but recovered thereafter (M6,
M7). These effects might be due to the adaption of the
individuals to the given living conditions, which were
much more limited with respect to space and privacy in
group 240.

Thus the neuroendocrine consequences of long-term
confinement appear to be initially dependent on the
chamber space and privacy available for each subject.
After longer periods of confinement (group 240), adap-
tion may occur. Interestingly, return to normal life
seems to be a very stressful event, irrespective of the
time and conditions of confinement.

Confinement and Immunological Changes

Pre vs. Post. The unspecific as well as the specific
immune system play an essential role in protecting the
body against microorganisms as the host’s defense.
Because no lymphoid tissue could be obtained, analyses
of the immune system were merely based on sam-
ples of circulating blood cells and the interaction of
specific and unspecific immune cells with intrader-
ally injected standardized antigens by the DTH skin
reaction.

The comparision of Pre to Post values of leukocyte
and granulocyte counts and associated functional pa-
rameters suggests activation of the innate part of the
immune system. Accordingly, the number of circulat-
ing PMNL was increased and the expression of β2-
integrins on PMNL was elevated. However, spontaneous
as well as FMLP-stimulated receptor-mediated O2
production did not change, reflecting a maintained
physiological activity of PMNL.

The role of CD56+CD16+ NK cells in normal immu-
nity is not yet clearly established, although they can
kill target cells (virus-infected cells or tumor cells) in a
relatively indiscriminate fashion (35). Hence, NK cells,
despite being lymphocytes, are considered to contrib-
ute to innate immunity. After confinement no changes
in the number of circulating NK cells was observed.

In contrast to the slightly activated functional state
of the innate part of the immune system (increased
PMNL counts, enhanced expression of β2-integrins),
variables that reflect specific immunity showed a mi-
nor depression. Although the total number of blood
lymphocytes increased, the ratio of T-helper (CD3+/
CD4+) to T-suppressor (CD3+CD8+) lymphocytes de-
creased because of the increased number of CD3+CD8+
lymphocytes.

To search for signs of possible functional conse-
quences of neutrophil activation and depression of the
CD4+/CD8+ ratio of circulating lymphocytes, the DTH
reaction was evaluated by a standardized recall anti-
gen multitest skin test (Immignost). This assay can be
performed repeatedly without sensibilization and with high reproducibility (16). The skin reaction against intracutaneously administered antigens reflects an orchestrated group of related responses to antigens that are essentially dependent on CD4-positive lymphocytes, on various proinflammatory cytokines located in the tissue, as well as on the activation of phagocytes as effector cells. When CD4+ T-cell or phagocyte functions are compromised, the entire sequence of cell-mediated responses is affected, which hence results in an immunocompromised state (4). A physiological reaction in men is defined as a mean DTH erythema diameter >10 mm (3), which was observed before and also after confinement in all subjects (Table 5). Thus the slight depression of the CD4+/CD8+ ratio appears to have no major effects on the functional state of the specific cellular immunoreactivity against recall antigens in either group 110 or group 240.

During confinement. In group 240, additional blood samples were withdrawn monthly (M1–M7), demonstrating an increase in the PMNL counts and in the expression of β2-integrins already in the first months of confinement. This activation seemed not to be due to an inflammatory process, because other highly sensitive parameters of systemic inflammation [e.g., concentrations of IL-6 (14, 22) and CRP (39)] remained very low at any time point (Table 4). No changes in the specific part of the immune system were observed during confinement. However, when subjects returned to “normal” life the specific immune system appeared to be altered because the ratio of T-helper to T-suppressor cells decreased (M7 ~ 2.3 vs. Post ~ 1.6). This was, however, without any effect on the DTH.

Thus, as concluded for the changes observed in neuroendocrine parameters, immunological alterations, especially of the nonspecific limb of the immune system, appear to be more pronounced when less space per subject is available during confinement.

Return to normal life, however, had a clear effect on immunological parameters, irrespective of how long or under which conditions subjects were confined.

Conclusions

The study presents the effects of long-term confinement for 110 and 240 days on Earth in working and living modules that are built to mimic the conditions on the ISS.

During confinement for 110 days, subjects lived and worked in a spacious chamber (200 m³). Under these conditions, no psychoneuroendocrine stress response could be observed. In contrast, when living and working space were reduced to almost half the size (group 240), thereby leading to a lack of privacy, a chronic stress response developed during confinement because of slightly increased psychic stress, and alteration of the circadian rhythm of cortisol secretion and prolactin plasma concentrations occurred. In addition, excretion of catecholamines increased during the first months after confinement but ceased thereafter, which might be due to adaptation to the restricted living conditions.

The innate part of the immune response became activated, as suggested by the increase in the number of PMNL and of the expression of β2-integrins, whereas no enumerative changes occurred in lymphocyte subpopulations.

When confinement was terminated in both groups, parameters of psychic stress and sympathoadrenergic stimulation increased, reflecting a stressful readaptation to normal life.

These Post confinement living conditions are supposed to have a strong impact on each individual. However, the changes in living conditions were comparable to those that are encountered by astronauts and cosmonauts on return to Earth. Like astronauts and cosmonauts, the volunteers subjected to confinement had to perform almost the same follow-up experiments and medical examinations. This return to “normality” obviously also affected the specific immune system, as proven by a decrease in the ratio of T-helper to T-suppressor cells in both groups. However, the decrease in CD4+ T cells did not alter the DTH.

Altogether, these results demonstrate moderate and distinct reactions of the neuroendocrine and immunological systems which from the laboratory point of view can be considered negative. Thus confinement caused changes of variables that reflect physiological rather than pathological adaptive processes. However, besides space-dependent environmental conditions, other variables like low gravitation and radiation may render astronauts and cosmonauts more sensitive to long-term confinement and social interactions occurring in a small group of humans living and working under hostile conditions.

We thank Dr. M. Vogeser and G. Grüger for help and skilful assistance in the determination of catecholamines and cytokines and B. Lobstein for the revision of the manuscript.

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