# EFFECT OF PHYTONCIDE FROM TREES ON HUMAN NATURAL KILLER CELL FUNCTION

Q. LI, M. KOBAYASHI, Y. WAKAYAMA, H. INAGAKI, M. KATSUMATA, Y. HIRATA, K. HIRATA, T. SHIMIZU, T. KAWADA, B.J. PARK[1], T. OHIRA[2], T. KAGAWA[2] and Y. MIYAZAKI[1]

*Department of Hygiene and Public Health, Nippon Medical School, Tokyo; [1]Chiba University, Chiba; [2]Forestry and Forest Products Research Institute, Tsukuba, Japan*

We previously reported that the forest environment enhanced human natural killer (NK) cell activity, the number of NK cells, and intracellular anti-cancer proteins in lymphocytes, and that the increased NK activity lasted for more than 7 days after trips to forests both in male and female subjects. To explore the factors in the forest environment that activated human NK cells, in the present study we investigate the effect of essential oils from trees on human immune function in twelve healthy male subjects, age 37-60 years, who stayed at an urban hotel for 3 nights from 7.00p.m. to 8.00a.m. Aromatic volatile substances (phytoncides) were produced by vaporizing *Chamaecyparis obtusa* (hinoki cypress) stem oil with a humidifier in the hotel room during the night stay. Blood samples were taken on the last day and urine samples were analysed every day during the stay. NK activity, the percentages of NK and T cells, and granulysin, perforin, granzyme A/B-expressing lymphocytes in blood, and the concentrations of adrenaline and noradrenaline in urine were measured. Similar control measurements were made before the stay on a normal working day. The concentrations of phytoncides in the hotel room air were measured. Phytoncide exposure significantly increased NK activity and the percentages of NK, perforin, granulysin, and granzyme A/B-expressing cells, and significantly decreased the percentage of T cells, and the concentrations of adrenaline and noradrenaline in urine. Phytoncides, such as α-pinene and β-pinene, were detected in the hotel room air. These findings indicate that phytoncide exposure and decreased stress hormone levels may partially contribute to increased NK activity.

The forest environment has been enjoyed by humans for a long time because of the quiet atmosphere, beautiful scenery, mild climate, and clean fresh air. We previously reported that the forest environment enhanced human natural killer (NK) cell activity, the number of NK and NKT cells, and intracellular anti-cancer proteins in lymphocytes, and that the increased NK activity lasted for more than 7 days after the trips to forests both in male and female subjects (1-5). However, it is not clear what kind of factors in the forest environment activated human NK cells. We speculate that aromatic volatile substances derived from trees, including monoterpenes and sesquiterpenes, called phytoncides, such as α-pinene and limonene (6), may play an important role. Thus, the effects of phytoncides, such as α-pinene,

*Mailing address:* Qing Li, MD, Ph.D
Department of Hygiene and Public Health,
Nippon Medical School,
1-1-5 Sendagi, Bunkyo-ku,
Tokyo 113-8602, Japan
Tel: ++81 3 3822 2131 Fax: ++81 3 5685 3065
e-mail: qing-li@nms.ac.jp

*d*-limonene, and essential oils extracted from trees including *Cryptomeria japonica* (Japanese cedar, sugi in Japanese) and *Chamaecyparis obtusa* (hinoki cypress, hinoki in Japanese), on NK activity and intracellular levels of perforin, granzyme A (GrA), and granulysin (GRN) in NK cells were studied *in vitro*. It was found that phytoncides significantly increased the NK activity in a dose-dependent manner and significantly increased the intracellular levels of perforin, GrA, and GRN in NK cells (6). Komori et al. (7) reported that citrus fragrance affected the human endocrine and immune systems as analyzed by the measurement of urinary cortisol and dopamine levels, NK activity, and CD4/8 ratios. The above-mentioned findings strongly suggest that phytoncides have beneficial effects on human immune functions. Thus, in the present study, we investigate the effect of tree-derived phytoncide exposure on human immune function in male subjects.

## MATERIALS AND METHODS

### Subjects

Twelve healthy male subjects, aged 37-60 years (51.8±7.3) from a medical school in Tokyo were enrolled in the study. The sociodemographic information on the subjects, including age and lifestyle habits, was obtained by means of a self-administered questionnaire and has been reported previously (1-5, 8). None of the subjects had any signs or symptoms of infectious disease, used drugs that might affect immunological analysis, or were taking any medication at the time of the study. Written informed consent was obtained from all subjects after a full explanation of the study procedures. The Ethics Committee of the Nippon Medical School approved this study (approval No. 16-1).

### Hotel Experiment

The subjects stayed in an urban hotel in Tokyo, Japan for three consecutive nights. On the first day, urine samples were taken at 7.00a.m. at their homes, blood samples were taken at 8.30a.m. at a hospital, and several questionnaires, including the Profile of Mood States (POMS), were completed as a control before the stay. The subjects then worked as usual. From 7.00p.m. all subjects stayed at the same hotel in the same type of room. During the 3 nights, aromatic volatile substances (phytoncides) were produced by vaporizing *Chamaecyparis obtusa* (hinoki in Japanese) stem oil with a humidifier in the hotel room for 3 consecutive nights. All subjects went to bed

at 11.00p.m. On the second and third days, the subjects got up at 7.00a.m., took a urine sample, completed the questionnaires and ate breakfast, and then went to their workplace and worked as usual. On the last day, the subjects got up at 7.00a.m., took a urinary sample, completed the questionnaires and gave blood samples at 8.30a.m. at the hospital, finished the experiment and returned to their workplace. Daily physical activity of the subjects was monitored with a pedometer (1-3). The duration of sleep was measured with a piezo-electric accelerometer, Actiwatch (R) (Mini Mitter Co. Inc., Sunriver) (1-3). Since it has been reported that human NK cell activity shows circadian rhythms (9), all samples were obtained at 8.30a.m. All blood samples were placed in an ice/water box at 4°C and assays were performed within 2 hours of the blood being taken. White blood cell (WBC) counts, NK activity, proportions of NK and T cells, and GRN, perforin, and granzymes A/B-expressing cells in peripheral blood lymphocytes (PBLs) were measured. Adrenaline and noradrenaline concentrations in urine were also determined.

### NK activity

PBLs were separated from peripheral blood with a BD Vacutainer CPT (Becton Dickinson, Franklin Lakes, NJ, USA), and then adjusted to $4 \times 10^6$ cells/ml for the assay of NK activity. NK activity was assayed according to a standard method (1-3). Briefly, K-562 target cells were labeled with a sodium $^{51}$Cr-chromate solution (Perkin Elmer, Boston, MA, USA) for 60 min at 37°C and washed 4 times in RPMI-1640 containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA). The target cells were plated into round-bottomed 96-well microplates, then PBLs at $4 \times 10^6$, $2 \times 10^6$, and $1 \times 10^6$ cells/ml in 100 µl were added to the wells in triplicate at E:T ratios of 40:1, 20:1, and 10:1. Following a 4-h incubation at 37°C in 5% $CO_2$, the microplates were centrifuged and 100 µl of supernatant from each well was collected and measured in a gamma counter. Then, the NK activity was calculated as described previously (1-3).

### Cell staining and flow cytometric analysis

The surface markers of PBLs were stained with fluorescein isothiocynate (FITC)/phycoerythrin (PE)-CD16 and PerCP-Cy5.5-CD3 monoclonal antibodies (BD PharMingen, San Diego, CA, USA) for NK and T cells, and FITC/PE/PerCP-Cy5.5-mouse IgG1 as negative controls, for 30 min in the dark. Then, the cells were fixed/permeablized with Cytofix/cytoperm solution (BD PharMingen) for 20 min at 4°C, and then intracellular perforin and GrA/B were stained with FITC- anti-human perforin and FITC-GrA/B antibodies, respectively, with FITC-IgG2b for perforin and FITC-IgG1 for GrA/B as

negative controls (BD PharMingen) for 30 min at 4°C according to the manufacturer's instructions. Intracellular GRN was stained with a rabbit anti-human GRN polyclonal antibody and rabbit serum as the negative control (1-3, 6, 8, 10) after fixation/permeablization with Cytofix/cytoperm solution, and then stained with FITC-goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA) for 30 minutes at 4°C in the dark. After staining, the cells were washed twice with fixative solution and once with PBS containing 1% FBS. Flow cytometric analysis was performed with a FACScan flow cytometer as described previously (1-3, 6, 8, 10). Lymphocytes were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude dead cells and granulocytes. The fluorescence gates were set using negative controls.

### Urinary adrenaline and noradrenaline measurements

The levels of adrenaline and noradrenaline in urine were measured by an HPLC method using an HLC-725CAII analyzer as described previously (2-3).

### WBC count

WBC, RBC, and platelet counts, the percentages of granulocytes, lymphocytes, and macrophages in peripheral blood, and the concentration of Hb, Hct, MCV, MCH, and MHCH were determined by an automatic cell counter (LC-550, Horiba Co., LTD. Kyoto, Japan) as described previously (1-3).

### POMS test

The Profile of Mood States (POMS) test was used to examine mood changes of each subject before, during and after the hotel stay using the POMS test in Japanese (1, 3).

### Measurements of phytoncides and environmental temperature/humidity in the hotel rooms

The concentration of volatile organic compounds (phytoncides), temperature, and humidity in the hotel rooms during the investigation were measured as reported previously (1-3).
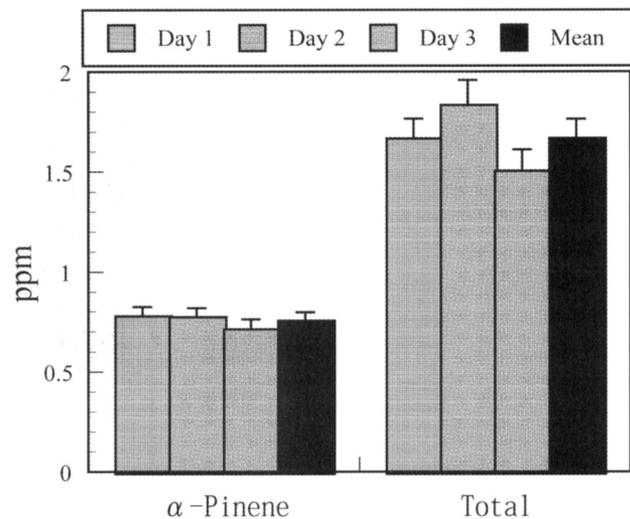
### Statistical analysis

Comparisons between different days were made with the paired t-test and performed with the Microsoft Excel software package for Windows. The significance level for p values was set at < 0.05.

## RESULTS

### Concentrations of α-pinene and the total phytoncides in the hotel room air

Phytoncides, such as α-pinene, β-pinene, β-



**Fig. 1.** *Concentrations of α-pinene and the total phytoncides in the hotel room air.*
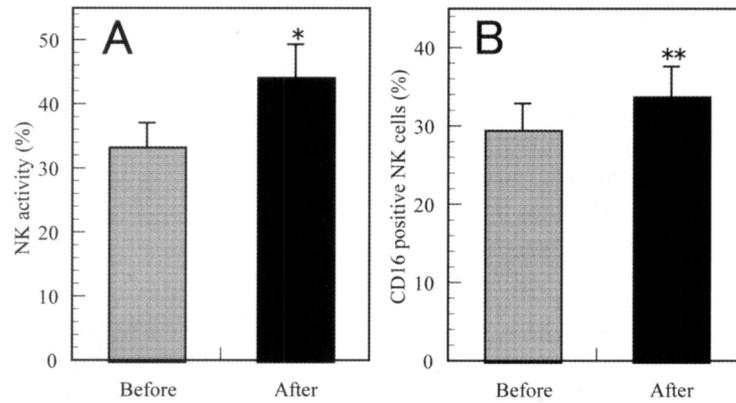*Data are presented as the mean+SE (n=12).*

cadinene, and limonene, were detected in the hotel room air during the experiment, and α-pinene was the main phytoncide (approximately 50%). There was no significant difference in the concentrations of α-pinene and the total phytoncides in the hotel room air between the different days and the different rooms (Fig. 1). The average temperature and humidity in the hotel rooms were 24.2±1.1°C and 55.9±5.5%, respectively.

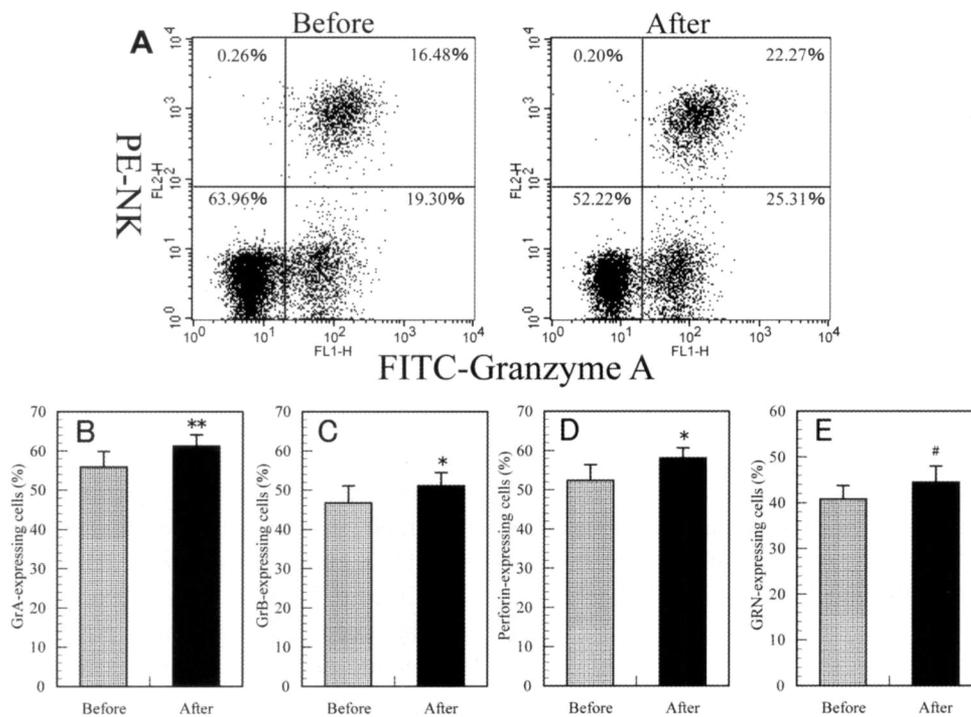### Effect of phytoncide exposure on NK activity and NK cells

Phytoncide exposure significantly increased human NK cell activity (Fig. 2A) and the percentage of CD16^{+} NK cells (Fig. 2B). There was no significant difference in the absolute number of NK cells between before and after phytoncide exposure. Phytoncide exposure did not affect lymphocyte or WBC counts.

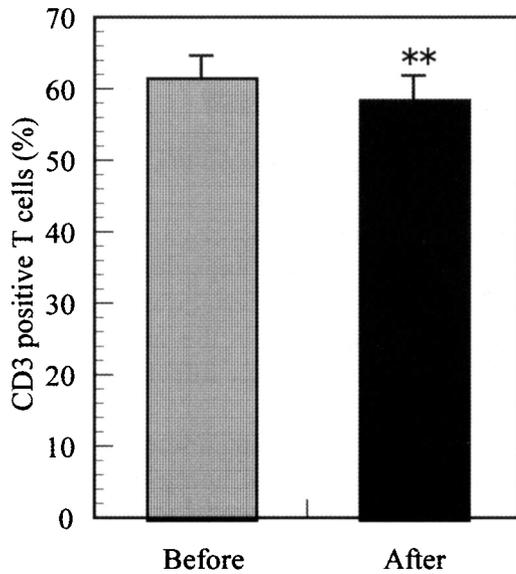### Effect of phytoncide exposure on the percentage of cells expressing cytolytic molecules

Fig. 3A shows sample diagrams of FITC-GrA/PE-CD16 in PBLs of a subject before and after phytoncide exposure, respectively. In this subject, the total of GrA^{+} cells increased from 35.78 to 47.58% after phytoncide exposure. As shown in Fig. 3B-E, phytoncide exposure significantly
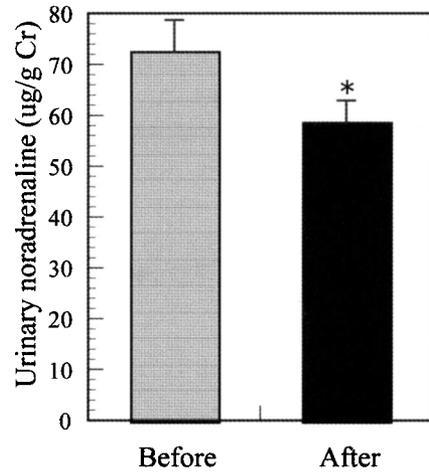
**Fig. 2.** *Effect of phytoncide exposure on human NK cell activity (A) and the percentage of CD16⁺ NK cells (B). Data are presented as the mean+SE (n=12). \*: p<0.05, \*\*: p<0.01 significantly different from before the exposure by paired t-test. The activity values for an E/T ratio of 20/1 are shown, and similar results were also obtained with E/T ratios of 40/1 and 10/1. The columns labeled "Before" indicate NK activity and CD16⁺ cells determined before phytoncide exposure; the columns labeled "After" indicate NK activity and CD16⁺ cells determined after phytoncide exposure.*
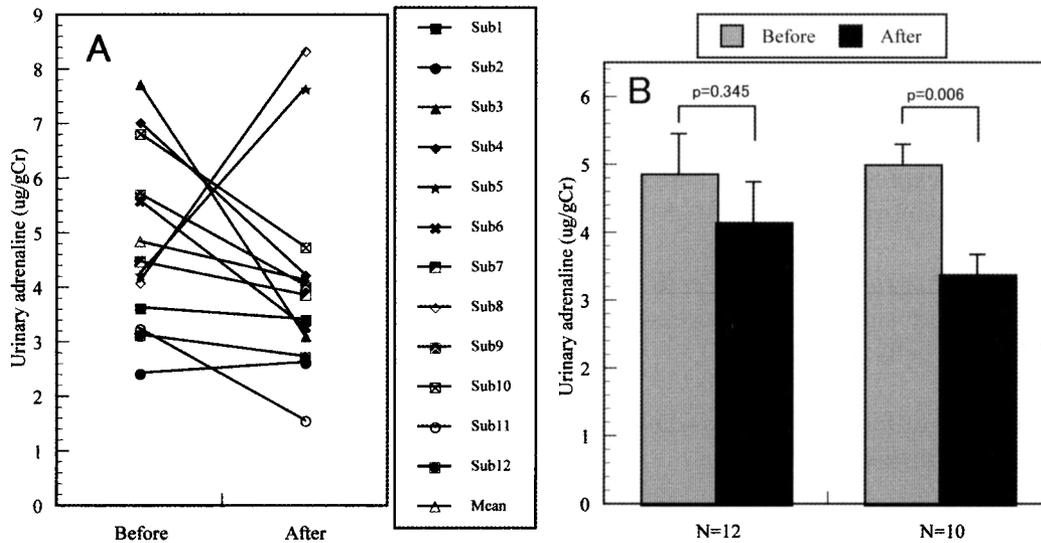


**Fig. 3.** *Effect of phytoncide exposure on GrA/B- (A-C), perforin (D)-, and GRN (E)- expressing cells in PBLs. A: Dot plots of FITC-GrA/PE-CD16-positive cells in PBLs of a subject before (left) and after (right) phytoncide exposure. The horizontal axis shows the intensity of fluorescence of FITC-GrA, while the vertical axis shows the intensity of fluorescence of PE-CD16 (NK cells). Percentages in quadrants 2 and 3 show GrA⁺/ CD16⁺ and GrA⁺/ CD16⁻ cells, respectively. Data are presented as the mean+SE (n=12). \*: p<0.05, \*\*: p<0.01, significantly different from before the exposure by paired t-test. #: p=0.081 different from before the exposure by paired t-test. The columns labeled "Before" indicate GrA/B, perforin and GRN-expressing cells determined before phytoncide exposure; the columns labeled "After" indicate GrA/B, perforin and GRN-expressing cells determined after phytoncide exposure.*
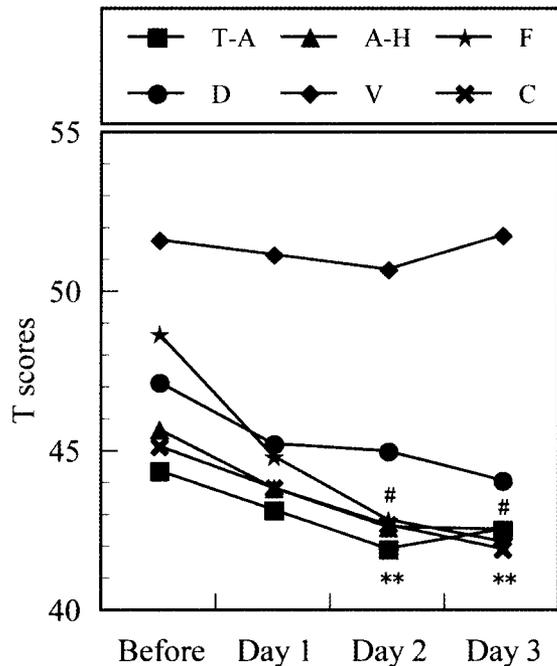
**Fig. 4**. *Effect of phytoncide exposure on the percentage of CD3⁺ T cells. Data are presented as the mean+SE (n=12). \*\*: p<0.01 significantly different from before the exposure by paired t-test. The column labeled "Before" indicates CD3⁺ cells determined before phytoncide exposure; the column labeled "After" indicates CD3⁺ cells determined after phytoncide exposure.*



**Fig. 6**. *Effect of phytoncide exposure on urinary noradrenaline. Data are presented as the mean+SE (n=12). \*: p<0.05 significantly different from before the exposure by paired t-test. The column labeled "Before" indicates urinary noradrenaline determined before phytoncide exposure; the column labeled "After" indicates urinary noradrenaline determined after phytoncide exposure.*



**Fig. 5**. *Effect of phytoncide exposure on urinary adrenaline. A: urinary adrenaline shown in 12 subjects, B: Data are presented as the mean+SE (n=12 for all subjects and n=10 for subjects who showed a decreased urinary adrenaline). Statistical significances were analyzed by paired t-test. The columns labeled "Before" indicate urinary adrenaline determined before phytoncide exposure; the columns labeled "After" indicate urinary adrenaline determined after phytoncide exposure.*

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**Fig. 7.** *Effect of phytoncide exposure on POMS scores. Data are presented as the means (n=12). \*\*: p<0.01, significantly different from before the exposure for fatigue, #: p<0.1 for confusion by paired t-test. T-A: Tension-anxiety, A-H: anger-hostility, F: fatigue, D: depression, V: vigor and C: confusion.*

increased the percentages of GrA/B (Figs. 3B, 3C)-, and perforin (Fig. 3D)-expressing cells in PBLs. Although phytoncide exposure also increased the percentages of GRN-expressing cells in PBLs (Fig. 3E), this increase was not significant (p=0.081).

*Effect of phytoncide exposure on CD3$^+$ T cells*

Phytoncide exposure significantly decreased the percentage of CD3$^+$ T cells (Fig. 4).

*Effect of phytoncide exposure on adrenaline and noradrenaline concentrations in urine*

Although phytoncide exposure decreased the concentrations of adrenaline, this decrease was not significant (p=0.345). However, when the subjects were divided into two groups [the increased group (2 subjects) and the decreased group (10 subjects)], it was found that there was a significant decrease in the decreased group (10 subjects, p=0.006) (Fig. 5b), indicating individual differences on response to

phytoncide exposure. On the other hand, phytoncide exposure significantly decreased the concentrations of noradrenaline in urine (Fig. 6).

*Effect of phytoncide exposure on the score of POMS test*

Phytoncide exposure decreased the scores for tension/anxiety, depression, anger/hostility, fatigue, and confusion in the POMS test; however, there was a significant decrease only in the score of fatigue. Phytoncide exposure did not affect the score for vigor (Fig. 7).

There were no significant differences in daily physical activity before and during the stays (data not shown). The hours of sleep increased during the hotel stays compared with control days (data not shown).

## DISCUSSION

We previously found that a forest bathing trip, but not a city visit, significantly increased human NK cell activity, the number of NK and NKT cells, and intracellular levels of anti-cancer proteins in PBLs in both male and female subjects (1-5). However, it is not clear what kind of factors in the forest environment played this important role. It has been reported that aromatic volatile substances derived from trees, called phytoncides, such as α-pinene and limonene significantly increased NK activity and the intracellular levels of anti-cancer proteins in NK cells *in vitro* (6). Thus, in the present study we investigated whether phytoncide exposure *in vivo* affects human immune function. We released phytoncides in the hotel room air (Fig. 1). We found that phytoncide exposure significantly enhances human NK cell activity and the percentage of CD16$^+$ NK cells. Komori et al. (7) reported that citrus fragrance affects the urinary cortisol and dopamine levels, NK activity, and CD4/8 ratios in humans. Santos et al (11) also found that β-carotene induced enhancement of NK activity in elderly men, suggesting that terpenes can activate NK cells. These findings suggest that phytoncides contributed to the enhanced NK activity during the stay at the hotel.

NK cells kill tumor or virus-infected cells by the release of perforin, granzymes (10, 12-14), and GRN (15-16) via the granule exocytosis pathway.

In order to explore the mechanism of enhancement of NK activity by phytoncide exposure, we investigated the effect of phytoncide exposure on the intracellular levels of perforin, GRN, and GrA/B in PBLs. We found that phytoncide exposure significantly increased the proportion of PBLs expressing these effector molecules. These cytolytic molecules contribute to NK and anti-tumor activity (16). Taken together, phytoncide exposure increased human NK activity at least mediated by increased percentages of CD16+ cells and GrA/B- and perforin-expressing lymphocytes.

The concentrations of adrenaline and noradrenaline in urine have been used to evaluate work-related stress in nurses (17), lorry drivers (18), and psychosocial stress (19). We found that phytoncide exposure significantly decreased the concentrations of adrenaline and noradrenaline in urine, suggesting that the subjects were under conditions of lower stress during the hotel stay. Haze et al (20) also found that fragrance inhalation of rose oil decrease adrenaline concentration in plasma in normal adults, supporting our findings. In addition, phytoncide exposure significantly decreased the score for fatigue and confusion in the POMS test, suggesting that the subjects were physiologically relaxed during the hotel stay. Schiffman et al. (21) also found that use of pleasant odors improved the mood of males at midlife in the POMS test. It has been reported that both adrenaline and noradrenaline inhibit human NK cell activity (22-23). We found previously that physical and/or psychological stress decreased NK activity, NK receptor levels, and mRNA transcription of granzymes and perforin in mice (24). The increase in NK activity during phytoncide exposure may be related to an attenuated stress hormone response (adrenaline, noradrenaline).

Phytoncide exposure significantly decreased T cells in male subjects. We previously found that the forest environment also significantly decreased T cells in both male and female subjects (1-3). It has been reported that mental stress increased T cells in PBLs (25-26). We have also found that people with an unhealthy lifestyle showed a higher percentage of T cells than people with a healthy lifestyle (8); therefore, we speculate that the proportion of T cells in PBLs may reflect the stress status (3).

Many factors, including circadian variation (9),

physical exercise (8), and alcohol consumption (8, 27) can affect human NK activity. In order to control the effect of circadian rhythm on NK activity, we took blood samples at 8.30a.m. on all days. To control for the effect of physical exercise on NK activity, we limited the walking steps during the experiment to the averaged normal workday distances. To control the effect of alcohol on NK activity, the subjects did not consume alcohol for 2 days before providing the blood sample during the study period.

The Swedish occupational exposure value for a mixture of monoterpenes (phytoncides) and a single monoterpene is 150 mg/m³ (27 ppm) and the Finnish occupational exposure limit is 570 mg/m³ (102 ppm) (28-29). The concentrations of α-pinene (less than 1 ppm) and total phytoncides (less than 2 ppm) in the hotel room air in the present study were far less than the occupational exposure values, suggesting that phytoncide exposure will not have any adverse effects on human health, including immune function, and that phytoncide exposure at the present concentration should be safe for human health.

We previously found that staying in an urban hotel room in the absence of phytoncide exposure for 2 nights did not affect NK activity, the numbers of NK cells and perforin-, GRN-, and GrA/B-expressing lymphocytes, or urinary adrenaline and noradrenaline (2), suggesting that the increased NK activity in the present study was not due to the stay at the hotel, but to phytoncide exposure.

Although we observed beneficial activity exerted by short-term exposure to phytoncide, it is necessary to conduct epidemiological studies or perspective studies in a population exposed to phytoncide in daily life to confirm its beneficial effect on human immune function in the future.

In summary, phytoncides from trees can increase NK activity, the percentage of NK cells, and the expression of intracellular perforin, GrA/B, and GRN in male subjects. Phytoncides in forest air may partially contribute to the increased NK activity in subjects visiting a forest (1-3).

## ACKNOWLEDGEMENTS

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