Functional changes in neutrophils and psychoneuroendocrine responses during 105 days of confinement


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SPACEFLIGHT CORRESPONDS TO environmental stressors (e.g., microgravity, prolonged isolation, and confinement) that are known to affect and alter multiple human physiological systems and their interactions [e.g., the immune system and the psychoneuroendocrine axis (7, 14)]. An essential tenet of good general health is that the innate immune system is able to serve as a first line of defense against pathogenic microorganisms, and that neutrophils are the system’s major components. Several spaceflight studies have coherently shown that the number of neutrophils increases after spaceflight. This increase is most likely associated with the stress of landing (6, 38). Furthermore, alterations in cell number but also in cell function were detected when the microbicidal and cytotoxic abilities of neutrophils were examined. Depending on mission duration, their ability to generate reactive oxygen species after activation and to phagocytose hostile microorganisms was significantly reduced (23). Additionally, more recent research using astronauts aboard the International Space Station showed a dysregulation in the cytokine release pattern (7). To elaborate these findings, space analogs (e.g., confinement studies) were initiated to mimic spaceflight conditions. Confinement studies of different duration detected an activation of the innate immune system, emphasizing the end of confinement as a crucial event (4, 34).

Moreover, these studies also analyzed either the catecholamine and glucocorticoid systems or an attained psychic stress level to detect possible modulating links with these immune changes. Confinement impaired the neuroendocrine response depending on the space and privacy available with the common point being that the end of isolation always initiated neuroendocrine stress responses. The correlation between immune answers and psychoneuroendocrine functions was further demonstrated in a clinical study that showed inhibited shedding of CD62L and inhibited microbicidal function of neutrophils when increasing catecholamine levels (19).

On the basis of these reported results we hypothesize a dysregulation of the innate immune system during 105 days of confinement and a potentially reversed relation with possible detectable responses of the psychoneuroendocrine system.

MATERIALS AND METHODS

Study Protocol

The international confinement and isolation study Mars-105 was carried out at the Russian Institute for Biomedical Problems in Moscow. It partly simulated a long-duration mission with 105 days of isolation in a ground-based space module. The space module consists of five interconnected compartments with an overall space of about 500 m³. It is hermetically sealed from the external environment. Environmental factors (e.g., air/oxygen partial pressures) in the modules were kept at normal range. Microgravity and cosmic radiation were not simulated. A balanced nutritional supply was guaranteed. A 24/7-h mission control center was established. It had only restricted written and oral communication links with the confined crew according to conditions of planned interplanetary missions. The candidate selection process included broad medical and psychological testing in accordance with actual astronaut/cosmonaut selection criteria. After written informed consent was received from each participant and approval from the ethical/medical boards of the Russian Federation and the European Space Agency had been obtained, four Russian and German participants were selected for the study.
Hydrogen peroxide (H$_2$O$_2$) production by PMNLs was stimulated by a mixture of tumor necrosis factor-alpha (TNF-α) and N-formyl-methionyl-leucyl-phenylalanine (fMLP), expressed as relative fluorescence units (RFU). Data are means ± SE.

Cytotoxic capabilities of polymorphonuclear leukocytes (PMNLs) were analyzed separately from EDTA-anticoagulated whole blood specimens in a Coulter Cell Counter T540 (Coulter Electronics, Luton, UK). PMNL competencies. We analyzed 1) the hydrogen peroxide (H$_2$O$_2$) production of PMNLs stimulated by a mixture of tumor necrosis factor-alpha (TNF-α) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) and 2) the adhesive and phagocytic capabilities of PMNLs after incubation with fluorescein isothiocyanate (FITC)-labeled zymosan particles and addition of hydrochloric acid. The method utilized has been described before (21).

L-selectin. A quantitative analysis of the shedding of the cell adhesion molecule L-selectin (also known as CD62L) on PMNLs was carried out using flow cytometry for 1) a control specimen at 37°C body temperature and 2) a specimen stimulated with the chemotactic fMLP as described previously (11, 39).

Cytokines. Plasma concentrations of cytokines and colony-stimulating factors were determined from EDTA-anticoagulated blood specimens. Quantitative analyses were performed by Luminex xMAP technology (Bioplex) according to the manufacturer’s guidelines.

Psychoneuroendocrine Responses

Cortisol. Saliva samples were collected in the morning and evening to mirror the circadian rhythm of cortisol. A cotton swab was chewed for 30–45 s, stored in a SALIVETTE tube (Sarstedt, Nürnberg, Germany), and stored frozen at −80°C. Quantification of free cortisol was performed by an automated immunoassay system based on the principle of electrochemiluminescence (Elecsys Cortisol; Roche Diagnostics, Mannheim, Germany).

Catecholamines. The catecholamines epinephrine and norepinephrine were analyzed by high-performance liquid chromatography (Chromosystem, Martinsried, Germany) from 24-h urine samples, and the ratio of norepinephrine to epinephrine was calculated.

Evaluation of stress. To quantify the intensity level of the emotional strain and to describe the subjects with regards to anxiety, we analyzed three different kinds of questionnaires applied intermittently during the observational period: a short questionnaire to assess current stress; the Spielberger State and Trait Anxiety Inventory (STAI); and the General Health Questionnaire (GHQ). The test setups and score ranges are described in literature and the result section (13, 25, 30, 37).

Statistics

Normal distribution of the experimental data was tested by the Shapiro-Wilk test using SigmaPlot software (version 12.0; Systat Software, San Jose, CA). To enable repeated measurements within groups a one-way repeated measure ANOVA was applied followed by the post hoc Fisher least significant difference test. Results were statistically significant if $P < 0.05$. Results are expressed as means ± SD and figures show means ± SE.

RESULTS

PMNL number and competence. Leukocyte and PMNL cell concentrations showed no significant changes during the entire experimental period (Table 1).

Table 1. **White blood cell counts**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>BDC</th>
<th>14 Days</th>
<th>34 Days</th>
<th>66 Days</th>
<th>81 Days</th>
<th>R+14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>4.53</td>
<td>4.77</td>
<td>4.78</td>
<td>4.24</td>
<td>5.05</td>
<td>4.37</td>
</tr>
<tr>
<td>PMNL</td>
<td>2.57</td>
<td>1.93</td>
<td>2.50</td>
<td>1.98</td>
<td>2.00</td>
<td>2.57</td>
</tr>
</tbody>
</table>

Values are mean ± SD; units are G/l; BDC, baseline data collection; R + 14, 14 days after end of confinement; PMNL, polymorphonuclear leukocytes.

![Stimulated H$_2$O$_2$ production of PMNL](image1.png)

**Fig. 1.** Cytotoxic capabilities of polymorphonuclear leukocytes (PMNLs). Hydrogen peroxide (H$_2$O$_2$) production by PMNLs was stimulated by a mixture of tumor necrosis factor-alpha and N-formyl-methionyl-leucyl-phenylalanine (fMLP), expressed as relative fluorescence units (RFU). Data are means ± SE; $n = 6$; *$P < 0.05$ vs. baseline data collection (BDC).

![Phagocytic and adhesive capabilities of PMNL](image2.png)

**Fig. 2.** Phagocytic and adhesive capabilities of PMNLs. Gray columns show adhesion of FITC-labeled zymosan particles to PMNLs, black columns show phagocytosis of FITC-labeled zymosan particles by PMNLs. Data are means ± SE and given in % of PMNLs; $n = 5–6$, *$P < 0.05$ vs. BDC.
Shedding of CD62 L

Fig. 3. Shedding of CD62L by PMNLs. Gray columns indicate the control group at 37°C; black columns indicate shedding after stimulation with fMLP. Data are means ± SE and expressed as RFU; n = 3–6. *P < 0.05 vs. BDC.

study period (Table 1). Spontaneous H₂O₂ production revealed moderate changes (data not shown), whereas activation by TNF-α/fMLP significantly enhanced H₂O₂ production by PMNLs during the isolation period (days 34 to 66 vs. BDC, P = 0.003/0.017). After confinement a significant decrease in stimulated H₂O₂ production was measurable [R+14 (= 14 days after end of confinement) significant vs. BDC (baseline data collection)] (Fig. 1). The phagocytic abilities of PMNLs were significantly suppressed during the entire isolation period. They remained suppressed after isolation (all time points significant vs. BDC), whereas their adhesive functions remained stable compared with baseline values (Fig. 2).

Unstimulated PMNLs showed only minor shedding of CD62L during the observation period. A higher degree of shedding was quantified only at day 66. However, activation of PMNLs with fMLP induced a strong and highly significant and persistent enforced shedding of CD62L over several time points during the confinement period. The most pronounced effect was observed at day 66 vs. BDC. The fMLP-induced shedding of CD62L showed a slow recovery at the end of the study without regaining basic values (R+14 vs. BDC) (Fig. 3).

Humoral markers of inflammation. Analysis of various cytokine and colony-stimulating factor concentrations (interleukins 1β, 2, 4, 5, 6, 7, 8, 10, 12, and 13; granulocyte colony-stimulating factor, granulocyte macrophage-colony stimulating factor, interferon-γ, monocyte chemotactic protein-1, macrophage inflammatory protein-1β, TNF-α, and transforming growth factor-β) in subject plasma revealed only low individual variation and no significant increase at any time point in the study (data not shown).

Psychoneuroendocrine Responses

The psychoneuroendocrine axis revealed no significant alterations when tested by the KAB, the STAI, or the GHQ (Table 2). Higher test scores generally indicate a higher perceived stress level. STAI threshold values indicating psych disequilibrium and anxiety vary dependent on age and sex [state anxiety for men total 36.83 ± SD 9.82, trait anxiety for men total 34.45 ± SD 8.83 (25)]. A GHQ score above 23 is associated with growing psychic stress and disorder.

Stress hormone levels showed no significant changes and their excretion modus did not alter significantly (Tables 3 and 4).

DISCUSSION

Our study aimed to 1) confirm that a 105-day confinement affects the innate immune system and the psychoneuroendocrine responses, 2) clarify how those systems are influenced, and 3) demonstrate their potential interactions.

In contrast to former confinement studies (4, 34, 35), we could not detect significant changes in leukocyte cell count or its PMNL subpopulation. Changes in leukocyte distribution are known to be influenced by stimulation of the autonomic nervous system via its second-messenger catecholamines or glucocorticoids (9). These messengers mobilize neutrophils from the bone marrow and lymphatic tissue to increase their number in the bloodstream (2, 28). We verified this relationship by assessing emotional strain and anxiety during confinement: none of the questionnaires indicated stress and neither glucocorticoid nor catecholamine concentrations were changed, thus potentially explaining the stability of the leukocyte count and distribution. But even high cortisol levels may not necessarily explain a shifted leukocyte distribution as demonstrated in recently published results from the subsequent Mars-500 study: high cortisol levels as a consequence of chronic stress were not correlated to a shifted leukocyte distribution (lymphocytosis) (42). This finding is potentially attributed to glucocorticoid receptor resistance of the immune cells, which is known to develop when facing chronic stress (5, 29). The duration of 105

Table 2. Psychological stress evaluation

<table>
<thead>
<tr>
<th>Time Point</th>
<th>BDC</th>
<th>14 Days</th>
<th>34 Days</th>
<th>66 Days</th>
<th>81 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morning</td>
<td>2.33 ± 0.47</td>
<td>1.89 ± 0.56</td>
<td>1.97 ± 0.60</td>
<td>2.10 ± 0.89</td>
<td>2.36 ± 0.83</td>
</tr>
<tr>
<td>Evening</td>
<td>2.33 ± 0.88</td>
<td>2.11 ± 0.63</td>
<td>1.86 ± 0.65</td>
<td>1.93 ± 0.93</td>
<td>2.19 ± 0.77</td>
</tr>
<tr>
<td>STAI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trait</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State</td>
<td>26.83 ± 4.88</td>
<td>27.83 ± 5.57</td>
<td>29.17 ± 7.55</td>
<td>29.00 ± 5.51</td>
<td>32.33 ± 4.03</td>
</tr>
<tr>
<td>Trait</td>
<td>36.00 ± 7.43</td>
<td>34.83 ± 5.74</td>
<td>34.17 ± 5.71</td>
<td>32.33 ± 4.03</td>
<td></td>
</tr>
<tr>
<td>STAI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHQ 28 total</td>
<td>9.17 ± 1.94</td>
<td>10.00 ± 2.10</td>
<td>10.17 ± 1.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SD; units are points. BDC, baseline data collection; KAB, Kurzfragebogen zur aktuellen Beanspruchung (current stress test); STAI, Spielberger State and Trait Anxiety Inventory; GHQ, general health questionnaire; GHQ 28 total, total test score of 28 questions.

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days of confinement seems not to be sufficient to cause a chronic psychoneuroendocrine stress response in highly selected volunteers compared with 520 days of isolation. Choukér et al. (4) presumed that the chamber space and privacy available for each subject account for the stress level: four subjects shared 200 m$^3$ for 110 days but only 100 m$^3$ for 240 days, which led to an initial stress increase. The relationship between little space and perceived stress was also demonstrated by Shimamiya et al. (34), who found low mood at the beginning of confinement and improvement toward its end. In our study, six subjects lived for 105 days in ~500 m$^3$, which provided each individual with more living space and a shorter confinement period than in the other two studies.

In contrast to the 110-day confinement experiment (4), we analyzed immune cell functions not only before and after confinement but also during the confinement period to better understand its influence on immune answers. As hypothesized, we observed a dysregulation of the innate immune response but the modifications we detected were not consistent but rather oppositional. Activated by TNF-$\alpha$/fMLP, the cytotoxic properties (H$_2$O$_2$ production) of PMNLs were enhanced during confinement, whereas their microbicidal functions were significantly restricted.

To evaluate the clinical relevance of these observations and the extent to which they may influence health, we analyzed previous studies that detected similar results. However, it became clear that the findings of other researchers seem to be due to specific clinical disease conditions, therefore limiting the possibility of transferring them to other circumstances. Enhanced cytotoxic and reduced microbicidal functions were detected in several animal experiments mimicking infection and sepsis (16, 24, 40) and in patients with severe inflammatory states (15, 41). Kaufmann et al. noticed a correlation between decreased phagocytosis and increased cytotoxic functions of PMNLS in patients with increased sepsis severity, presuming the development of a PMNL dysfunction syndrome due to infection (20). Comparable immune changes have been evidenced after trauma (18, 26). Here, the alterations were assumed to be part of a regulatory mechanism to minimize deleterious effects of the increased neutrophil oxidative activity (18).

Apparentlly, the stated immune changes frequently occurred in clinical settings in combination with inflammatory responses, which in contrast, did not apply in our study. However, the reasons for this remain unclear.

Simultaneously, we were able to show an enforced shedding of the adhesion molecule CD62L during isolation after stimulation with TNF-$\alpha$/fMLP. L-selectin is highly important for the process of leukocyte adherence to the endothelium during inflammation (17, 36). A decreased expression of CD62L can be observed in patients with stress or with fibromyalgia (22), potentially compromising immune defense. Controversial data exist concerning the shedding of CD62L and the levels of its soluble form in plasma. On the one hand, elevated levels of soluble adhesion molecules in sepsis were positively linked with severity of illness and mortality (1, 33). On the other hand, evidence could be provided indicating the protective role of enforced shedding of CD62L and increased levels of soluble L-selectin during inflammation (3, 8, 10, 32). On the basis of previous studies (12, 27), Zonneveld et al. (43) propose that the shedding of adhesion molecules regulates the inflammation process in a beneficial way.

Evidently, some functional innate immune alterations during long-term confinement are to a certain extent similar to those occurring under pathologic conditions. However, this study revealed a stable leukocyte count and showed no rise in any cytokine population. This is because all subjects stayed healthy throughout the study. Therefore, an acute inflammatory state as cause for the immune alterations seems improbable. The divergent immune results in combination with a stable hypothalamic-pituitary-adrenal axis and the absence of quantifiable and documented psychic stress contrast with the formerly stated positive correlation between increased catecholamine levels and a globally suppressed innate immune answer (19). In a similar way, living factors (space, privacy, duration of confinement) previously accounted only for changes in stress parameters and leukocyte count, but not for changes in PMNL function (4). Therefore, the enforced cytotoxic, reduced phagocytic innate immune functions, and a simultaneously enhanced shedding of CD62L, have different (patho-) physiological reasons.

On the one hand, it can be assumed that external environmental influences such as the prevailing microbial flora in confined habitats may have played a role in the observed changes. Classification of the isolated microbial load on surfaces and in the air of the artificial Mars-500 habitat revealed a colonization with mainly human-associated microorganisms. This led to the assumption that the human inhabitants represent the major source for possible contamination during confinement periods (31). However, the measured microbial load in the habitat ranged within the limits set for the International Functional Changes in Neutrophils during Confinement • Streve C et al. 1125

Table 3. Morning and evening cortisol levels

<table>
<thead>
<tr>
<th>Time Point</th>
<th>BDC</th>
<th>14 Days</th>
<th>34 Days</th>
<th>66 Days</th>
<th>81 Days</th>
<th>R+14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morning</td>
<td>0.40 ± 0.26</td>
<td>0.54 ± 0.27</td>
<td>0.54 ± 0.31</td>
<td>0.66 ± 0.44</td>
<td>0.54 ± 0.22</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>Evening</td>
<td>0.38 ± 0.33</td>
<td>0.27 ± 0.07</td>
<td>0.24 ± 0.08</td>
<td>0.33 ± 0.13</td>
<td>0.29 ± 0.12</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Ratio m/e</td>
<td>1.56 ± 1.04</td>
<td>2.37 ± 1.61</td>
<td>2.49 ± 1.39</td>
<td>2.27 ± 1.57</td>
<td>2.05 ± 0.81</td>
<td>2.09 ± 1.19</td>
</tr>
</tbody>
</table>

Values are mean ± SD; units are µg/dl. Ratio m/e, ratio morning to evening.

Table 4. Catecholamine levels

<table>
<thead>
<tr>
<th>Time Point</th>
<th>BDC</th>
<th>14 Days</th>
<th>34 Days</th>
<th>66 Days</th>
<th>81 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>42.51 ± 16.76</td>
<td>28.24 ± 10.20</td>
<td>37.89 ± 10.06</td>
<td>36.99 ± 8.70</td>
<td>29.64 ± 1.07</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>3.72 ± 2.26</td>
<td>4.18 ± 1.63</td>
<td>4.19 ± 1.05</td>
<td>5.50 ± 1.08</td>
<td>4.60 ± 2.71</td>
</tr>
<tr>
<td>Ratio Nor./Epi.</td>
<td>15.26 ± 9.90</td>
<td>7.48 ± 3.95</td>
<td>9.30 ± 2.32</td>
<td>7.12 ± 2.77</td>
<td>9.72 ± 7.23</td>
</tr>
</tbody>
</table>

Values are mean ± SD; units are µg/24 h. Ratio Nor./Epi., ratio norepinephrine to epinephrine.

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Space Station and was only moderate compared with nonconfined control rooms such as office buildings, hospitals, or commercial airline cabins. So it is likely that it did not have enough impact to affect immune answers as stated.

On the other hand, the subjects were entirely shielded from outside infectious agents during the confinement period. Therefore, when taking into account that no inflammatory response was observed, the stated immune changes might constitute an appropriate adaption of the human immune system to altered immune challenges in a confined environment without any pathological significance.

Conclusion

Our hypothesis that 105 days of confinement would lead to a dysregulation of innate immune answers was confirmed; however, a relationship with the psychoneuroendocrine response could not be verified. Moreover, innate immune responses did not alter consistently, but rather described differential reactions concerning their microbicidal and phagocytic abilities.

Former study results were consulted, but they could only partially elucidate the causes for these findings. Yet unknown and unexplored influences seem to affect the different human physiological systems and their interactions, and it seems likely that their complex variety and cross-linking triggers the observed changes.

ACKNOWLEDGMENTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


