J Appl Physiol 97: 920–924, 2004.
First published May 14, 2004; 10.1152/japplphysiol.00043.2004.

Effects of 10-day confinement on the immune system and psychological aspects in humans

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Submitted 15 January 2004; accepted in final form 12 May 2004

Shimamiya, Tamiyasu, Nobuyuki Terada, Yoshimitsu Hiejima, Sonoe Wakabayashi, Hirotake Kasai, and Motohiko Mohri. Effects of 10-day confinement on the immune system and psychological aspects in humans. *J Appl Physiol* 97: 920–924, 2004. First published May 14, 2004; 10.1152/japplphysiol.00043.2004.—We investigated the changes in percentages of leukocyte subpopulations, natural killer (NK) cells, CD69-expressing lymphocytes, and psychological aspects in 10 subjects who participated in a 10-day confinement study. Suppression of lymphocyte proliferative reaction and changes in leukocyte distribution are known to occur in space. These responses are similar to those induced by psychological stress. Ground-based confinement studies are suitable for validating the effects of stress arising only due to confinement. Two groups, consisting of five male subjects (ages 20–27 yr, mean 22.8 yr) each, participated in a 10-day confinement study. Blood samples were taken once before, three times during, and once after the confinement and activated with an anti-CD2 agonistic antibody cocktail. The percentages of leukocyte subpopulations, NK ($CD45^+CD56^+$) cells, and activated lymphocytes ($CD45^+CD69^+$) were measured by flow cytometric assay. The face scale test was used to measure psychological aspects. The percentage of $CD69^+$ lymphocytes decreased during the period of confinement. This was mostly caused by changes in the ratio between NK and non-NK lymphocytes. The face scale showed that the subjects' moods improved toward the postconfinement period. Consistent with the face scale, the percentages of innate immune cells, such as NK cells and granulocytes, increased during the postconfinement period. We concluded that the changes in the distribution of immune cells caused by stress plays an important role in suppression of proliferative reactivity. The observed physiological reactions were specific to the confined environment, and the stress caused by confinement plays a role in the immune changes observed in space.

CD69; mitogenic reaction; stress; granulocyte; natural killer cell

ENDOCRINOLOGICAL CHANGES CAUSED by psychological stress are known to affect immune status. These effects include recruitment of natural killer (NK) cells and granulocytes to peripheral blood through stimulation of sympathetic nerves (1, 3, 8) and suppression of proliferative reaction of lymphocytes through β-adrenergic or glucocorticoid receptors (1, 2, 9, 10). Similar changes are known to occur both during and after spaceflight. Proliferative reactions of isolated peripheral blood mononuclear cells to mitogens, such as phytohemagglutinin or concanavalin A, have been reported to be suppressed (6, 21). Increases have also been reported in number and percentage of granulocytes (7, 15, 20, 21, 23). The causes of these changes include not only physical factors, such as microgravity, cosmic radiation, and decompression, but also psychological stress, including anxiety regarding danger, interpersonal conflict, and confinement (6, 7, 15, 20, 21, 23). CD69, which is known as an early activation antigen on lymphocytes, is expressed in the first 2–4 h in proliferative reactions (14, 22). We analyzed CD69 because it can be detected while blood is still fresh and in almost in vivo status. There have been no previous reports regarding the effects of confinement on CD69 expression.

The purpose of this study was to assess the effects of confinement on immune status. If confinement stress is not responsible for immune changes in space, no similar effects should be observed in ground-based confinement studies. Thus we investigated the changes in percentage of leukocyte subpopulations, NK cells, CD69-expressing lymphocytes, and psychological aspects in a 10-day confined environment that is periodically relevant to space shuttle missions.

METHODS

Confinement profile. Ten Japanese men (age 20–27 yr, mean 22.8 yr) who had not met before the experiment volunteered to participate in this study. Applicants who had an academic interest in the experiment were recruited. Their majors at university were economics, psychology, education, agriculture, and veterinary medicine. We could not recruit female examinees because there were no female applicants. We conducted two identical 18-day experiments, each of which included a 10-day confinement period. The first was conducted in August 2000, whereas the other was in February 2002. Five-day preconfinement and 3-day postconfinement periods were arranged. Five volunteers were recruited for each experiment. The study protocol was approved in advance by the Japan Marine Science and Technology Center (JAMSTEC; Yokosuka, Japan) committee. Each subject provided written, informed consent before participating in the study. Subjects met 5 days before the confinement and began to inhabit a dive simulation chamber at JAMSTEC (Fig. 1). The total habitation area was 34.1 m^2. Subjects were allowed to leave the chamber in the preconfinement period and received medical checkups, training, and instructions. Then the hatch was closed, and the 10-day confinement period started. The subjects spent time in the chamber taking psychological tests, measuring their physiological data, and performing moderate exercise (20 min/day). After this period, the hatch was opened and the 3-day postconfinement period started. For 3 days, the volunteers continued the same schedule as in the confinement period. Blood samples were taken into heparinized test tubes (Vacutainer, Becton Dickinson, San Jose, CA) at 7:00 AM on sample-taking days. To avoid contact with outside personnel, a "service lock" consisting of a small port was used for drawing blood (Fig. 1).

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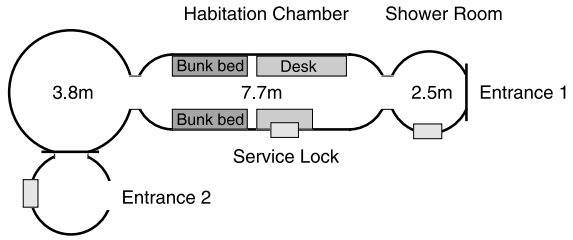

Fig. 1. Dive simulation chamber.

Samples were taken once before, three times during, and once after confinement.

Activation of lymphocytes. The blood samples were transported from Yokosuka to Yamanashi at 4°C. Six hours after the blood samples were taken, 20 μg/ml CD2 agonistic antibody solution CD2/CD2R (Becton Dickinson) was added to the whole blood. Then the samples were incubated for 4 h at 37°C. Samples without CD2/CDR were prepared as controls. CD2/CD2R contains two kinds of anti-CD2 antibodies, which recognize different epitopes in the CD2 molecule and activate NK and T cells specifically (9, 12, 16). Total leukocyte number was counted with a Sysmex microcell counter F-300 (Sysmex, Kobe, Japan).

Reaction of fluorochrome-labeled antibody. Aliquots of 50 μl of activated blood samples were incubated with 20 μl of fluorochrome-labeled antibody cocktail solution (FASTImmune, Becton Dickinson) for 20 min. The solution included FITC-labeled anti-CD56 antibody, phycoerythrin-labeled anti-CD69 antibody, and peridinin chlorophyll protein-labeled anti-CD45 antibody. CD56, CD69, and CD45 antigens are specific surface markers expressed on NK cells, activated lymphocytes, and leukocytes, respectively. After hemolysis and fixation with 450 μl of fluorescence-activated cell sorting lysing solution (Becton Dickinson), samples were analyzed by a flow cytometer (FACSCalibur, Becton Dickinson).

Flow cytometric analysis. Instrument settings for measurements were in accordance with the manufacturer's instructions. Cell Quest software (Becton Dickinson) was used for data acquisition and analysis. A total of 10,000 events, which passed a gate set on a lymphocyte subpopulation in a side-scatter vs. CD45 dot plot, were analyzed on a CD69 vs. CD56 dot plot. By referring to a nonactivated sample, a quadrant marker separating the CD69- or $CD56^+$ subpopulation was set on the plot. NK cells ($CD56^+$) were shown on the right, whereas non-NK cells, consisting of T and B cells, were shown on the left. In activated samples, $CD69^+$ cells were seen in the upper area. The four areas on the plot sum to 100%. The percentage in each area was taken relative to the whole 10,000 events of $CD45^+$ lymphocytes gated at the side-scatter vs. CD45 plot. The $CD69^+$ percentage among NK cells was calculated by dividing the upper right area by the whole right area. The upper left was divided by the whole left for that of non-NK cells. Percentages of granulocytes, monocytes, and lymphocytes were analyzed on a forward-scatter vs. side-scatter dot plot.

Face scale test. A very brief, pictorial scale of mood, which uses a sequence of 20 faces and does not require reading literacy, was employed to measure psychological aspects. The face scale was devised by Lorish and Maisiak (13) as a means to estimate quality of life in arthritis patients. The face scale consists of 20 drawings of a single face, arranged in serial order by rows, with each face depicting a slightly different mood state. Subjects were shown a face scale test sheet and instructed to "mark the face that best shows your mood."

Statistical analysis. Descriptive statistics were performed on each time point. After multivariate ANOVA concerning each variable was conducted, Dunnett's multiple comparisons between preconfinement and other time points were performed. Because one blood sample failed to be taken on *day 15*, analyses were conducted on the basis of the results for 9 samples. Data are expressed as means ± SE. JMP software (SAS Institute, Cary, NC) was used for statistical analysis.

RESULTS

NK, non-NK cells, and their $CD69^+$ percentages among lymphocytes. The $CD69^+$ percentages of NK and non-NK cells relative to whole lymphocytes showed different changes throughout the experiment (Fig. 2, *A* and *B*). As the percentages of NK and non-NK cells sum to 100%, both showed complementary alterations (Fig. 2, *C* and *D*). The ratio of NK to non-NK cells increased toward the end of confinement. $CD69^+$ NK cell percentage and NK cell percentage in whole lymphocytes showed coordinated change (Fig. 2, *B* and *D*). $CD69^+$ non-NK cell percentage and non-NK cell percentage in whole lymphocytes decreased in the first half of the experiment (Fig. 2, *A* and *C*). No statistically significant differences were observed when the $CD69^+$ percentages in whole lymphocytes were divided by the non-NK or NK cell percentage (Fig. 2, *E* and *F*). This indicated that $CD69^+$ percentages in NK or non-NK cells were mostly constant and tended to decrease during the period of confinement.

Granulocyte, monocyte, and lymphocyte percentage. The granulocytes increased from the beginning of the confinement period toward the postconfinement period (Fig. 3*A*). The monocytes showed an increase at the beginning of confinement, but no changes were seen in the other periods (Fig. 3*B*). The lymphocytes decreased toward the postconfinement period (Fig. 3*C*).

Total leukocyte counts. Mean numbers of total leukocyte count per microliter were as follows: 5,480 ± 150 ($n = 5$) in the preconfinement period; then 6,120 ± 403 ($n = 10$), 6,140 ± 391 ($n = 10$) and 6,244 ± 370 ($n = 9$) during the confinement period; and 6,544 ± 570 ($n = 9$) in the postconfinement period. Because the number of complete data was small, statistical analyses were not performed.

Face scale test. The face scale test was conducted to assess subject's psychological aspect. In this test, higher numbers in the face scale are associated with a better mood of the subject. The mean face scale value was 12.8 ± 1.03 in the preconfinement period, then 13.6 ± 0.76, 14.6 ± 0.58, and 14.1 ± 0.59 during the confinement, and 16.3 ± 0.67 in the postconfinement period. The *P* value in the postconfinement period compared with the preconfinement period was 0.0016 ($n = 10$). These observations indicated that the subjects' moods were low at the beginning and then improved toward the end of the experiment.

DISCUSSION

Different changes were observed in $CD69^+$ percentage between NK and non-NK cells (Fig. 2, *A* and *B*). However, the changes were mostly coordinated with those in NK and non-NK cell percentages (Fig. 2, *A–D*), and no significant differences were seen in $CD69^+$ percentages in NK and non-NK cells (Fig. 2, *E* and *F*). Thus these observations indicated that the change in ratio between NK and non-NK cells was responsible for the alteration of $CD69^+$ percentage. Suppression of proliferative reaction occurred when non-NK

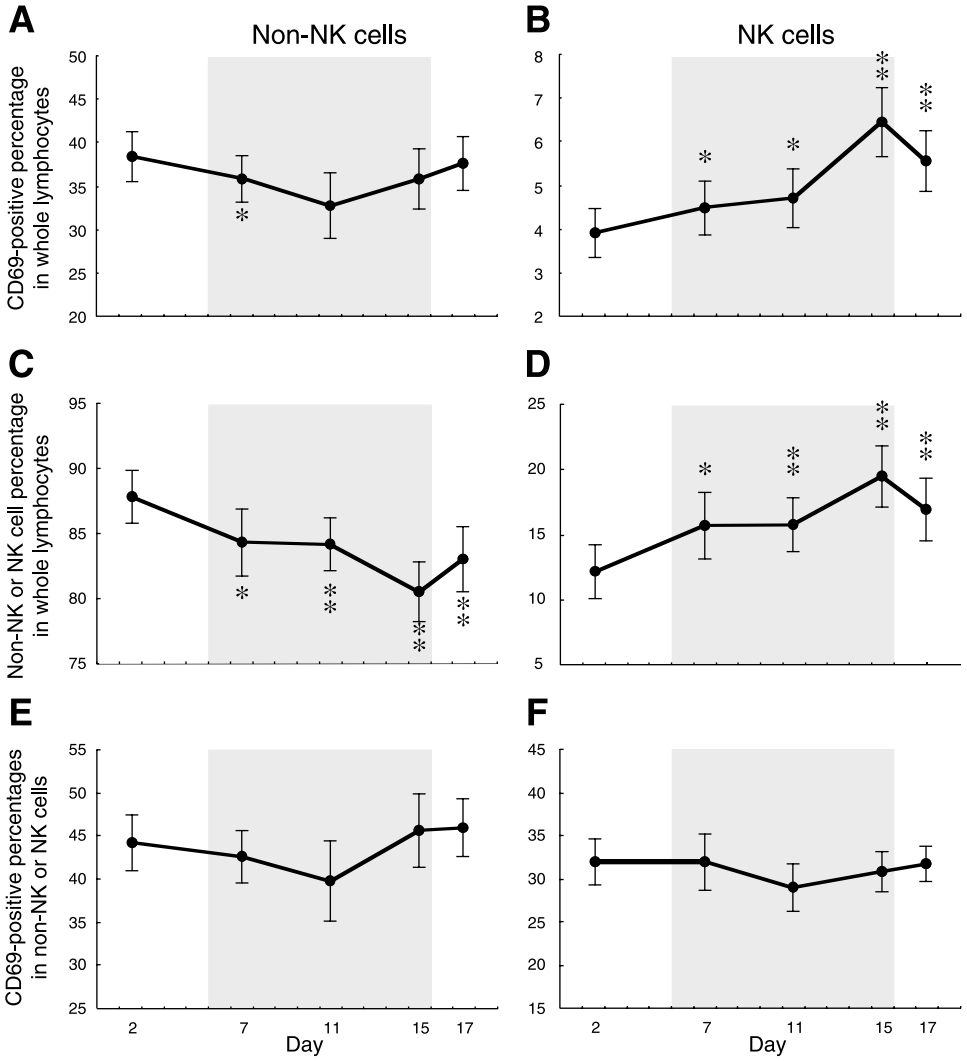

Fig. 2. Natural killer (NK) and non-NK cells, and their $CD69^+$ percentages among lymphocytes. $CD69^+$ NK and non-NK cell percentages in whole lymphocytes are plotted in *A* and *B*, respectively. NK and non-NK cell percentages relative to whole lymphocytes are plotted in *C* and *D*, respectively. As the NK and non-NK cell percentages sum to 100%, they showed complementary changes. $CD69^+$ percentages in NK and non-NK cells are plotted in *E* and *F*, respectively. The gray area indicates the confinement period. Values are means ± SE ($n = 9$). Significant differences between preconfinement and other time points: $*P < 0.05$; $**P < 0.01$.

cell percentage decreased. We speculated that a compensatory increase in percentage of NK cells and their $CD69^+$ percentage indicated elevation of innate immunity.

Lymphocytes are known to express both β-adrenergic and glucocorticoid receptors. Catecholamines and glucocorticoids regulate the proliferative reaction of lymphocytes through these receptors (1, 8, 9). The effects of stress on the immune system include not only suppression of proliferative reaction but also changes in leukocyte distribution (1, 3, 4, 17). Enhancement of the autonomic nervous system seems to elevate innate immunity. Stimulation of sympathetic nerves and β-adrenergic control of lymphatic smooth muscle are thought to control the rapid mobilization of NK cells and granulocytes from marginal pools and the spleen (8). Increases in neutrophil or granulocyte number were reported not only postspaceflight but also in a ground-based long-term confinement study (5). Many reports have shown that both the number and percentage of NK cells in peripheral blood are increased by acute laboratory stress, which necessitates a rise in heart rate (1, 3, 4, 17). On the other hand, long-lasting chronic stress, such as caregiving, bereavement, and examinations in medical students (11), causes decreases in the number, percentage, and cytotoxity of NK cells. Similar changes have been observed in postspaceflight astronauts who experienced prolonged stress during the reentry procedure (7, 20, 23). As mentioned, the NK cell percentage increased toward the end of confinement (Fig. 2*D*). The ratio of granulocytes to lymphocytes and monocytes also increased toward the end (Fig. 3). The face scale test showed that the subjects' moods improved toward the end of the period of confinement. Therefore, a possible explanation for the increases in percentages of NK cells and granulocytes is that subjects' autonomic nervous systems were influenced by an exalted mood, because they expected the forthcoming completion of the experiment. In addition, the low NK cell percentage shown in the preconfinement period (Fig. 2*D*) was caused by prolonged anxiety before the experiment because face scale was also low at the beginning.

Cytokine and hormone status are known to affect immune reactions in host defense (1, 8, 9, 15). [^{3}H]thymidine incorporation assays have been used in not only ground-based confinement studies simulating the environment of a space station or a long-term mission to another planet but also many studies of the effects of stress on the immune system. This method has mostly been used for analysis of phytohemagglutinin-stimu-

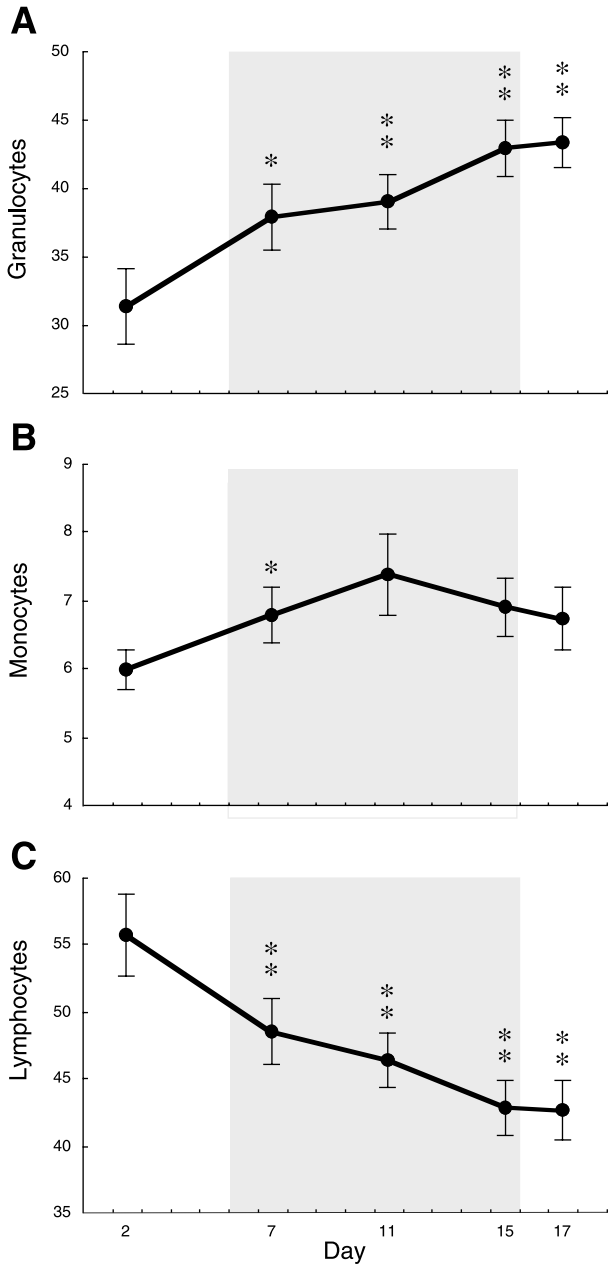

Fig. 3. Subpopulation percentages in whole leukocytes of granulocytes (*A*), monocytes (*B*), and lymphocytes (*C*). The gray area indicates the confinement period. Values are means ± SE ($n = 9$). Significant differences: *$P < 0.05$; **$P < 0.01$.

lated peripheral blood mononuclear cells (1, 2, 18). In this method, blood constituents that should function in the reaction are removed in advance. Furthermore, because [^{3}H]thymidine incorporation assay takes 2–3 days to show a proliferative response, the data shown may not reflect the actual status in vivo. CD69 is an early activation marker of lymphocytes expressed within 2–4 h on the way to proliferation (14, 22). The expression of CD69 was reported to be correlated with [^{3}H]thymidine incorporation (14). Analysis of CD69 to monitor proliferation response in whole blood may provide results that reflect the effects of stress, because the data can be obtained while blood samples are still fresh and retain in vivo status. Moreover, because flow cytometric assay is available for measurement of CD69 antigen expression, information was obtained on a subpopulation basis. This enabled us to observe the relation between the suppression of proliferation and the distribution changes in lymphocytes. We also employed a CD2 agonistic antibody to initiate the proliferative reaction. CD2 is specifically expressed on T and NK cells (12, 16), which are responsible for triggering proliferation and have a high degree of sensitivity to stress hormones (1, 8, 9, 15). We employed CD2/CD2R to stimulate these stress-sensitive cells selectively.

Because a time-matched control group was not prepared, we regarded the preconfinement time point as reference. Although statistical analyses were not conducted, no major change was seen in the total leukocyte count. Thus the percentages seemed to represent the number of cells. The subjects participating in this experiment were all university students in their 20s and were therefore different from well-trained personnel, such as astronauts. Adolescents might be more sensitive both psychologically and immunologically. Previous confinement studies in which immune responses were analyzed were relatively long and varied from 28 to 240 days (5, 18, 19). In a 240-day confinement study conducted in Russia, urinary norepinephrine levels were elevated in the first month of confinement and in the postconfinement period. This was considered due to stress caused by adaptation to confinement and readaptation on returning to normal conditions. Therefore, environmental changes, rather than long-term confinement, are thought to have a significant influence (5). Our 10-day confinement data may have shown the first susceptible state before adapting to the confined environment. There have been few previous reports of short-term confinement studies, with confinement over short periods, such as 10 days. As the duration of space shuttle missions in which peripheral blood mononuclear cell suppression has been reported is mostly ~1–2 wk (7, 21, 23), a 10-day confinement period seemed to be an appropriate reference. The other major factor that may influence psychological aspect is experience. To further substantiate the adaptation to confinement stress, it would be of interest to perform similar experiments with career astronauts as subjects or to repeat the confinement experiment with the same subjects to determine whether the immune system can become accustomed to a confined environment in a coordinated manner with respect to psychological aspects.

In conclusion, the expression of the early activation marker CD69 was suppressed during the period of confinement. This was caused by changes in the distribution of NK and non-NK cells. Coordinated with changes in the face scale, the percentage of innate immune cells, such as NK cells and granulocytes, increased toward the end of confinement. These distribution changes may have been mediated by the activated autonomic nervous system, which was caused by the exalted mood in subjects anticipating completion of the experiment. We concluded that changes in the distribution of immune cells caused by stress play an important role in suppression of proliferative reactivity. The observed physiological reactions were shown to be specific to the confined environment, and the stress caused by confinement plays a role in the immune changes in space.

ACKNOWLEDGMENTS

We thank Taishi Onodera and Kenichi Umezawa for assistance with sample preparation and analysis.

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