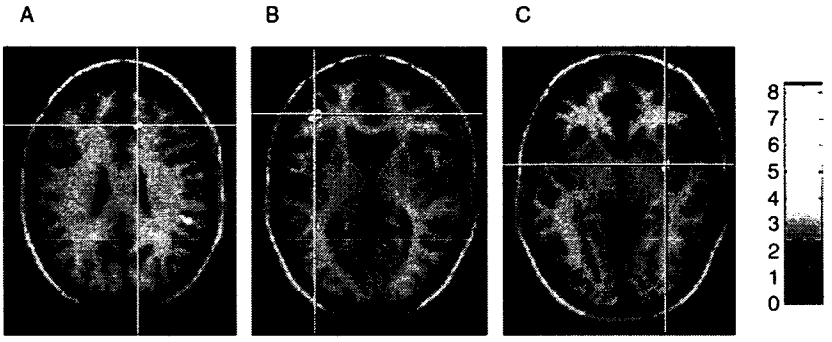

Figure 5 Conditioning effects on regional cerebral blood flow (rCBF). Parametric maps of regional cerebral blood flow increase during the postconditioning CS-alone trial compared with the preconditioning CS-alone trial is superimposed on Talairach–Tournoux stereotactic space. Sagittal, coronal and axial views are centred at 12, 34, 30, in the right anterior cingulate cortex (left; A), at −42, 40 and 10, in the left prefrontal cortex (middle; B) and at 36, 2, −4, in the right insula (right; C), respectively. In the view of A, activation of the right parietal cortex also can be seen. Significant changes are marked with a split-grey scale ($P \leq 0.001$, uncorrected).

Table 3 Areas of rCBF significantly increased in the CS with full intensity US trial compared to the preconditioning CS-alone trial ($P \leq 0.001$, uncorrected)

Area (Broadmann area)	Hemi-sphere	Talairach co-ordinate			Z-score
		x	Y	z	
Primary sensory cortex (1)	Left	−34	−28	56	3.8
Primary sensory cortex (2)	Left	−48	−22	36	3.4
	Right	52	−22	30	3.4
Orbitofrontal cortex (11)	Left	−34	36	−16	4.1
Precentral gyrus (6)	Left	−38	2	26	3.9
Posterior cingurate cortex (31)	Left	−18	−52	20	3.6
Middle temporal gyrus (22)	Left	−42	−42	6	3.8
Occipital cortex (18)	Left	−20	−82	24	4.0
Pons	Left	−8	−40	−40	4.2
	Right	16	−24	−34	3.8

rCBF, regional cerebral blood flow.

Table 4 Areas of rCBF significantly increased in the CS with weak intensity US trial compared to the preconditioning CS-alone trial ($P \leq 0.001$, uncorrected)

Area (Broadmann area)	Hemi-sphere	Talairach co-ordinate			Z-score
		x	Y	z	
Primary somatosensory cortex (2)	Left	−34	−32	40	3.4
Prefrontal cortex (9)	Left	−40	8	30	3.8
Prefrontal cortex (10)	Left	−36	40	20	3.8
Orbitofrontal cortex (11)	Left	−34	34	−16	3.6
Anterior cingulate cortex (24)	Left	−4	10	36	4.6
Insura (13)	Left	−42	−16	4	3.7
Parietal cortex (40)	Left	−50	−30	46	3.9
Superior frontal gyrus (6)	Left	−6	6	58	3.7
Middle frontal gyrus (6)	Left	−10	−6	50	3.7
Precentral gyrus (6)	Left	−56	0	20	3.7
	Right	54	2	14	3.5
Primary motor cortex (4)	Left	−34	−24	60	3.4
Putamen	Left	−28	−22	−4	4.5
Pons	Left	2	−20	−20	4.2

rCBF, regional cerebral blood flow.

the conditioned group, showing anticipatory fear, actually had a large sympathetic increase that was partly masked by a simultaneous parasympathetic increase.[28,29] Considering the previous studies on autonomic nervous activity during anticipation, increase in colorectal motility observed following a series of painful stimuli to the hand may result as dominant parasympathetic arousal. Further pharmacological intervention should be needed to confirm this hypothesis.

On the other hand, the anticipatory rectosigmoid motor responses could represent nonspecific arousal or anxiety. It has been reported that psychological stress induces significant changes in gastrointestinal motility, which were associated with alterations in autonomic nervous activity.[30,31] However, nonspecific arousal or anxiety cannot explain main effects of the conditioned phasic contractions of the rectosigmoid colon observed in this study for two reasons: (i) the overall anxiety score which is considered to be very global did not show a significant difference between before and after the series of painful stimuli. (ii) The increases in PVEs were limited to the poststimulus interval and were not seen during the baseline interval in the postconditioning CS-alone trial although this baseline interval was preceded by a series of painful stimuli.

Considering the conditioning effect in the brain, our findings of the brain imaging (Table 2 and Fig. 5) were in accordance with previous studies showing cerebral activation in the frontal and parietal cortices following Pavlovian conditioning.[12–14] Activation of the prefrontal cortex was seen during somatic stimulus, and has been implicated in cognitive appraisal of the stimulus.[32] In addition, significant cortical activation in the anterior cingulate cortex (ACC), which is believed to play a role in mediating the affective qualities of the pain experience[33,34] and expectation of pain,[35] and in the insula which serves as limbic integration cortex[36] was also seen as anticipatory responses in this study. Therefore, our results support that activation of the cognitive- and affective-related brain regions may contribute to the learned anticipatory responses and that this learned process was confirmed after the conditional trials in this experimental model. However, this model has been set up to study the anticipatory colonic motor response and brain activation patterns that have been identified only reflected that. The direct relationships between the brain activation and the gastrointestinal response during anticipation have not been clarified with this model.

When comparing brain-imaging data between the postconditioning buzzer with high- or low-mA stimulus and the preconditioning buzzer-alone trials, increased rCBF not only in the left primary somatosensory cortex but also in the prefrontal cortex was observed (Tables 3 and 4). These findings were in concordance with the previous reports on the central processing of painful stimulus using with brain-imaging studies,[32,35,37] Thus, in the present study, these comparisons revealed that the transcutaneus electrical stimulation to the right hand induced painful sensation. Furthermore, activation of the cognitive- and affective-related brain regions was observed in common, suggesting that cerebral responses involved in painful/fearful anticipation might be different from the nociceptive process.[38]

There are some limitations in this study. First of all, participants of the study were limited to small group of healthy volunteers from a local university. Lee *et al.* have investigated differences in gastrointestinal symptom severity in males *vs* females and variations with menstrual cycles.[39] Female subjects might show different patterns in the brain and/or colonic motility function for the conditioned responses. Secondly, additional examinations such as an anorectal morphological study were not explored. However, existence of severe megarectum[40] and/or the other anorectal disorders,[41] which might affect rectal wall motor function, were unlikely because no subjects had been reported any problems of bowel movement and any abnormal physical findings. Finally, the reliable visceral sensory tests (e.g. the ascending method of limits and/or the random sequence)[19] were not used because of the limited time in the PET scanning room. Despite of these limitations, we believe that this study could be worth to report the conditioned phenomena in this model as a first step to understand anticipatory colonic motility responses to somatosensory painful stimulus in humans. The available data on conditioned gastrointestinal responses are very limited and deserve further studies.

In summary, the Pavlovian conditioning study is significant because of positive findings that the conditioned phenomenon in this model is a first step to understand the anticipatory colonic motility responses. Significant increases in colonic phasic contractions and significant increases in cerebral blood flow in the cognitive- and affective-related cortical regions were observed in this study. This conditioning paradigm could be a model to investigate anticipatory responses in gastrointestinal motility and brain function, which may contribute to development of functional gastrointestinal disorders. We concluded that the rectosigmoid motility could become conditioned by pairing a painful somatosensory stimulus with a neutral stimulus in humans.

ACKNOWLEDGMENTS

This study was supported by Grant-in-Aid for Scientific Research Nos 833-5020-11680778, 622-5637-13897021 and 7201-3400-15390218 from the Ministry of Education, Science and Culture of Japan and Grant-in-Aid for Scientific Research Nos H11-Nou-003, H13-Chouju-028 and H14-Si-9 from the Ministry of Health, Welfare and Labour of Japan (PI: Shin Fukudo).

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BIOLOGICAL PSYCHOLOGY
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Temporal variation of acute stress responses in sympathetic nervous and immune systems

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Received 16 June 2004; accepted 17 December 2004
Available online 23 May 2005

Abstract

Sympathetic nervous activity plays a prominent role in acute stress responses in the immune system, enhancement of innate immunity and suppression of specific immunity. The present study was conducted to examine the temporal characteristics of such immune responses to acute stress and to determine their association with sympathetic activity in detail. For this purpose, 15 female undergraduates engaged in a continuous mental arithmetic task for 14 min, and we collected their blood samples for immune indices (CD3+ T cells, CD4+ T cells, NK cells) each 3 min during the task and saliva samples before and after the task. Our results showed that the proportion of Natural Killer cells (NK cells) increased even 2 min after initiation of the task, whereas proportions of CD3+ and CD4+ lymphocytes decreased 8 min after initiation of the task. Moreover, we found significant correlations between cardiovascular activity and the variations of immune indices.

Keywords: Stress; Sympathetic nervous; Mental arithmetic; Psychoneuroimmunology

1. *Introduction*

A variety of experimental protocols for the induction of psychological acute stress in laboratory settings (Bachen et al., 1992; Herbert et al., 1994; Breznitz et al., 1998; Gerra et al., 2001; Atanackovic et al., 2002; Scarpa and Luscher, 2002; Willemsen et al., 2002; Kunz-Ebrecht et al., 2003; Lutgendorf et al., 2004) have indicated that the function of innate immunity represented by Natural Killer cell (NK cell) number was enhanced while the function of specific immunity including B cells and CD4+ helper T cells was suppressed in acute stress situations, especially in active coping situations (Delahanty et al., 1996; Mills et al., 1996; Pike et al., 1997; Burleson et al., 1998; Marsland et al., 2001; Willemsen et al., 2002; Isowa et al., 2004). Furthermore, the volume of secretory immunogloulin A (s-IgA), which is a dominant antibody in mucosal immunity, increases in such acute stress situations (Willemsen et al., 1998; Ring et al., 1999, 2000; Bosch et al., 2001; Winzer et al., 1999).

An important cause of immune modulation by stress is the activity of the hypothalamus–pituitary–adrenal cortex (HPA) axis, which secretes cortisol from the adrenal cortex, resulting in immunosuppression (Rupprecht et al., 1990; Garvy and Fraker, 1991; Northrop et al., 1992; Slade and Hepburn, 1983). However, at least in acute stress situations, immunomodulation should be affected more by autonomic nervous system activity than by HPA axis activity because secretion of cortisol begins about 15 min after the initiation of stressful tasks and reaches a peak about 20 min later (Kuhn, 1989). In spite of the delay in the rise of cortisol secretion, even brief stress tasks for 6 or 4 min influenced the immune cells in peripheral blood (Willemsen et al., 2002). Considering those findings, the autonomic nervous system should contribute to stress responses of immune cells at least in acute stress situations. The fact that adrenalin and noradrenalin secreted during excitation of the sympathetic nervous system can influence the surface receptors of immune cells in blood supports this idea (Benschop et al., 1994, 1996). However, little is known about the characteristics of temporal changes in immune functions during acute stress.

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0301-0511/$ – see front matter
doi:10.1016/j.biopsycho.2004.12.006

In previous studies, variations in immune indices were typically evaluated before and after tasks, and the duration of tasks varied among studies. Tasks used for this purpose included performing mental arithmetic for 8 min, experiencing 4 min of a cold pressor (immersion of the forearm in cold water) and performing public speaking for 6 min (Farag et al., 2002; Willemsen et al., 2002). The fact that activation of cardiovascular activities can occur within a second of exposure to stress tasks (Kuhn, 1989) suggests that variation in immune functions can be evoked at the early stage of such stress situations. Unfortunately, the simple pre-post design adopted in previous studies prevented study authors from making a detailed examination of temporal variations in immune functions during acute stress. The aim of the present study, therefore, was to examine at which stage the indices of the immune system change during an acute stress situation and to determine the influence of sympathetic nervous activity in these changes. For this purpose, we also focused on a peripheral manifestation of sympathetic nervous system activity as revealed by heart rate variability (HRV) because it is necessary to confirm whether our stress task admittedly elicits the activation of sympathetic nervous system. HRV is determined by the balance of parasympathetic and sympathetic nervous system activity regulating the heart, and the ratio of the spectrally derived low-frequency to high-frequency band power (LF/HF ratio) is considered to be a reliable index of sympathetic activation (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).

In the present study, therefore, we measured proportions of subsets of blood lymphocytes (CD3+ T cell, CD3+CD4+ helper T cell, CD3–CD16+CD56+ NK cell) and concentration of salivary secretory immunogloblin A as indices of natural, specific and mucosal immune functions every 3 min during a mental arithmetic task that lasted 14 min. Additionally, to examine the autonomic–endocrine–immune association, we evaluated the concentration of salivary cortisol as an endocrine index before and after the task and measured the heart rate and blood pressure and HRV as autonomic indices continuously during the task. But because our research interest was paid to the sympathetic nervous activity and immune changes to the acute stress, with consideration of a participant's burden, blood sampling at short intervals was performed only during the task and taken only for the immune indices.

2. Methods

2.1. Subjects

Fifteen female undergraduates in Mie Prefectual College of Nursing (age range 20–26 years; mean = 21.67; S.D. = 1.88) participated in the present study. The mean body-mass index of the subjects was 20.88 kg/m^2 (S.D. = 2.48). Since the stress of the cannula insertion itself was taken into consideration, only the students of nursing who are usually familiar with the cannula insertion participated. None was suffering from any chronic or oral illness, and none was taking medication known to influence immunity. In addition, no subjects were using oral contraceptives. Although there were several reports that differences in the balance of the sex hormone changing with the menstrual cycle influenced on an immune system (Pehlivanoglu et al., 2001), significant difference in immune measures about a menstrual cycle was not observed in this research (data not shown).

All subjects signed informed consents to participate in the study, which was approved by the Ethics Committee of Mie Prefectural College of Nursing.

2.2. Immunological measures

Blood samples for immunological testing were collected in heparinized tubes. The numbers of total white blood cells (WBC), lymphocytes, monocytes and granulocytes per sample were determined by standard means. Percentages of lymphocyte subsets were determined by flow cytometry (FACS Calibur, Becton-Dickinson). A whole-blood lysis method was used to stain the cells with the following pairs of Fluorescein isothiocyanate (FITC)/Phycoerythrin (PE)-conjugated monoclonal antibodies (DAKO, Inc.). The isotype-matched antibodies used were Mouse IgG1, CD3+/CD4+ indicating helper T cells and CD3−/CD16+/CD56+ indicating NK cells.

To determine the volume of secreted saliva and the concentration of s-IgA, we collected samples of unstimulated saliva using cotton swabs (Salivettes, Sarstedt Ltd.). A cotton swab was placed underneath the tongue of each participant for 3 min. After that, the cotton swab was removed and saliva was extracted from the cotton by centrifugation at 3.5×10^3 rpm for 10 min. Saliva was stored frozen in capped test tubes at −20 °C until assay. We determined the s-IgA concentration in saliva (in micrograms per milliliter) by an enzyme-linked immunoabsorbent assay (using IgA test; MBL, Inc.). The thawed saliva aliquots (10 μl) were diluted 40 times. Saliva samples were reacted with polystyrene beads that labeled the antihuman secretary component. After incubation at 37 °C for 1 h, the beads were washed twice and reacted with peroxidase standard antihuman IgA (rabbit IgG/Fab') (secondary reaction). After incubation at room temperature for 1 h, the beads were washed three times, and then enzyme metrical fluid (orthophenylenediamine + 4 mM H_2O_2) (third reaction) was added. After incubation at room temperature for 30 min, the reaction was stopped by the addition of H_2SO_4. The reaction product was quantified spectrophotometrically at 492 nm with a microplate reader (Bio-Rad, Inc., Model 550). The s-IgA secretion rate (in micrograms per minute) was calculated as the product of s-IgA concentration and saliva flow rate.

2.3. Endocrine measures

Samples of saliva for measurement of the concentration of salivary cortisol were obtained by the same method as

for s-IgA and stored at −20 °C until assay. The concentration of cortisol in the saliva (in micrograms per milliliter) was determined by an enzyme-linked immunoabsorbent assay. Ninety-six-well Costar™ microplates pre-coated with anticortisol rabbit antibody were prepared. A saliva sample of 50 μl was added to each well in triplicate, and then an enzyme conjugate of 50 μl was added to each well and samples were incubated at room temperature for 1 h. After incubation, each well was washed three times. A substrate of 50 μl was added to each well and incubated at room temperature for 30 min. After that, 1N HCL 50 μl was added to each well to stop the enzyme reaction. To measure the concentration of cortisol the plate was read at 450 nm with a microplate reader (Bio-Rad, Inc., Model 550).

2.4. Cardiovascular measures

Cardiodynamic activity was recorded using electrocardiography (ECG) and non-invasive finger blood pressure (FINAP) measurements. ECG was recorded using a MP 100 system (Biopac Systems, Inc.). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded through a finger cuff of a Portapres Model 2 (TNO Biomedical Instrumentation, Inc.) attached to the third finger of the non-dominant hand of each subject. Each indicator was recorded continuously during the task and rest periods. The ECG data were subsequently analyzed to yield HRV. The data were first subject to ocular inspection and only completely artifact-free data were used for estimation of the R–R intervals. The R–R interval data were resampled at 4 Hz to obtain equidistant time series values. A power spectrum density was then obtained through a fast Fourier transformation of the tachogram. In connection with the fast Fourier transformation, the data were detrended linearly and filtered through a rectangular window. The integral of the power spectrum was studied in two major frequency bands: the high-frequency band (0.15–0.5 Hz) and the low-frequency band (0.05–0.15 Hz). The former related to respiratory sinus arrhythmia and is exclusively attributable to parasympathetic influence reflecting vagal activity and in the latter case, it mirrors the baroreceptor feedback loop that controls blood pressure and appears to reflect both sympathetic and parasympathetic activity. Consequently, the HF component and the relative contributions of LF and HF power (LF/HF) which reflect sympathovagal balance, were considered in the present paper. Analyses of ECGs and FINAP waveforms were performed using AcqKnowledge software for the MP 100.

2.5. Psychological measures

The subjects were asked to evaluate subjectively the intensity of the stress on analog-visual scales (0–100%). In addition, they completed a Japanese version (Katsuharu and Tadanobu, 1982) of the State-Trait Anxiety Inventory (STAI) (Speilberger et al., 1970). The STAI is composed to measure state anxiety and consists of 40 items.

2.6. Stress task

The subjects performed mental arithmetic for 14 min. The subjects were told to add the currently displayed number (from 2 to 9) to the next one displayed on the PC monitor, and to answer only one digit of the current answer by pressing a key (from 0 to 9). Each number was displayed for 500 ms and followed by a 1300 ms interval of no number display. The task included 460 numbers. During the task, once participants responded by key after each set, O or x was shown on the monitor as feedback indicating whether the participant's previous answer was correct or wrong.

2.7. Procedure

The subjects had been instructed to eat a light breakfast on the morning of the experiment; caffeine-containing beverages were not allowed. Also, the subjects had been instructed to paste a monoanesthetic seal (PENLES; Wyeth Lederle, Inc.) at the location of the cannula insertion in their arms about 1 h before the experimental sessions to reduce pain. Subjects suffering from an infectious illness within 2 weeks of the experiment were rescheduled.

The experimental sessions were composed of a mental arithmetic task and two rest periods. The subjects were tested individually in a temperature- and humidity-controlled room. After each subject entered the experiment room, a cannula was inserted into the forearm vein of her non-dominant arm. Next, electrodes for electrocardiographic measurements and a finger cuff for blood pressure recording were attached. For the next 15 min, the subjects filled in psychological questionnaires. Then, instruction was given for the mental arithmetic task and the subjects were allowed to practice the mental arithmetic task for 1 min. During the instruction period, the subjects were told that four blood samples would be taken during the arithmetic task but they were to perform the task continuously and not stop concentrating on it.

After a rest period of 10 min, the first blood sample (for assays of immunological parameters) and the first saliva sample (for assays of s-IgA and cortisol) were taken as a baseline sample, and the subjects were asked to fill in the questionnaire. After that the subjects performed the mental arithmetic task for 14 min. While subjects performed the task, the four blood samples were taken at 2, 5, 8 and 11 min.

Immediately after the task, the sixth blood and the second saliva sample were taken and each subject filled in the questionnaire again. After a second rest period of 15 min, the seventh blood and the third saliva sample were taken and the questionnaires were filled in again. Autonomic indices (ECG and BP) were measured continuously throughout the experimental session. After the end of the procedure, the electrodes, blood pressure cuff and cannula were removed,

and the subjects were fully debriefed and thanked. Each subject was paid 800 Japanese yen for their participation.

2.8. Statistical analysis

Prior to statistical analysis, mean values of HR, SBP and DBP data were calculated for the last 2 min of the pre-experimental baseline period, periods during the stress task (1–2, 4–5, 7–8, 10–11 and 13–14 min), and the last 2 min of each rest period after the task. The cardiovascular data were analyzed using repeated-measures analyses of variance (ANOVAs) with a within-subjects factor of periods (baseline, $stress_{2\ min}$, $stress_{5\ min}$, $stress_{8\ min}$, $stress_{11\ min}$, $stress_{14\ min}$, rest). Immune data excluding s-IgA were analyzed using repeated-measures ANOVAs with a within-subjects factor of period (baseline, $stress_{2\ min}$, $stress_{5\ min}$, $stress_{8\ min}$, $stress_{11\ min}$, $stress_{14\ min}$ rest). Cortisol, s-IgA, HF component of HRV, LF/HF ratio and psychological data were analyzed using repeated-measures ANOVAs with a within-subjects factor of period (baseline, stress, rest). The Greenhouse–Geisser epsilon correction factor, ε (Jennings and Wood, 1976), was used where appropriate. In cases where significant main effects were found in the ANOVAs, post hoc analyses using LSD tests ($p < .05$) were conducted to examine which combinations of data points differed significantly. Pearson correlation coefficients were computed among changed scores (scores at the stress period–scores at the baseline) of these indexes to examine the relationship between immune, cardiovascular and endocrine reactivity.

3. Results

3.1. Immunological measures

The data including WBCs, lymphocytes, monocytes and granulocytes at the baseline, stress (2, 5, 8, 11, 14 min) and rest periods are summarized in Table 1. Fig. 1 illustrates changes in the percentages of CD3+ T cells, CD4+ T cells and NK cells.

The main effects of period, reflecting temporal variation of indices during the acute stress task, were significant for granulocytes, $F(6, 84) = 3.61, p < .01$; CD3+T cells, $F(2.63, 36.81) = 4.97, p < .01$; CD4+ T cells, $F(3.06, 42.83) = 5.12, p < .01$; NK cells, $F(2.59, 36.28) = 8.97, p < .01$. Post hoc comparisons indicated that CD3+ T cells and CD4+ T cells decreased, and granulocytes and NK cells increased during the acute stress task. Furthermore, CD3+ T cells decreased significantly at 5 min and CD4+ T cells showed significant decreases at 8 min after the start of the task and remained at a level lower than that at baseline during the task. The increments of granulocytes and NK cells were found to be significant at 2 min from the start of the task and were maintained throughout the acute stress task. However, there were no significant main effects between baseline and rest in NK cells. Therefore, it can be deduced that, as predicted, the acute stress task influenced immune indices, which returned to normal a short time after the task. Meanwhile, ANOVAs yielded no significant main effects of task for WBCs, $F(1.75, 24.56) = 2.53$, ns, lymphocytes, $F(3.46, 48.48) = 1.53$, ns or monocytes, $F(3.14, 43.99) = 1.41$, ns.

3.2. Salivary measures

The changes in salivary flow, s-IgA concentrations and concentrations of cortisol at three points (baseline, stress, rest) are shown in Table 2, which also shows that an ANOVA showed tendency of main effects of period about s-IgA $F(2, 28) = 2.74$, $p < .10$. This indicates that s-IgA tended to increase in response to an acute stress task. There were no significant main effects of period in connection with the other two indices.

3.3. Cardiovascular measures

Changes in cardiovascular indices are illustrated in Fig. 2. ANOVAs yielded a significant main effect of period for HR, $F(3.83, 53.60) = 18.05$, $p < .01$; SBP, $F(2.07, 28.92) = 14.19, p < .01$; DBP, $F(2.55, 35.62) = 25.67, p < .01$. These results mean that all of these cardiovascular indices showed higher levels during execution of the stress task than at baseline or during the rest periods. Moreover, there were significantly maintained increments of these indices during all the time of the task. While the increments of HR and DBP returned to the baseline level after the acute stress task, SBP did not return and remained higher than baseline. On the other hand, the HF component of HRV was significantly reduced during task period compared with the baseline and rest periods $F(2, 28) = 13.47, p < .01$. And the LF/HF ratio showed significant increase in the task period, $F(1.43, 20.01) = 6.50$, $p < .05$, but no difference was observed between baseline and rest periods. The decrease in HF

Table 1
Means (S.D.s) of immunological measures and results of ANOVAs

	Baseline	Task (2 min)	Task (5 min)	Task (8 min)	Task (11 min)	Task (14 min)	Rest	Main effect
WBC ($\times 10^3$, μl)	52.07 (17.04)	54.60 (17.82)	54.67 (17.78)	54.13 (18.30)	54.53 (18.00)	54.80 (18.04)	54.00 (16.00)	ns
Lymphocyte ($\times 10^3$, μl)	15.62 (4.87)	15.40 (5.26)	15.47 (5.65)	15.87 (5.45)	16.02 (5.77)	15.84 (4.98)	15.84 (4.98)	ns
Monocyte ($\times 10^3$, μl)	3.20 (1.35)	3.48 (1.52)	3.55 (1.50)	3.53 (1.49)	3.49 (1.40)	3.37 (1.33)	3.45 (1.20)	ns
Granulocyte ($\times 10^3$, μl)	32.04 (12.98)	34.40 (13.66)[a]	34.37 (13.01)[a]	33.57 (14.03)[a]	33.78 (13.71)[a]	34.40 (14.26)[a]	34.64 (13.11)[a]	Period**

Main effects as results of ANOVAs.
[a] Significant different from baseline at this time point.
** $p < .01$

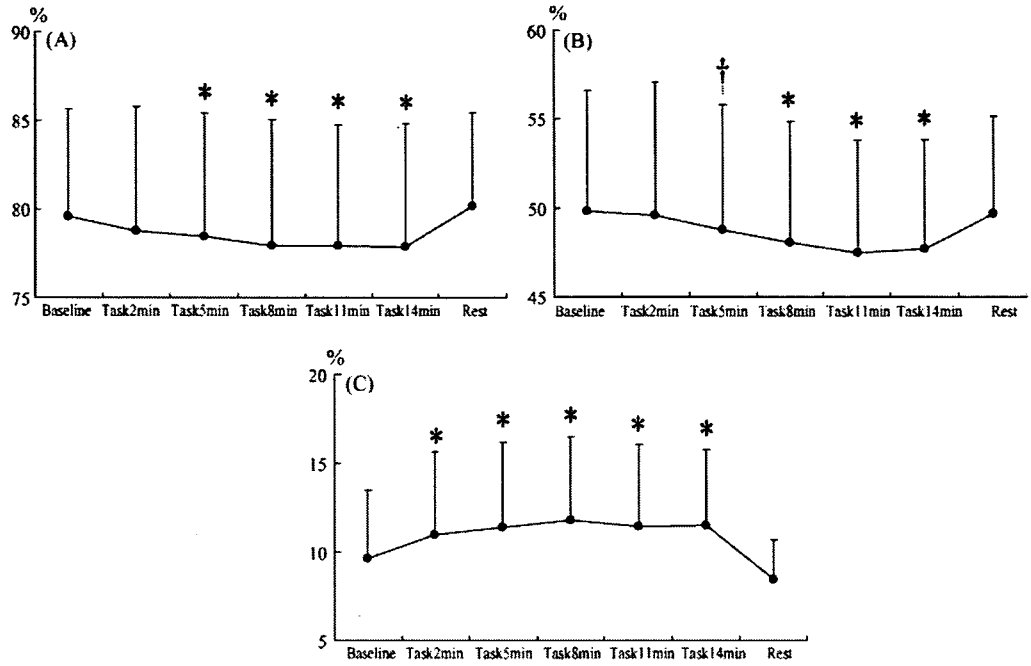

Fig. 1. Changes in percentages of CD3+ T cells (A), CD4+ T cells (B) and CD16+CD56+ NK cells (C) at each measurement point. Error bars indicate standard deviations. Symbol (*) denotes a significant ($p < .01$) difference of each index in comparison to baseline. Symbol (†) denotes a tendency toward a significant difference ($p < .1$) of each index in comparison to the baseline.

Table 2
Means (S.D.s) of salivary measures and results of ANOVAs

	Baseline	Task (14 min)	Rest	Main effect
Saliva flow rate (μl/3 min)	709.93 (713.16)	871.00 (823.59)	756.13 (776.02)	ns
s-IgA concentration (μg/ml)	61.58 (13.31)	67.58 (21.16)	58.12 (11.41)	†
Cortisol (ng/ml)	9.83 (6.69)	9.83 (5.76)	10.70 (6.44)	ns

Main effects as results of ANOVAs.
† $p < .1$.

component and the increase in LF/HF ratio could suggest that our mental arithmetic task induced the dominance state of sympathetic nervous system. The data derived from HRV are presented in Table 3.

3.4. Psychological measures

The psychological data at the baseline, stress and rest periods are presented in Table 4. In the table, perception of stress is shown on a visual analog scale. From the results of ANOVAs, significant differences were observed in perception of stress $F(1.72, 24.08) = 8.77$, $p < .01$ and state of anxiety $F(1.50, 21.01) = 7.16$, $p < .01$. Post hoc comparisons revealed that perception of stress and state of anxiety were higher in the stress period than baseline and rest periods. These results could show the task used in this study functioned as acute stress task for participants.

Table 3
Means (S.D.s) of HF component of HRV, LF/HF ratio and results of ANOVAs

	Baseline	Stress	Rest	Main effect
HF (%)	53.74 (23.39)	37.43 (20.53)	53.46 (19.90)	Period**
LF/HF ratio	1.37 (1.38)	2.76 (2.67)	1.32 (1.52)	Period*

Main effects as results of ANOVAs.
* $p < .05$.
** $p < .01$.

3.5. Associations between immune and cardiovascular measures

Correlations between changes in immune and cardiovascular parameters were computed for the whole sample (see Table 5). The results indicated that, for the mental arithmetic task, systolic and diastolic blood pressure in measures of

Table 4
Means (S.D.s) of psychological measures and results of ANOVAs

	Baseline	Stress	Rest	Main effect
Perception of stress	29.80 (20.02)	43.07 (13.77)	25.13 (13.96)	Period**
STAI State anxiety	42.00 (6.67)	44.70 (5.80)	38.93 (4.56)	Period**

Main effects as results of ANOVAs.
** $p < .01$.

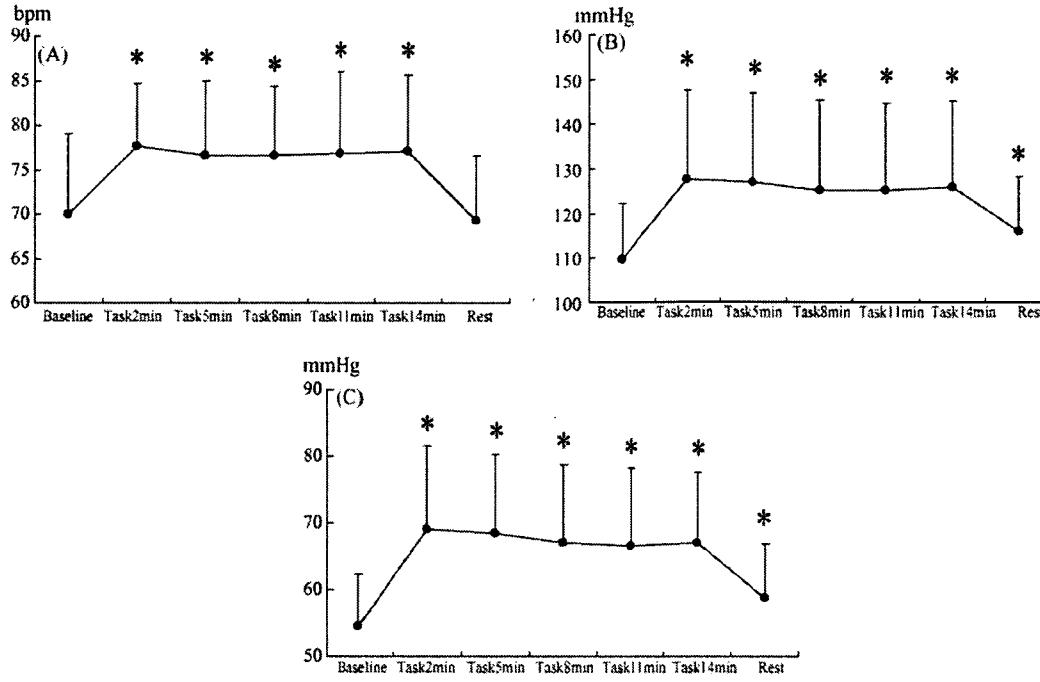

Fig. 2. Changes of HR (A), SBP (B) and DBP (C) at seven measurement points. Error bars indicate standard deviations. Symbol (*) denotes a significant ($p < .01$) difference of each index in comparison to the baseline.

Table 5
Correlations between changes in immune and cardiovascular measures

	HR	SBP	DBP	HF	LF/HF ratio
WBC	.588**				
Lymphocyte					
Monocyte					
Granulocyte	.728**				
CD3+		−.865**	−.739**		
CD4+		−.622*			−.535*
NK cell		.861**	.732**		
s-IgA					
Cortisol					

* $p < .05$.
** $p < .01$.

cardiovascular activity correlated negatively with change in the percentage of CD3+ T cells and CD4+ T cells but positively with change in the percentage of NK cells. And LF/HF ratio in HRV data showed significant negative correlation only with CD4+ T cells. Interestingly, NK cells and CD3+ T cells correlated with the cardiovascular measurements immediately after the task was started. On the other hand, the significant correlations in CD4+ T cells were observed at 8 min after the beginning of the task (data not shown). Additionally, significant positive correlation between heart rate and granulocytes or WBCs was also indicated.

4. Discussion

The present study replicated and confirmed previous findings of an increased proportion of NK cells and a reduction of that of CD3+CD4+ helper T cells, suggesting enhancement of innate immunity and suppression of specific immunity in an acute stress challenge (Delahanty et al., 1996; Pike et al., 1997; Burleson et al., 1998; Willemsen et al., 2002; Isowa et al., 2004). The main contribution of the present study is the finding, for the first time, that such typical immune responses to acute stress can happen within 2 min from initiation of the task, especially concerning NK cells. This finding suggests that variations of immune functions can happen in a very early stage of acute stress situations.

Another important point in the present study is our finding of a time lag in reactivity to acute stress of NK cells, CD3+ T cells and CD4+ T cells. We observed a significant increase of NK cells immediately after initiation of the task (2 min), whereas reductions of CD3+ T cells and CD3+CD4+ helper T cells reached significant levels 5 or 8 min after initiation of the task. In previous studies (Peters et al., 1999; Pehlivanoglu et al., 2001), changes of immune functions have been evaluated by indices of proportions of subsets of lymphocytes. Because such proportion measures are substantially relative, it is not clear whether increases of NK cells and decreases of T cells (especially helper T cells) are independent processes. By examining temporal variations of both immune measures, we were able in the present study to offer evidence that enhancement of innate immunity and suppression of specific immunity in acute stress should be dissociable processes. Those present findings are consistent with a previous finding that absolute numbers, not relative proportions, of NK cells and T cells were independently influenced by acute stress (Schmid-Ott et al., 2001; Adler et al., 2002; Mills et al., 2003; Steptoe et al., 2004).

Observed highly significant correlations between cardiovascular and immune indices and lack of a significant change of salivary cortisol suggest that sympathetic activity rather than the HPA activity mediates the temporal variations in immune functions during acute stress. The increments of heart rate and systolic and diastolic blood pressure observed in the present study are part of a typical pattern of cardiovascular activities. This type of response has been observed in previous studies using acute stress tasks including mental arithmetic tasks (Willemsen et al., 1998, 2002; Ring et al., 2000; Isowa et al., 2004), public speaking tasks and Stroop tasks (Owen and Steptoe, 2003; Redwine et al., 2003). The common pattern of this response in these studies is characterized by dominant sympathetic nervous system, especially beta-adrenergic activity compared to alpha-adrenergic activity (Ring et al., 2000; Isowa et al., 2004). Our HRV data might confirm evidently that the task used in this study has elicited the pre-dominance state of sympathetic nervous system. During this kind of stress response, adrenaline or noradrenalin is secreted from sympathetic glands and activates NK cells located on the vascular endothelium via beta-adrenergic receptors on their surface, resulting in an increase in the number of NK cells in peripheral blood (Benschop et al., 1994, 1996). Elevated blood pressure and increased bloodstream elicited by sympathetic activity might drop NK cells into the peripheral blood physically (Benschop et al., 1993). Since the increment of blood pressure influences immune cells directly from this kind of mechanism, it might be considered that the correlations with immunological measures were more strongly observed in blood pressure than in HRV. Moreover, the observed result showed that the increment of granulocytes and significant correlation with heart rate was also related to beta-adrenergic activity. Granulocytes, especially neutrophils, have a number of receptor types expressed on their surface, in particular beta-adrenergic ones (Dhabhar et al., 1996). The present result is consistent with a previous finding that activation of beta-adrenergic receptors induces a functional rise of neutrophils (Ellard et al., 2001). Thus, the observed correlation between WBC and heart rate could be also interpreted by the increment of neutrophils, which is a type of granulocyte via beta-adrenergic receptor activation. But this speculation needs more empirical research.

The dissociation of temporal activity between NK cells and T cells reported above can also be interpreted as resulting from a difference of their sensitivity to noradrenalin and adrenalin. Both NK cells and CD3+ T cells and CD3+CD4+ helper T cells express beta2-adrenergic receptors on their surface and therefore can be influenced by noradrenalin and adrenalin. However, NK cells express more beta2-adrenergic receptors than do CD3+CD4+ helper T cells (Landmann et al., 1984; Van Tits et al., 1990; Wahle et al., 2001). Since there are relatively few distributions of beta-adrenergic receptors in T cells, their sensitivity to adrenaline and noradrenaline might be lower than that of NK cells. Therefore, the influence of these substances could not take place easily in T cells (Schedlowski et al., 1996; Kohm and Sanders, 2001). That might be a reason why the reduction of proportions of CD3+ T cells and CD4+ helper T cells appeared in the later stage of the acute stress task. However, these speculations need further examination.

Several limitations in this study must be recognized. First, the number of participants ($N = 15$) was relatively small. As indicated in the results, however, the subjects experienced robust temporal variations in immune parameters and correlations between autonomic and immune indices. A second limitation was that we examined only female subjects, whereas the previous study (Willemsen et al., 2002) reported sex differences in some immune responses to acute stress. Thus, the generalizability of the present findings must be further tested using a larger sample composed of both sexes. A third limitation was that, contrary to previous studies (Willemsen et al., 2000; Bosch et al., 2001), the concentration of salivary s-IgA did not increase significantly after the acute stress task, although we observed a tendency for the concentration to increase. This unexpected result might has been caused by the small number of participants in our study. On the other hand, although we found strong association between immune and sympathetic nervous system, this study was a correlational design. We speculated the mechanism indirectly from cardiovascular responses but did not measure catecholamine itself. Further study measuring catecholamine or using adrenergic receptor blocker is needed for more precise inspection. Moreover, blood sampling at short intervals were performed only in the task period. There is, however, not only an acute stress response but researches which suggest the importance of the recovery period (Steptoe et al., 2003; Gold et al., 2004). It is thought that performing precise evaluation in recovery period is necessary for understanding the mechanism of an immune system and other systems. Further studies to overcome these limitations are awaited.

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Biological Psychology 71 (2006) 202–213
BIOLOGICAL PSYCHOLOGY
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Immune, endocrine and cardiovascular responses to controllable and uncontrollable acute stress

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Received 23 September 2004; accepted 6 April 2005
Available online 27 June 2005

Abstract

This study, using a triadic-yoked design, clarified the effects of controllability of acute stress on responses of immune, cardiovascular (heart rate and blood pressure), and cortisol activities. Forty-three women in their follicular phase completed a mental arithmetic task as a stressor in which controllability was manipulated by correct or yoked-bogus feedback. The task decreased proportions of CD3+ T cells, CD4+ T cells, and CD19+ B cells, whereas it increased the numbers of white blood cells, lymphocytes, natural killer (NK) cells, and NK cell activity (NKCA). Our main hypothesis that greater immune and cardiovascular responses to the task would be obtained under the uncontrollable condition than under the controllable condition was not supported. However, the uncontrollable stress condition, but not the controllable situation, led to higher correlations between heart rate or blood pressure, and various immune parameters. On the other hand, parameters of heart rate variability reflecting sympathetic and parasympathetic activities showed significant correlations only with NKCA. These results suggest that immune responses were most directly associated with cardiovascular activities under the uncontrollable condition.

Keywords: Stress; Controllability; Mental arithmetic; Psychoneuroimmunology

1. Introduction

There is much evidence that experimental acute stress tasks such as mental arithmetic influence the function of peripheral immunity. Previous studies have reported that innate immunity and mucosal immunity (e.g., the number of natural killer (NK) cells, NK cell activity (NKCA), and salivary secretory immunoglobulin A (S-IgA)[1]) are enhanced, whereas specific or acquired immunity (e.g., numbers of helper T cells and B cells) either does not change or decreases during mental arithmetic (Delahanty et al., 1996; Pike et al., 1997; Burleson et al., 1998; Willemsen et al., 1998, 2002; Ring et al., 1999, 2000; Bosch et al., 2001, 2002; Isowa et al., 2004; Kimura et al., in press; for review, see Segerstrom and Miller, 2004).

In addition, numerous studies have provided evidence that the nature and magnitude of the immune, endocrine, and cardiovascular responses induced by acute stress may depend on specific situational determinants such as controllability over stressors (Laudenslager et al., 1983; Maier et al., 1986; Weisse et al., 1990; Sieber et al., 1992; Peters et al., 1998, 1999, 2003). The effects of controllability have been widely studied in animal models using a triadic-yoked design (Seligman, 1975). Some previous studies using animal such as rat found that controllability of stress task was effective to physiological responses to it (Laudenslager et al., 1983; Nakata et al., 1996).

Several researchers have tried to extend these findings to human subjects (Weisse et al., 1990; Sieber et al., 1992; Gomez et al., 1994; Peters et al., 1998, 1999, 2003), though the results of these studies have been inconsistent. Weisse et al. (1990) reported decreased lymphocyte proliferation in

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[1] The concentration or secretion rate of S-IgA is an index of specific immunity function in local mucosal sites. However, the roles and characteristics of S-IgA are quite unique compared to those of general specific immunity represented by the production of the other subsets of immunoglobulin. Thus, we specifically refer to mucosal immunity for the value of S-IgA.

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doi:10.1016/j.biopsycho.2005.04.002

response to concanavalin A (ConA) and PHA in subjects who had control over a stress task. Sieber et al. (1992) found decreased NKCA after uncontrollable stress but not after controllable stress. Gomez et al. (1994) found no effect of uncontrollability on various immune parameters. One possible explanation for the contradictory results may be that these experiments employed slightly different stress tasks of different durations. Furthermore, because most of the previous studies did not measure parameters of all three of the aspects of homeostasis (autonomic nervous, endocrine, and immune systems), it is difficult to examine in detail the interaction of these systems during controllable and uncontrollable acute stress. To our knowledge, only one study by Peters et al. (1999) examined the effects of controllability on the cardiovascular, endocrine, and immune systems of humans. They found that in vitro production of cytokine interleukin-6 (IL-6) was decreased after an uncontrollable stressor, and concluded that this phenomenon was induced by activation of the hypothalamus-pituitary-adrenocortex (HPA) axis. Although their finding is very valuable and important for examining the effect of controllability in acute stress, regrettably, the mediation of activity of the autonomic nervous system in the effects of controllability has not previously been reported.

Many previous experiments have demonstrated that the autonomic nervous system can react more rapidly than the HPA axis (Isowa et al., 2004; Burleson et al., 1998; Pike et al., 1997; Fokkema et al., 1988; Bohous et al., 1987). Moreover, Swenson and Vogel (1983) found that plasma NE and E levels in rats exposed to inescapable shock were higher than those in rats exposed to escapable shock, whereas the corticosterone level was the same in the two groups. Peters et al. (1998) reported that under an uncontrollable stressful condition, the level of plasma NE in humans increased more than it did under a controllable stressful condition, and that blood pressure under the uncontrollable condition was higher than that under the controllable condition. Such evidence suggests that controllability of an acute stressor will have a greater impact on autonomic parameters than on parameters of the HPA axis. In turn, it can be predicted that the autonomic activity induced by the controllability will have a rapid effect on peripheral immune functions. However, no study has directly examined such processes in an experimentally manipulated uncontrollable stressful situation.

Therefore, using a triadic-yoked design, we examined the effects of controllability on the responses of the human autonomic, endocrine, and immune systems to acute stress. For this purpose, we estimated the autonomic (sympathetic versus parasympathetic) activity through heart rate variability (HRV) and examined the effects of cardiovascular activity itself (heart rate (HR) and blood pressure (BP)) on immune parameters. On the basis of the previous studies described above, we predicted that the acute stress task would elicit prompt activation of the autonomic nervous system, and that this system, in turn, would mediate the enhancement of innate immunity (NKCA and proportions of NK cells in blood) and the suppression of specific immunity (proportions of T cells and helper T cells in blood). Specifically, we anticipated that the magnitude of the responses of the autonomic and immune parameters under the uncontrollable condition would be larger than that under the controllable condition.

2. Methods

2.1. Subjects

Forty-three female undergraduates in the Mie Prefectural College of Nursing (age range, 19–34 years; mean = 21.51, S.D. = 2.66) participated in the present study. Each participant was randomly assigned to one of three groups: a controllable stressors (C), an uncontrollable stressors (UC), or a no stressors (control) group. The C and UC groups were assigned 18 subjects, and the control group 7 subjects.

The mean BMI of the subjects was 21.03 kg/m^2 (S.D. = 2.22). None of the subjects were suffering from any chronic or oral illness, and none were taking medication known to influence immunity. In addition, no subjects were using oral contraceptives. Considering the effects of the menstrual cycle on the immune system, all subjects were required to measure their basal body temperature (BBT) daily for more than 3 months before the experimental sessions. They participated in the experiments during the late luteal and early follicular phases. In these periods, secretion of female sex hormones (progesterone and estrogen) is at low levels; thus, the influence of these hormones on the autonomic nervous and immune systems was minimized. For confirmation of the periods of the menstrual cycle, participants reported about both their current menstrual cycles and their BBT, and serum levels of estradiol (E_2) and progesterone were measured in all subjects on the days of the experiment. The mean value of estradiol was 50.87 pg/ml (S.D. = 55.31), and the mean value of progesterone was 0.96 ng/ml (S.D. = 1.78). In most cases, the hormone levels matched the levels expected based on the participant's self report. All subjects provided written informed consent. The Ethics Committee of the Mie Prefectural College of Nursing approved the present study.

2.2. Immunological measures

Blood samples for immunological determinations were collected in heparinized tubes. The numbers of total white blood cells (WBC), lymphocytes, monocytes, and granulocytes per sample were determined by standard means. Percentages of lymphocyte subsets were determined by flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA). A whole-blood lysis method was used to stain the cell with the following pairs of Fluorescein isothiocyanate (FITC)/Phycoerythrin (PE) conjugated, isotype-matched

monoclonal antibodies (DAKO Inc., Carpinteria, CA): mouse IgG1, CD3+ T cells, CD3+/CD4+ helper T cells, CD3+/CD8+ cytotoxic T cells, CD3+/CD19+ B cells, and CD3+/CD16+/CD56+ NK cells.

A chromium release assay was used to determine natural killer cell activity (NKCA). Effector and ^{51}Cr-labeled K562 target cells were incubated for 3.5 h in 96-well round-bottomed plates. Wells contained effector and target cells at ratios of 20:1. Wells with K562 in medium alone or with 1N HCL were used to assess spontaneous and maximum release. Radioactivity was counted in a γ-counter and the percentage-specific lysis was determined according to the formula: (mean experimental cpm − mean spont. release cmp)/(mean maximal cmp − mean spont. release cmp) × 100.

To determine the volume of secreted saliva and the concentration of S-IgA, samples of unstimulated saliva were collected using cotton swabs (Salivettes; Sarstedt, Ltd., Leicester, UK). A cotton swab was placed underneath the tongue of each participant for 5 min. Subsequently, the cotton swab was removed and saliva was extracted from the cotton by centrifugation at 3.5×10^3 rpm for 10 min. Saliva was stored frozen in capped test tubes at −20 °C until assay. The S-IgA concentration (in salivain micrograms per milliliter) was determined by enzyme-linked immunoabsorbent assay using an IgA test (MBL Inc., Nagoya, Japan). The thawed saliva aliquots (10 μl) were diluted 40 times. Saliva samples were reacted with polystyrene beads that labeled the antihuman secretory component. After incubation at 37 °C for 1 h, the beads were washed twice and reacted with peroxidase standard antihuman IgA (rabbit IgG/Fab′) (second reaction). After incubation at room temperature for 1 h, the beads were washed three times, and then enzyme metrical fluid (orthophenylenediamine + 4 mM H_2O_2) (third reaction) was added. After incubation at room temperature for 30 min, the reaction was stopped by the addition of H_2SO_4. The reaction product was quantified spectrophotometrically at 492 nm with a microplate reader (model 550; Bio-Rad Inc., Hercules, CA). The S-IgA secretion rate (in micrograms per min) was calculated as the product of S-IgA concentration and saliva flow rate.

In this study, we measured the proportions of lymphocyte subsets and S-IgA at five points (baseline, just after the task, and 15 min, 30 min, and 1 h after the task) and NKCA at three points (baseline, just after the task, and 1 h after the task). Sampling points for NKCA were fewer mainly due to the limits of the Mie Prefectural College of Nursing on the total allowable quantity of blood drawn: 5 ml blood is necessary for the assay of NKCA, but only 1 ml blood is necessary to measure other immune indices. In addition, the sampling timing for the NKCA assay was determined based on a previous study in which NKCA was increased immediately after a 15-min stress task and decreased 15 min after termination of the task (Peters et al., 1999).

2.3. Cortisol measures

Samples of saliva for measurement of the concentration of salivary cortisol were obtained by the same method as that used for S-IgA and stored at −20 °C until assay. The concentration of cortisol in the saliva (in micrograms per milliliter) was determined by an enzyme-linked immunoabsorbent assay. Ninety-six-well Costar™ microplates precoated with anti-Cortisol rabbit antibody were prepared. A saliva sample of 50 μl was added to each well in triplicate. Then an enzyme conjugate of 50 μl was added to each well and incubated at room temperature for 1 h. After incubation, each well was washed three times. A substrate of 50 μl was added to each well and incubated at room temperature for 30 min. Subsequently, 1N HCL 50 μl was added to each well to stop the enzyme reaction. To measure the concentration of cortisol, the plate was read at 450 nm with a microplate reader (model 550; Bio-Rad Inc.).

2.4. Cardiovascular measures

Cardiodynamic activity was recorded by electrocardiography (ECG) and non-invasive finger blood pressure (FINAP) measurements. To determine HR, ECG was recorded using an MP 100 system (BIOPAC Systems Inc., Santa Barbara, CA). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded through the finger cuff of a Portapres Model 2 (TNO Biomedical Instrumentation Inc., Amsterdam, The Netherlands) attached to the third finger of the non-dominant arm of each subject. Each indicator was recorded continuously during the task and the rest periods. The ECG data were subsequently analyzed to yield HRV. The data were first subject to visual inspection, and only completely artifact-free data were used for estimation of the R–R intervals. The R–R interval data were resampled at 4 Hz to obtain equidistant time series values. A power spectrum density was then obtained through a fast Fourier transformation of the tachogram. In connection with the fast Fourier transformation, the data were detrended linearly and filtered through a rectangular window. We studied the integral of the power spectrum in two major frequency bands, a high frequency band (HF, 0.15–0.5 Hz) and a low frequency band (LF, 0.05–0.15 Hz). The former is correlated with respiratory sinus arrhythmia and is exclusively attributable to parasympathetic influence, and the latter mirrors the baroreceptor feedback loop that controls blood pressure and appears to reflect both sympathetic and parasympathetic activity (Sayers, 1973). In the present study, we examined the HF component expressed as a percentage of the total power in the spectrum (Perini et al., 2000) and the relative contributions of LF and HF power (LF/HF), which reflects sympathovagal balance. Two subjects in the C group, two subjects in the UC group, all subject in the control group were excluded from analyses of HRV due to technical problems. Thus the HRV analyses were performed for 16

subjects in the C group and 16 subjects in the UC group. Analyses of ECGs and FINAP waveforms were performed using the software package AcqKnowledge for the MP 100.

2.5. Psychological measures

The subjects were asked to evaluate subjectively the intensity of their stress, physical fatigue, and mental fatigue on visual–analog scales (0–100%). Additionally, they were asked to rate their sense of control over the task on a scale from 0% (not controllable at all) to 100% (perfectly controllable). In addition, they completed a Japanese version (Kazuhito et al., 1990) of the Profile of Mood States (POMS) (Usala and Hertzog, 1989) and a Japanese version (Katsuharu and Tadanobu, 1982) of the State-Trait Anxiety Inventory (STAI) (Speilberger et al., 1970). POMS is composed of six sub-scales (Tension-Anxiety (T-A), Depression-Dejection (D), Anger-Hostility (A-H), Vigor (V), Fatigue (F), and Confusion (C); 65 items). STAI is composed of two sub-scales to measure state and trait anxiety and consists of 40 items.

2.6. Stress task and manipulation of controllability

The subjects in the C and the UC groups performed mental arithmetic for 15 min. The subjects were told to add the currently displayed number (from 2 to 9) to the next one shown on the PC monitor, and to indicate the last digit of the resulting number by pressing a key (from 0 to 9). Each number was displayed for 500 ms and followed by a 1500-ms interval. The task included 34 sets (one set consists of 10 answers). During the task, bursts of aversive noise (approximately 100 dB) were delivered continuously when the error rate exceeded 20% in a set, and the noise was stopped when the rate of correct answers exceeded 80% in the next set. Subjects in the C and UC groups were told that they had to calculate a correct answer 90% of the time, and that if their accuracy rate was lower than this, none of their data would be used for the analysis.

As a manipulation of controllability, the noise was administered to the subjects in the C group. On the other hand, in the UC group the noise yoked to the C group was administered irrespective of the subjects' performance. Thus, subjects in the UC group could not stop the noise by achieving a high rate of correct answers. The subjects in the control group did not perform the mental arithmetic task and thus received no aversive noise.

2.7. Procedure

The subjects were instructed to eat a light breakfast on the morning of the experiment, and caffeine-containing beverages were not allowed. Also, the subjects were told to paste a monoanesthetic seal (PENLES; Wyeth Lederle Inc., Tokyo, Japan) at the location of the cannula insertion in their arms about 1 h before the experimental sessions to reduce pain. Subjects suffering from an infectious illness within 2 weeks of the experiment were rescheduled.

The experimental sessions were composed of a mental arithmetic task and four rest periods. The subjects were tested individually between 9:00 a.m. and 12:00 p.m. in a temperature- and humidity-controlled room. After a subject entered the experiment room, a cannula was inserted into the forearm vein of her non-dominant arm. Next, electrodes for electrocardiographic measurements and a finger cuff for blood pressure recording were attached. For the next 15 min, the subject filled out psychological questionnaires. After a rest period of 15 min, the first blood sample (for assays of female sex hormones and immunological parameters) and the first saliva sample (for assays of S-IgA and cortisol) were taken. Next, instructions were given for the mental arithmetic task, and the subjects were allowed to practice the mental arithmetic task for 2 min. The subjects then performed the mental arithmetic task for 15 min. Immediately after the task, the second blood and saliva samples were taken, and each subject filled out the questionnaire again. After each rest period (15, 30, and 60 min), the third, fourth, and fifth blood and saliva samples were taken, respectively, and the questionnaires were filled out. Autonomic indices (ECG and BP) were measured continuously throughout the experimental session. After the end of the procedure, the electrodes, blood-pressure cuff, and cannula were removed, and the subjects were fully debriefed and thanked. Each subject was paid 2400 Japanese yen for participating in the study.

2.8. Statistical analysis

Prior to statistical analysis, the mean values of HR, SBP, and DBP were calculated for the last 5 min of the pre-experimental baseline period, for the periods during the stress task (0–5 min, 5–10 min, and 10–15 min), and for the last 5 min of each rest period after the task. Means of the cardiovascular parameters were determined every 5 min to examine their temporal variations during the stress task (e.g., habituation to the task). The cardiovascular data were analyzed using repeated-measures analyses of variance (ANOVAs): Group (C, UC, and control group) × Period (baseline, $\text{stress}_{5\ \text{min}}$, $\text{stress}_{10\ \text{min}}$, $\text{stress}_{15\ \text{min}}$, $\text{rest}_{15\ \text{min}}$, $\text{rest}_{30\ \text{min}}$, $\text{rest}_{60\ \text{min}}$). The immune, endocrine, and psychological measures and the HF components of HRV and the LF/HF ratio were analyzed using repeated-measures ANOVAs: Group × Period (baseline, stress, $\text{rest}_{15\ \text{min}}$, $\text{rest}_{30\ \text{min}}$, $\text{rest}_{60\ \text{min}}$). The components of HRV were calculated during 15-min epochs of each baseline, stress and rest period. The Greenhouse–Geisser epsilon correction factor, ε (Jennings and Wood, 1976), was used where appropriate. Corrected degrees of freedom are reported; the P-values reflect the epsilon correction. In cases where significant interactions were found in the ANOVAs, post hoc analyses using LSD tests ($P < 0.05$) were conducted to examine which combinations of data points differed significantly. For perception of subjective controllability,

a Student's t-test was used on only the C and UC groups, both of which had performed the mental arithmetic task. For each group, Pearson correlation coefficients were computed among change scores (scores at the stress period − scores at the baseline) of these indices to examine the relationship between immune, cardiovascular, and endocrine reactivity. Additionally, analyses comparing the strength of correlation coefficients between the C and UC groups were carried out using z-scores of the normal distribution for all correlation coefficients that showed significant correlations in the C group, the UC group, or both.

3. Results

3.1. Immunological measures

The immune data at the baseline, stress, and rest periods are summarized in Table 1. Fig. 1 illustrates changes in the percentages of CD3+ T cells, NK cells, B cells, and NKCA. The main effects of Period were significant for WBCs, lymphocytes, NK cells, NKCA, CD3+ T cells, CD19+ B cells, CD4+ T cells, and CD8+ T cells (Fs(1–2,34–113) = 9.47–50.11, Ps < 0.001).

A previous study has shown that salivary flow is often correlated with a change in the concentration of S-IgA (Stone et al., 1987). Because the present data also yielded a significant negative correlation between S-IgA concentration and salivation ($r(42) = -0.523$, $P < 0.01$), a finding in accord with previous research (Herbert and Choen, 1993), the secretion rate of S-IgA was calculated by multiplying the saliva S-IgA concentration (μg/ml) and saliva volume (ml/min) for statistical analysis. In parameters of mucosal immunity, the main effects of Period were significant for S-IgA concentration and S-IgA secretion rate ($F(34,109) = 2.78$, $P < 0.05$, $F(3,111) = 3.59$, $P < 0.05$). In addition, the main effects of Group were significant for S-IgA concentration ($F(2,36) = 5.65$, $P < 0.01$).

Table 1
Means (S.D.s) of immunological measures and results of ANOVAs

	Group	Baseline	Stress	Rest 15 min	Rest 30 min	Rest 60 min	N	Effect[a]
WBC ($\times 10^3/\mu l$)	C	5.01 (1.46)	5.29 (1.45)	5.19 (1.42)	5.37 (1.44)	5.38 (1.47)	17	
	UC	4.45 (1.10)	4.73 (1.06)	4.62 (1.03)	4.71 (1.03)	4.78 (1.04)	18	Period**
	Control	3.85 (0.45)	3.95 (0.48)	4.08 (0.46)	4.13 (0.50)	4.17 (0.46)	6	
Lymphocyte ($\times 10^3/\mu l$)	C	1.59 (0.45)	1.76 (0.51)	1.61 (0.46)	1.72 (0.50)	1.77 (0.55)	17	
	UC	1.44 (0.23)	1.66 (0.33)	1.57 (0.25)	1.59 (0.28)	1.69 (0.33)	18	Period**
	Control	1.15 (0.36)	1.23 (0.12)	1.33 (0.15)	1.38 (0.12)	1.69 (0.42)	6	
Monocyte ($\times 10^3/\mu l$)	C	0.46 (0.21)	0.51 (0.20)	0.47 (0.19)	0.45 (0.18)	0.47 (0.16)	17	
	UC	0.44 (0.18)	0.43 (0.19)	0.40 (0.15)	0.46 (0.19)	0.41 (0.16)	18	n.s.
	Control	0.43 (0.12)	0.43 (0.15)	0.42 (0.13)	0.40 (0.06)	0.30 (0.10)	6	
Granulocyte ($\times 10^3/\mu l$)	C	2.95 (1.08)	3.02 (1.01)	3.11 (1.11)	3.2 (1.16)	3.14 (1.11)	17	n.s.
	UC	2.56 (1.02)	2.64 (1.03)	2.65 (0.98)	2.66 (0.91)	2.67 (0.86)	18	
	Control	2.27 (0.42)	2.28 (0.41)	2.33 (0.48)	2.3 (0.49)	2.3 (0.43)	6	
CD3+ CD4+ (%)	C	49.68 (7.53)	42.47 (10.48)	47.09 (7.55)	45.90 (7.21)	44.85 (6.32)	17	
	UC	48.29 (6.68)	39.57 (7.78)	44.45 (7.03)	44.58 (8.07)	43.81 (6.98)	18	Period** Group ×
	Control	46.73 (3.45)	45.45 (4.28)	44.62 (3.33)	43.86 (3.68)	42.64 (4.34)	6	Period*
CD3+ CD4+ (%)	C	28.66 (6.85)	27.25 (6.16)	28.52 (6.21)	28.11 (5.84)	27.87 (5.72)	17	
	UC	30.41 (5.74)	29.09 (5.53)	30.20 (5.58)	29.59 (5.26)	29.40 (5.33)	18	Period*
	Control	28.69 (2.94)	27.38 (3.42)	27.92 (3.50)	27.58 (3.69)	27.40 (3.44)	6	
Saliva flow rate (ml/min)	C	0.24 (0.16)	0.29 (0.10)	0.27 (0.14)	0.22 (0.13)	0.21 (0.11)	17	
	UC	0.28 (0.14)	0.30 (0.10)	0.28 (0.12)	0.23 (0.12)	0.24 (0.14)	18	n.s.
	Control	0.20 (0.14)	0.17 (0.16)	0.13 (0.14)	0.15 (0.15)	0.22 (0.12)	7	
sIgA secretion rate (μg/min)	C	12.98 (7.41)	15.23 (4.45)	13.86 (6.55)	10.97 (5.97)	11.03 (5.57)	17	
	UC	15.92 (5.73)	16.03 (2.84)	14.96 (5.04)	13.11 (5.09)	14.62 (8.50)	16	Period*
	Control	11.96 (9.16)	11.17 (12.11)	7.81 (8.05)	8.25 (7.90)	13.24 (7.60)	7	
sIgA concentration (μg/ml)	C	56.54 (8.15)	54.59 (7.40)	52.18 (5.37)	52.46 (5.03)	54.39 (6.09)	17	
	UC	54.20 (8.19)	51.97 (4.83)	51.08 (3.87)	53.28 (4.93)	56.03 (8.02)	16	Group**, Period*
	Control	56.58 (9.14)	67.04 (16.64)	61.83 (11.25)	60.88 (11.12)	62.23 (9.93)	7	
Cortisol (ng/ml)	C	2.83 (0.67)	2.79 (0.59)	2.60 (0.76)	2.53 (0.76)	2.95 (0.58)	17	
	UC	2.79 (0.56)	2.61 (0.62)	2.52 (0.72)	2.68 (0.59)	2.68 (0.57)	17	Period*
	Control	2.89 (0.39)	2.73 (0.66)	2.52 (0.79)	2.48 (0.92)	2.77 (0.96)	5	

C: controllable group; UC: uncontrollable group; Control: control group.
[a] Main effects and interactions as results of ANOVAs.
* $P < 0.05$.
** $P < 0.01$.

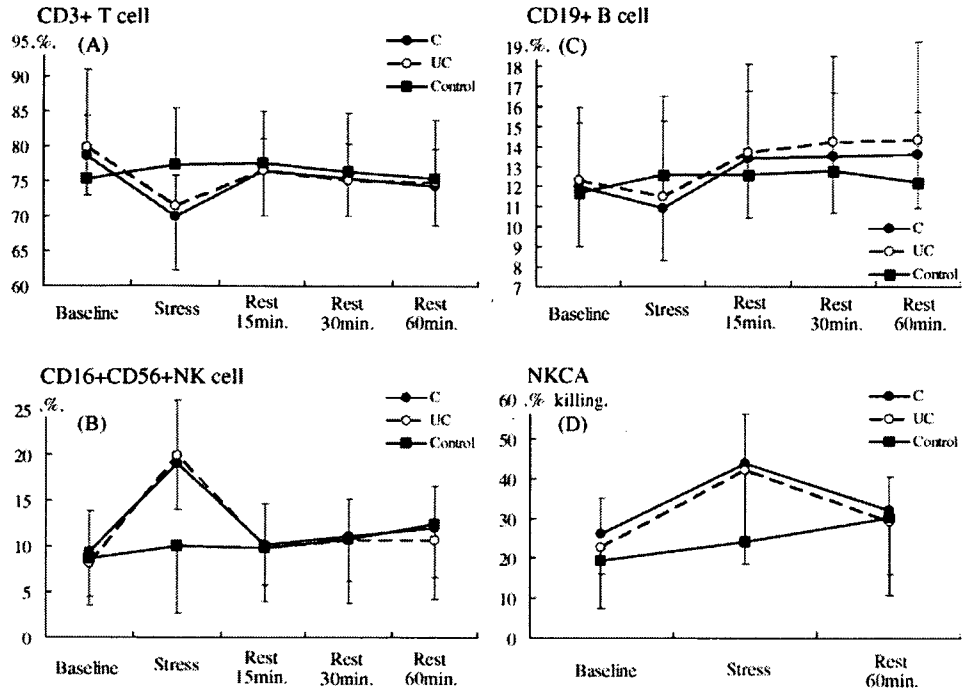

Fig. 1. Percentage of CD3+ (A); NK cells (B); CD19+ (C); and NKCA (D) at the five measurement points. Vertical bars indicate standard deviations. C: controllable group; UC: uncontrollable group; Control: control group.

In the C and the UC groups, indices of innate immunity (percentages of NK cells and NKCA) were significantly higher, and indices of specific immunity (percentages of CD3+, 19+, CD4+ and CD8 cells) were significantly lower, after the stress task than at the respective baseline (see Fig. 1). The C and UC groups differed significantly from the control group in all immune measures during the task periods ($Fs(4–8,34–156) = 2.28–9.56$, $Ps < 0.001–0.05$) but not during the rest periods ($Ps > 0.01$). Notably, there were no significant differences between the C and the UC groups in any indices.

3.2. Cardiovascular measures

Changes in cardiovascular indices are illustrated in Fig. 2. ANOVAs yielded significant main effects of Group for HR and DBP ($F(2,39) = 4.09$, $P < 0.05$, $F(2,39) = 4.90$, $P < 0.05$). Additionally, there were significant main effects of Period for HR, SBP, and DBP ($Fs(1–2,64–83) = 25.62–38.85$, $P < 0.001$). In the C and UC groups, all cardiovascular parameters were significantly higher during the stress task than at the respective baseline. These parameters remained at high levels and did not change during the task, suggesting that habituation did not take place during the 15-min task. The C and UC groups differed significantly from the control group in all cardiovascular measures during the task periods ($Fs(12,234) = 4.33–20.93$, $P < 0.001$) but not during the rest periods ($Ps > 0.01$). On the other hand, the main effects of Period were shown in HRV parameters: the HF component of HRV was significantly reduced during the task period compared with the baseline ($F(1,30) = 13.26$, $P < 0.01$), and the LF/HF ratio showed a significant increase in the task period ($F(1,30) = 6.07$, $P < 0.01$). The decrease in the HF component and the increase in the LF/HF ratio suggest that the mental arithmetic task induced a dominant state of sympathetic activity. For the cardiovascular measures, there were no significant differences between the C and the UC groups in any indices.

3.3. Cortisol measures

The cortisol levels at the baseline, after the stress task, and during the rest periods are presented in Table 1. In all groups, the cortisol level continued to significantly decrease to the $rest_{30\ min}$ compared with the baseline, and significantly increased after the $rest_{60\ min}$ compared with the baseline. There were no significant main effects of Group and no significant interactions between Group and Period. Notably, there were no significant differences between the C and the UC groups.

3.4. Psychological measures

The psychological data at baseline, after the stress task, and during the rest periods are presented in Table 2. Concerning the perceived controllability of the groups, the mean value of this parameter was 49.22 (S.D. = 17.20) in the C group, and 38.56 (S.D. = 16.64) in the UC group. That of the C group was marginally higher than that of the UC group ($t(34) = 1.77$, $P = 0.08$). ANOVAs yielded significant main effects of Group for perception of stress, state anxiety, and T-A, D, A-H, C of POMS ($Fs(2,34–39) = 3.53–6.72$,

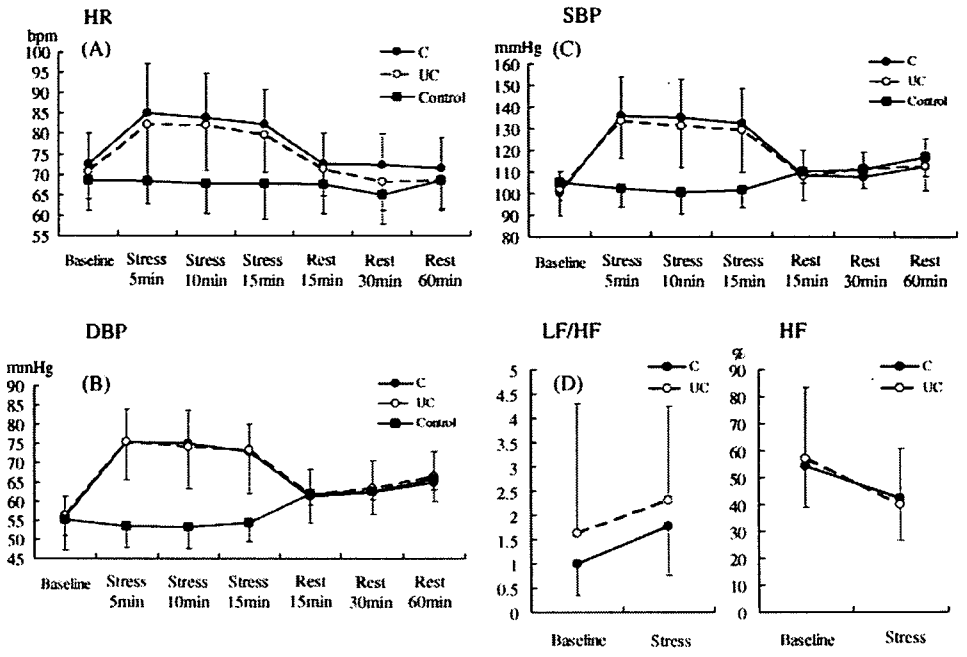

Fig. 2. HR (A); DBP (B); and SBP (C) at the seven measurement points; and HF and LF/HF (D) during baseline and task periods. Vertical bars indicate standard deviations. Vertical bars indicate standard deviations. C: controllable group; UC: uncontrollable group; Control: control group.

Ps < 0.01–0.05). In addition, there were significant main effects of Period for physical fatigue, mental fatigue, perception of stress, state anxiety, and T-A, D, A-H, V, C of POMS (*F*s(1–2,54–109) = 3.83–18.44, *Ps* < 0.001–0.05). Significant Group × Period interactions were observed for mental fatigue, perception of stress, state anxiety, and T-A, V, C of POMS (*F*s(4–8,68–156) = 2.72–4.92, *Ps* < 0.001–0.05). Post hoc analyses ($P < 0.05$) indicated that mental

Table 2
Means (S.D.s) of psychological measures and results of ANOVAs

	Group	Baseline	Stress	Rest 15min	Rest 30min	Rest 60min	*N*	Effect[a]
Perception of stress	C	26.29 (25.80)	46.59 (21.36)	35.59 (21.60)	36.29 (25.40)	38.89 (26.27)	17	Group*, Period**,
	UC	26.06 (21.53)	53.83 (27.89)	26.22 (15.44)	20.94 (14.74)	20.72 (17.83)	18	Group × Period**
	Control	10.43 (9.68)	16.86 (12.39)	18.29 (14.45)	19.43 (14.26)	18.43 (15.75)	7	
Mental fatigue	C	27.29 (23.59)	41.71 (21.67)	36.94 (20.64)	37.29 (24.89)	39.59 (24.62)	17	Period**,
	UC	33.67 (24.15)	53.33 (23.93)	33.22 (18.27)	25.83 (16.88)	25.39 (19.01)	18	Group × Period**
	Control	16.29 (14.60)	21.86 (17.49)	23.14 (18.22)	24.29 (17.54)	25.43 (17.89)	7	
STAI								
State-anxiety	C	41.82 (8.20)	48.53 (8.55)	40.76 (7.44)	40.41 (7.95)	39.35 (8.02)	17	Group**, Period**,
	UC	38.83 (5.26)	50.83 (10.06)	37.78 (4.81)	37.17 (4.63)	34.72 (5.49)	18	Group × Period**
	Control	36.57 (3.15)	33.86 (4.34)	32.71 (4.31)	33.14 (5.27)	31.14 (5.37)	7	
POMS								
Tension-anxiety	C	10.00 (6.77)	10.21 (7.01)	–	–	6.71 (5.89)	14	Group**, Period**,
	UC	13.19 (4.83)	18.13 (8.34)	–	–	7.75 (3.40)	16	Group × Period**
	Control	8.71 (5.47)	4.57 (2.57)	–	–	3.14 (2.79)	7	
Vigor	C	12.25 (6.07)	8.38 (6.04)	–	–	9.25 (6.14)	16	Period**,
	UC	13.76 (4.28)	8.35 (5.20)	–	–	12.41 (5.36)	17	Group × Period*
	Control	10.14 (4.41)	10.14 (4.41)	–	–	10.14 (3.53)	7	
Fatigue	C	8.06 (6.94)	7.56 (7.04)	–	–	8.13 (5.86)	16	Group × Period*
	UC	12.53 (7.09)	10.24 (7.00)	–	–	7.47 (4.62)	17	
	Control	4.86 (3.53)	5.29 (3.55)	–	–	5.14 (3.93)	7	

Only the parameters that interaction was found in is shown in Table 2; An effect of periods was found in the following parameters: Physical fatigue** and Depression-Dejection**, Confusion** and Anger-Hostility** of POMS; An effect of group was found in the following parameters: Depression-Dejection* and Anger-Hostility* of POMS: There was no significantly all effect for Trait-anxiety. C: controllable group; UC: uncontrollable group; Control: control group.

[a] Main effects and interactions as results of ANOVAs.

* $P < 0.05$.

** $P < 0.01$.

Table 3
Correlations between changes in immune, cardiovascular, and endocrine measures (each group)

Controllable	$HR_{5\ min}$	$HR_{10\ min}$	$HR_{15\ min}$	$SBP_{5\ min}$	$SBP_{10\ min}$	$SBP_{15\ min}$	$DBP_{5\ min}$	$DBP_{10\ min}$	$DBP_{15\ min}$	HF	Cortisol
Granulocyte											0.07
CD3+	0.01	−0.15		−0.04	−0.16	−0.14	0.00	−0.04	−0.04		
CD4+				−0.24	−0.28	−0.28	−0.14	−0.19	−0.16		
CD8+											
CD19+	−0.46	−0.43	−0.41	−0.35	−0.39	−0.42	−0.19	−0.12			
NKcell	0.19	0.33	0.32	0.19	0.33	0.32	0.08	0.10	0.15		0.20
NKCA	0.78*									0.23	
Cortisol	0.09	−0.04									
Granulocyte											−0.55*
CD3+	−0.54*	−0.51*		−0.63**	−0.66**	−0.64**	−0.55**	−0.69**	−0.68**		
CD4+				−0.58**	−0.74**	−0.70**	−0.51**	−0.70**	−0.73**		
CD8+											
CD19+	−0.46**	−0.44*	−0.42**	−0.43**	−0.45*	−0.42**	−0.32*	−0.31*			
NKcell	0.64**	0.58**	0.52**	0.65**	0.69**	0.68**	0.49*	0.62**	0.60**		0.59*
NKCA	0.33									−0.79**	
Cortisol	0.54*	0.53*									

The underline indicates significant differences between correlation coefficients in the controllable vs. uncontrollable groups. In control group, there were significant correlations between $SBP_{10\ min}$ and Monocyte ($r = 0.80^*$), CD19+ ($r = -0.77^*$), or NKCA ($r = 0.91^*$).
* $P < 0.05$.
** $P < 0.01$.

fatigue of the UC group and perception of stress and state anxiety of the C and the UC groups were higher than those of the control group after the task. In POMS, TA of the UC groups was higher than that of the C and control groups after the task.

3.5. Associations among immune, cardiovascular, and endocrine reactivity

Controllability was not shown to have any effect on cardiovascular or immune parameters by ANOVA. Thus, to further examine the effects of controllability on functional associations among the autonomic nervous, endocrine, and immune systems during the acute stress task, we performed correlation analyses among changes in cardiovascular, endocrine, and immune parameters in each experimental group separately. Furthermore, to examine the temporal characteristics of the influences of autonomic activity on the immune functions, we determined the mean changes of cardiovascular parameters in three time windows during the task: 0–5 min, 5–10 min, and 10–15 min; the correlations between these cardiovascular parameters and the immune parameters were then calculated for each time window. The results in the C and UC groups are presented in Table 3. There were no significant correlations except those between HR and NKCA in the C group. On the other hand, in the UC group, there were many strong correlations among the endocrine, cardiovascular, and immune measures (see Table 3). To prove that these correlations in the UC group were not merely artifacts, we performed a scatterplotting of the SBP and immune parameters in the UC and the C group, respectively, as shown in Fig. 3. All cardiovascular measures in the UC group correlated positively with the change in the percentage of NK cells, and negatively with the change in the percentage of CD3+ T cells and CD19+ B cells, and blood pressure correlated positively with the change in the percentage of NK cells, and negatively with the change in the percentages of CD3+ T cells, CD4+ T cells, and CD19+ B cells. Further, remarkably high correlations of the autonomic and the immune parameters were found continuously from the initiation to the end of the acute stress task. The HF component of HRV showed a remarkably significant negative correlation with NKCA ($r = -0.79$, $P < 0.01$) only in the UC group, suggesting that reduction of vagal activity led directly to upward regulation of the cytotoxicity of NK cells in the uncontrollable stress condition.

Analyses comparing the strength of the correlation coefficients between the C and UC groups showed that the correlation coefficients relating SBP or DBP at all time points and CD3+ T cells, CD4+ T cells, or NK cells in the UC group were significantly larger than those in the C group ($zs = 1.99$–2.39, $P < 0.05$).

4. Discussion

4.1. Cardiovascular and immune reactions to acute stress

During the mental arithmetic task, enhanced cardiovascular responses (HR, SBP, and DBP) were observed. Additionally, results of HRV parameters (LF/HF ratio and HF) suggested activation of the sympathetic nervous system and deactivation of the parasympathetic nervous system. Furthermore the proportion of NK cells in peripheral blood increased and the proportions of T cells, helper T cells, and B cells in blood decreased. Such lymphocyte trafficking by