Immune changes in humans during cold exposure: effects of prior heating and exercise

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1999.—This study examined the immunological responses to
cold exposure together with the effects of pretreatment with
either passive heating or exercise (with and without a ther-
mal clamp). On four separate occasions, seven healthy men
[mean age 24.0 $\pm$ 1.9 (SE) yr, peak oxygen consumption =
45.7 $\pm$ 2.0 ml·kg^{-1}·min^{-1}] sat for 2 h in a climatic chamber
maintained at 5°C. Before exposure, subjects participated in
one of four pretreatment conditions. For the thermoneutral
control condition, subjects remained seated for 1 h in a water
bath at 35°C. In another pretreatment, subjects were pas-
sively heated in a warm (38°C) water bath for 1 h. In two
other pretreatments, subjects exercised for 1 h at 55% peak
oxygen consumption (once immersed in 18°C water and once
in 35°C water). Core temperature rose by 1°C during passive
heating and during exercise in 35°C water and remained
stable during exercise in 18°C water (thermal clamping).
Subsequent cold exposure induced a leukocytosis and granu-
locytosis, an increase in natural killer cell count and activity,
and a rise in circulating levels of interleukin-6. Pretreatment
with exercise in 18°C water augmented the leukocyte, granu-
locyte, and monocyte response. These results indicate that
acute cold exposure has immunostimulating effects and that,
with thermal clamping, pretreatment with physical exercise
can enhance this response. Increases in levels of circulating
norepinephrine may account for the changes observed during
cold exposure and their modification by changes in initial
status.

passive heating; hypothermia; immune function; leukocytes;
natural killer cells; interleukin-6

BOTH ANECDOTAL (8, 21) and experimental reports (4, 29)
have suggested that cold exposure may increase an
organism's susceptibility to infection (42). Cold-induced
decrements in immunosurveillance can be a particular
problem for winter athletes or for military personnel
who must pursue physical activities in cold environ-
ments. Upper respiratory tract infections appear to be
the main cause of illness and reasons for missed
practice in elite cross-country skiers (5). Studies of
sustained military operations in the Canadian Arctic,
also, have reported an increased incidence and severity
of upper respiratory tract infections during patrols
involving high levels of energy expenditure and expo-
sure to cold conditions both day and night (39, 45).
Because the effective accomplishment of both athletic
endeavors and military operations depends on a high
level of human performance, maintenance of health is
essential in situations where athletes or troops are
exposed to adverse environmental conditions.

It remains unclear whether human viral susceptibil-
ity during and after cold exposure is attributable to a
cold-induced change in the function of the immune cells
or to other incidental consequences of the cold environ-
ment such as drying of the mucosal surface, a slowing of
tracheal cilia (17), or a deterioration of the normal
barrier function of the skin (19). Animal studies have
demonstrated that cold exposure induces changes in
both cellular and humoral aspects of immune function
(42), including a reduction in natural killer (NK) cell
count and cytolytic activity (1, 48), a decrease in
lymphocyte proliferation (18, 43), and (after several
days of cold exposure) an enhanced production of
proinflammatory cytokines (23). However, in many of
these studies, the animals were subjected to prolonged
periods of cold exposure or were placed in environments
to which they were unaccustomed (for example, cold-
water immersion). This would have placed additional
stress on the animals studied, and the psychological
stress rather than the cold exposure may have influ-
enced their immune status. Moreover, the results ob-
tained in animal studies are difficult to apply to hu-
mans because of interspecies differences.

To date, there has been minimal research examining
how cold exposure affects immune function in humans,
and no one has examined how any changes may be
modulated by prior passive heating or moderate exer-
cise (with or without a thermal clamp, a procedure that
maintains a constant core body temperature). It is
known that passive heating and exercise each recruits
leukocytes (granulocytes, lymphocytes, and lympho-
cyte subsets) into the circulation and enhances NK cell
function (6). Immersion of healthy young men in cold
(14°C) water induces a leukocytosis (22). Furthermore,
a brief bout (30 min) of cold (4°C)-air exposure in-
creases NK cell activity (27). We thus hypothesized that
exposure to cold air (5°C) for 2 h would have immuno-
stimulating effects and that pretreatment with exercise
or passive heating would have algebraically additive
effects on this response.

METHODS

Subjects

The subjects were seven healthy, moderately fit men aged 20–34 yr [mean age 24.0 ± 1.9 (SE) yr] recruited from a pool of military personnel in accord with a protocol approved by the Human Experimentation Committee at the US Army Research Institute of Environmental Medicine. Written informed consent was obtained before participation in the study. Exclusionary criteria included acute infection and a history of allergic conditions, prior cold injury, Raynaud's syndrome, cardiovascular disease, respiratory disease, or diabetes mellitus. Subjects were asked to refrain from consumption of alcohol, smoking, taking medication, and exercising 12 h before any testing session. Subjects were of average male height (1.76 ± 0.02 m), body mass (79.4 ± 4.7 kg), percent body fat (14.6 ± 1.6%), and aerobic power (45.7 ± 2.0 ml·kg^{-1}·min^{-1}).

Experimental Design

Each subject visited the laboratory on five occasions. At entry, anthropometric measurements [height, body mass, and determination of percent body fat by using dual-energy X-ray absorbitometry (model DPX-L, Lunar, Madison, WI)] were made, and peak oxygen consumption ($\dot{V}O_{2\,peak}$) was determined by using a progressive cycle ergometer test. The cycle ergometer protocol consisted of a warm-up (70 W for 2 min) followed by a 35-W increase in exercise intensity every 2 min until volitional exhaustion.

Subjects then completed four separate trials with different pretreatments followed by a standardized cold-air exposure. The pretreatments took place in a water bath, whereby subjects (submerged to the shoulder) either remained seated or performed semirecumbent exercise, pedaling (38–45 rpm) on a modified Monark cycle ergometer (41). A weighted waist belt allowed subjects to maintain their position underwater.

In the control condition, each subject sat at rest in approximately thermoneutral conditions (a 35°C water bath) for 60 min. During passive heating, each subject sat in a warm (38°C) water bath until his rise in core temperature matched that which had occurred during exercise in 35°C water (times ranging from 38 to 70.5 min, with an average of 59.8 min). During one pretreatment involving exercise, subjects performed cycle ergometer exercise for 60 min at an average of 55% of their $\dot{V}O_{2\,peak}$ while immersed in cold water (18°C); this maintained core body temperature constant (the "thermal clamp" condition). For the pretreatment involving exercise without a thermal clamp, each subject performed cycle ergometer exercise for 60 min at an average of 55% of his personal $\dot{V}O_{2\,peak}$ while immersed in water at 35°C. Immediately after each pretreatment, the subjects were dried and dressed in dry cotton shorts and cotton socks (~0.3 clo). Within 20 min, subjects were then transferred to a cold climatic chamber and exposed to cold air (5°C, 40% relative humidity, wind speed of 0.7 m/s) for 120 min. Subjects were assigned to each pretreatment according to the following order (exercise in 35°C water, sitting in warm water, exercise in cold water, sitting in thermoneutral water). This was done to match the rise in core temperature between the exercise trial (exercise in 35°C water) and the resting trial (sitting in warm water). Each experiment took place at the same time of day; individual experiments were separated by 1-wk intervals.

Physiological Measurements

Heart rate (electrocardiogram, CM-5 configuration) and rectal temperature (rectal thermistor inserted 0.1 m past the anal sphincter) were measured continuously during both water immersion and cold exposure. To avoid subject discomfort and resultant mouthpiece leakage, oxygen uptake was measured intermittently (after 15–20 min of immersion and at regular intervals during cold exposure), by using an online metabolic analysis system (model 2900, SensorMedics, Yorba Linda, CA).

Blood Sampling

Venous blood samples were collected from an indwelling venous catheter (Deseret Medical, Sandy, UT) that had been inserted into the medial antecubital vein of the left arm 30 min before each pretreatment. Patency of the catheter was maintained between sampling by means of a 1.0-ml heparin-saline lock (100 U/ml). Blood samples of 18 ml volume were taken before (pre) and at the end of exercise (post), and 23 ml samples were taken before (0 min), during (60 min), and at the end of cold exposure (120 min). The first 1.0 ml of each blood sample was discarded. Hemoglobin and complete blood counts were checked at the beginning of each experiment. All values proved normal, but the study design provided for more detailed hematologic evaluation and a delaying of further experiments if a hematologic deficit had persisted as a result of blood sampling during a prior treatment.

At the specified times, aliquots of blood were drawn into vacutainers (Becton-Dickinson, Oakville, ON). Complete blood counts were determined on K$_3$EDTA-treated specimens of whole blood by using a Cell Dyn Hematology System (Cell Dyn 3500, Abbott Laboratories, Abbott Park, IL). Heparinized whole blood (143 USP U sodium heparin) was used for the cytolytic functional assay. A separate vacutainer containing K$_3$EDTA-treated blood provided plasma samples for cytokine and cortisol analyses. Aliquots of blood for catecholamine analyses were drawn into 4.5-ml vacutainers containing K$_3$EDTA and reduced glutathione (Amersham, Arlington Heights, IL). Catecholamine and cortisol samples were obtained only during the cold-exposure component of the experiment. The method of Dill and Costill (12) was used to make appropriate adjustments of white cell counts, cytokine, and hormonal concentrations for changes in blood and plasma volumes relative to their respective baseline values.

Immunophenotyping

Immunophenotyping was performed by using 100-μl samples of K$_3$EDTA-treated whole blood. Samples were first washed with 2 ml of PBS saline containing 0.1% sodium azide (0.1% NaN$_3$-PBS). The cell pellet was then stained with 10 μl of the selected monoclonal antibodies (MAbs; Becton-Dickinson) conjugated with FITC, phycoerythrin (PE), or peridinin chlorophyll protein in the following staining combinations: anti-CD3 MAb (FITC)/anti-CD19 MAb (PE), anti-CD4 MAb (FITC)/anti-CD8 MAb (PE), anti-CD4 MAb (FITC)/anti-CD8 MAb (PE)/anti-CD3 MAb (peridinin chlorophyll protein) and anti-CD3 MAb (FITC)/anti-CD16,56 MAb (PE) (Becton-Dickinson, Mississauga, ON). After 30 min of incubation on ice in the dark, 2 ml of 10% fluoresence-activated cell sorting (FACS) lysing solution (Becton-Dickinson) was added and the tubes were vortexed. The tubes were then kept in the dark at room temperature (23°C) for a further 10 min to lyse the red cells. Nonlysed cells were separated by centrifuging at 300 g for 5 min at 4°C. Tubes were then washed twice with 2 ml of a cold 0.1% NaN$_3$-PBS solution and were centrifuged for a further 5 min at 4°C and 300 g after each wash. The resultant pellet was resuspended in 0.3 ml of cold 0.1% NaN$_3$-PBS with 3% formaldehyde, vortexed, and later analyzed by flow cytometer.

A FACS flow cytometer (Becton-Dickinson Immunosystems, Mountainview, CA) was used to examine the MAb-stained cell suspensions. First, the flow cytometer was calibrated with a mixture of monosized FITC- and PE-conjugated and unconjugated latex particles (~6.0-μm Calibrite beads, Becton-Dickinson) by using FACScomp software (Becton-Dickinson Immunocytometry Systems). An isotype-negative control was used to optimized the setting of the fluorescence detectors for each subject. Fluorescence compensation was adjusted by using a dual-stained anti-CD4 MAb (FITC)/anti-CD8 MAb (PE) sample. Gate settings for the lymphocyte population and boundaries for fluorescence intensity were determined by nonspecific staining, by using control tubes containing whole blood and mouse immunoglobulins IgG_{2a} MAb (FITC) and IgG_1 MAb (PE). The absolute count for a given lymphocyte subset was estimated by multiplying the observed percentage for that subset by the total number of lymphocytes in the peripheral blood, the latter value being adjusted for changes in blood volume.

Determination of NK Cell Activity

A ^{51}Cr-release assay assessed the total cytolytic activity of isolated peripheral blood mononuclear cells (PBMC). PBMC were first isolated by Ficoll-Hypaque centrifugation. Ten milliliters of heparinized venous blood were diluted with an equal volume of Dulbecco's PBS (Sigma Chemical, St. Louis, MO). For each blood sample, three 15-ml centrifuge tubes were used to layer 7 ml of diluted blood carefully over 5 ml of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The suspension was then centrifuged for 30 min at 20°C and 450 g. The mononuclear layer was removed and washed twice, first with PBS and then with RPMI 1640 culture medium containing L-glutamine (GIBCO, Burlington, ON). The cell suspension was centrifuged for 10 min at 10°C and 275 g after each wash. The washed PBMC were resuspended in 1 ml of RPMI 1640, supplemented with 10% fetal calf serum (GIBCO) (10% FCS-RPMI 1640). The total PBMC count was determined by means of an electronic cell counter (Coulter Counter model ZM, Luton, Beds, UK) and was then adjusted to 2×10^6 cells/ml, by using 10% FCS as the diluent.

A human erythroleukemic cell line (K562, American Type Culture Collection, Rockville, MD), maintained in suspension in our laboratory, was used for the ^{51}Cr-release assay. One million K562 cells, maintained in 10% FCS-RPMI 1640 medium (GIBCO) were labeled by mixing with 100 μl (3.7 MBq) of sodium chromate-51 (^{51}Cr) (New England Nuclear, Boston, MA) for 60 min at 37°C and 4% CO_2. The radiolabeled cells were washed 3 times with 4 ml of cold 10% FCS-RPMI 1640 medium and were then diluted with 10 ml of medium to achieve a final concentration of 1×10^5 cells/ml. The labeled cells were kept on ice until the assay was performed.

Triplicates of 100 μl of PBMC at concentrations of 2×10^6, 1×10^6, and 0.5×10^6 cells/ml were mixed with a 100-μl suspension of radiolabeled (^{51}Cr) target cells at 1×10^5 cells/ml and were then centrifuged (1 min at 37°C and 160 g) in a 96-well round-bottom microtiter plate (Sarstedt, St. Leonard, PQ). After incubation for 4 h at 37°C, 4% CO_2, the cell mixture was centrifuged for a further 5 min at 4°C and 225 g. One hundred microliters of supernatant were then withdrawn and transferred to polystyrene, round-bottom tubes (12 × 75 mm) (Falcon, Lincoln Park, NJ). The radioactivity of the supernatant was determined by a Cobra Automated Gamma Counter (model 5002, Packard Instruments, Downers Grove, IL). Spontaneous release of ^{51}Cr was assessed by incubating 100 μl of medium with 100 μl of target cells. The maximum potential release of radioactive material was determined by incubating 100 μl of 1% Triton X-100 (a biodegradable nonionic surfactant) (Sigma Chemical) with 100 μl of target cells. The percentage of ^{51}Cr release (cytolytic activity) was calculated by using the following formula: percentage lysis = [(test − spontaneous) cpm/(maximum − spontaneous) cpm] × 100%, where cpm is counts per minute. The exponential curve-fitting method of Pross et al. (36) was used to determine lytic units from the values obtained for percent lysis at three different effector-to-target ratios (20:1, 10:1, and 5:1). The lytic unit was defined as the number of effector cells required to lyse 20% of 10,000 target cells. Results were expressed as the number of lytic units contained in 1×10^6 PBMC (lytic units/10^6 PBMC) (36). Lytic units were also calculated on a per NK cell basis, by using the following formula: single cell cytolytic activity = lytic units/[% NK cells × (10^6 PBMC − monocytes)].

Biochemical Analysis

K_3-EDTA- and EGTA/reduced glutathione-treated vacutainers were mixed by gentle inversion, placed in an ice-water bath for no more than 30 min, and then centrifuged for 15 min at 4°C and 2,250 g. The plasma samples were separated from the packed red cells, transferred to Eppendorf tubes, and immediately frozen and stored at −70°C. Plasma concentrations of interleukin (IL)-6 were determined by using an enzyme immunoassay kit (R & D Systems, Minneapolis, MN). Total plasma concentrations of cortisol were determined by radioimmunoassay, with use of standard commercially available kits (Diagnostic Products, Los Angeles, CA). Plasma catecholamine concentrations were measured by using a gas chromatography-mass spectrometry system (49); values are reported as unbound norepinephrine and epinephrine concentrations.

Statistical Analysis

Results are expressed throughout as means ± SE. The statistical significance of changes in physiological, immunological, and hormonal parameters was analyzed by using one- and two-way (trial by time) repeated-measures analyses of variance. Comparison between the control condition and 38°C water immersion provided information on the effects of heat and subsequent response to cold exposure. Comparisons between the control condition vs. exercise in cold water and passive heating vs. exercise in 35°C water provided information on the effects of exercise and subsequent response to cold exposure. Last, comparisons between exercise pretreatments allowed for the analysis of the influence of an exercise-induced rise in core temperature. Specific post hoc contrasts were used to explore significant main effects and interactions. Bonferroni adjustments were made for multiple comparisons, a probability P value of < 0.001 being accepted as statistically significant.

From the data obtained during cold exposure, correlations between cell counts, heart rate, rectal temperature, and hormone levels were determined by using forward, stepwise multiple-regression analysis. Each cell type was analyzed as the dependent variable with the other variables (heart rate, rectal temperature, and hormonal levels) set as independent variables. In a separate analysis, IL-6 was analyzed as the dependent variable with four independent variables (rectal temperature, cortisol, norepinephrine, and epinephrine). The forward stepwise multiple-regression analysis sequentially determines nonsignificant variables that should be excluded in a predictive equation. Variables are progressively eliminated from the analysis, and a reduced equation is recalcu-

lated by using the remaining variables (44). These analyses were completed to gain an understanding of the mechanisms that may be involved in some of the changes observed. For each regression analysis, a P value of < 0.05 was accepted as statistically significant for individual terms in the equation. All statistical calculations were performed by using StatView and SuperANOVA microcomputer software packages (Abacus Concepts, Berkeley, CA).

RESULTS

Physiological Responses

A slight, although statistically significant, 0.3°C reduction in rectal temperature occurred when subjects sat in water at 35°C (Fig. 1). In contrast, rectal temperatures were significantly increased 0.7°C within 45 min of passive heating. Rectal temperatures were also significantly increased 0.6°C within 30 min of exercise in 35°C water and were not significantly altered when subjects exercised in 18°C water (hereafter described as exercise with a thermal clamp). For each of the pretreatment exercise conditions, subjects exercised on a modified underwater cycle ergometer at an average of 55% of their personal $\dot{V}o_{2\,peak}$.

Before entry into the cold chamber, rectal temperatures returned to pretreatment baseline levels after control condition (sitting in 35°C water) as well as after exercise with a thermal clamp. Rectal temperature was some 1°C higher after the pretreatments of passive heating or exercise in 35°C water (Fig. 1). After pretreatment with exercise with a thermal clamp, rectal temperatures were significantly reduced within 15 min of cold air exposure. Seventy-five minutes of cold-air exposure significantly reduced rectal temperatures not only in the control condition but also when subjects had been pretreated by exercise in water at 35°C. After 1 h and 45 min of cold exposure, rectal temperatures were significantly reduced irrespective of the pretreatment protocol. Rectal temperature reached its lowest value

when subjects had exercised with a thermal clamp before the cold-air exposure.

Heart rates rose during passive heating (pre $= 76 \pm 2$ beats/min; post $= 100 \pm 4$ beats/min) and were significantly elevated in response to exercise with and without a thermal clamp (exercise in water at 18°C: pre $= 77 \pm 5$ beats/min vs. post $= 123 \pm 5$ beats/min; exercise in water at 35°C: pre $= 84 \pm 3$ beats/min vs. post $= 150 \pm 6$ beats/min). Heart rates returned to baseline values immediately before cold exposure, irrespective of pretreatment.
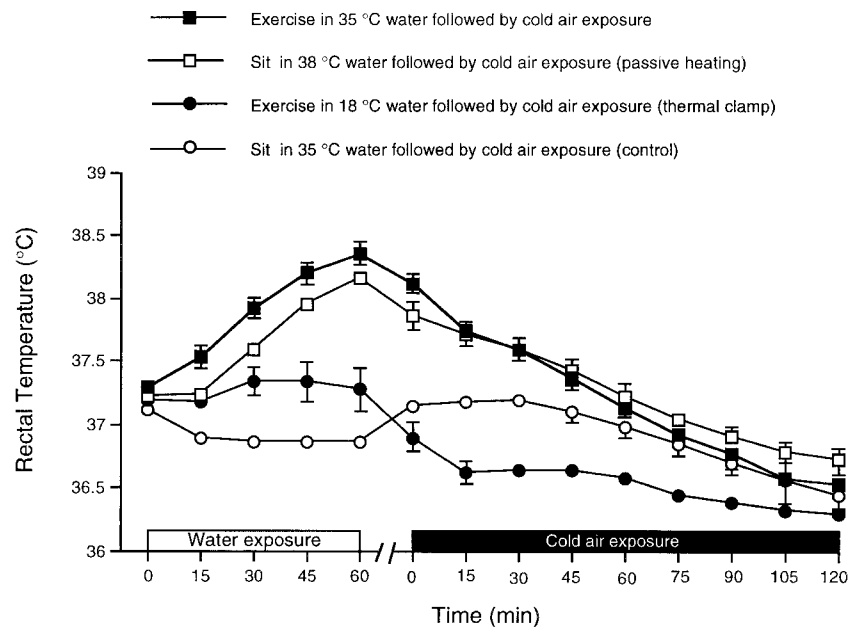
Total Leukocytes and Differentials

Before each treatment, the average leukocyte count (Fig. 2) was within the expected normal range of $4.5–11.0 \times 10^9$ cells/l (38). Cell counts were not significantly altered when the subjects remained seated in the 35 and 38°C water baths. In contrast, exercise with or without a thermal clamp induced a significant rise in leukocyte, granulocyte, and lymphocyte counts.

At entry into the cold chamber, total leukocyte counts were significantly higher when subjects had previously exercised with a thermal clamp. After 1 h of cold exposure, total white cell counts had increased significantly in all four pretreatment conditions, and values remained elevated at 2 h of cold exposure. Significantly more leukocytes were recruited into the circulation after 1 h of cold exposure, if the pretreatment was exercise with a thermal clamp rather than the control condition.

Granulocyte counts followed a similar pattern of response to the total leukocyte counts, increasing significantly within 1 h of cold-air exposure (Fig. 2). Compared with the control condition, a significantly greater number of granulocytes were recruited into the peripheral circulation when subjects had previously exercised with a thermal clamp for 1 h. Similarly, compared with

Fig. 1. Impact of the different experimental conditions on rectal temperatures. Values are means ± SE of data for 7 subjects. ○, Pretreatment with 1 h sitting in water at 35°C (thermoneutral control) before cold (5°C) exposure; ●, pretreatment with 1 h exercise at 55% peak oxygen consumption in cold water (18°C) (thermal clamp) before cold exposure; □, pretreatment with 1 h sitting in warm water (38°C) before cold exposure; ■, pretreatment with 1 h exercise at 55% peak oxygen consumption in water at 35°C before cold exposure.
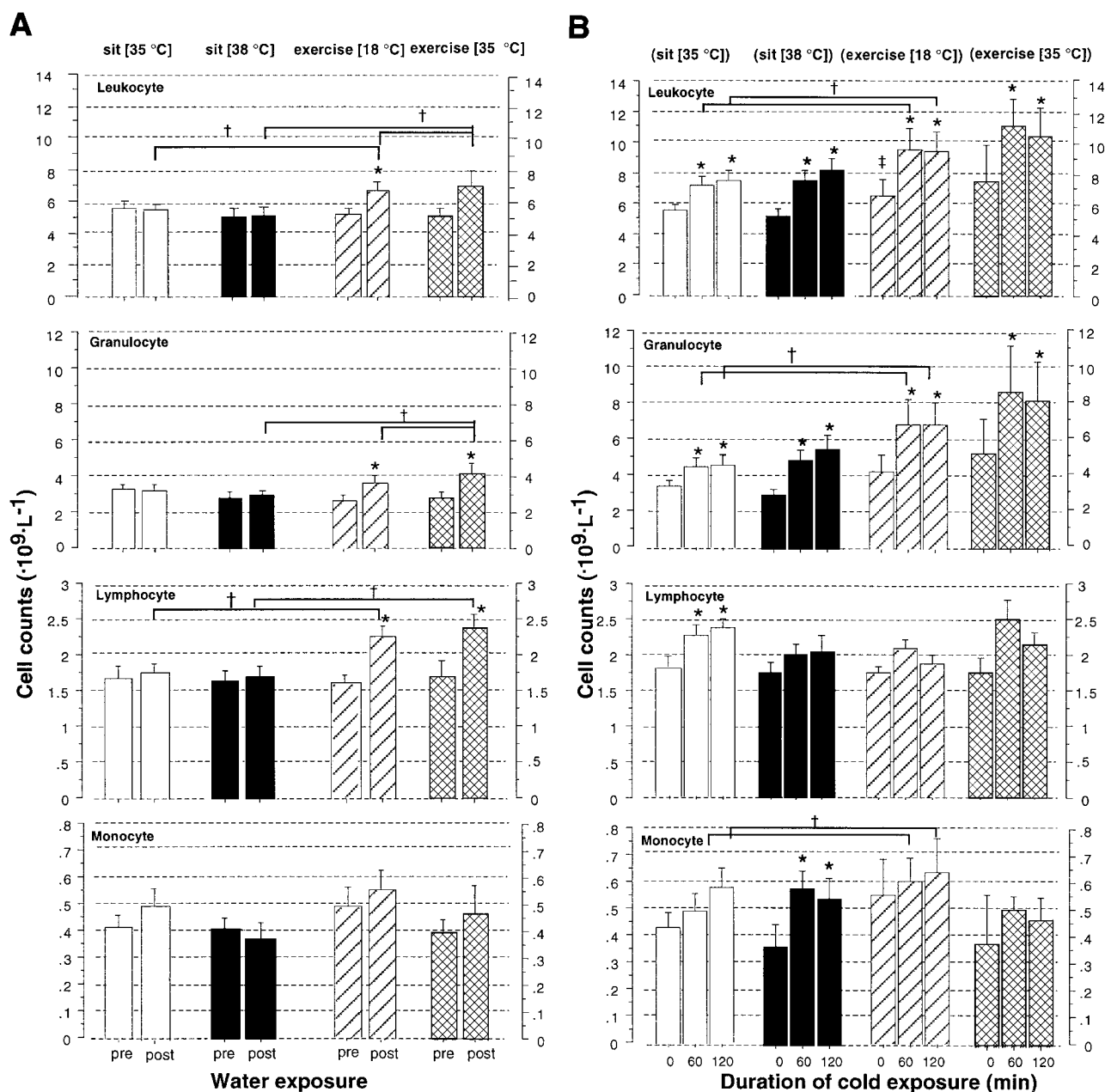
Fig. 2. Impact of pretreatment and subsequent cold exposure on leukocyte, granulocyte, lymphocyte, and monocyte counts. *A*: water-exposure treatment. *B*: cold exposure after 1-h pretreatment (water exposure). Values are means ± SE of data for 7 subjects. pre, Before water exposure treatment; post, at end of water exposure treatment. Open bars, pretreatment with 1 h sitting in water at 35°C before cold (5°C) exposure; solid bars, pretreatment with 1 h sitting in warm water (38°C) before cold exposure; hatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in cold water (18°C) before cold exposure; crosshatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in water at 35°C before cold exposure. *Significant difference from initial measurement (pre vs. post; *time 0* vs. *time 60* or *time 0* vs. *time 120*), $P < 0.05$. †Significant difference from condition marked by bracket, $P < 0.05$. ‡Significant difference from initial baseline values with same condition in *A*, $P < 0.05$.

the passive heating pretreatment condition, granulocyte counts tended to be higher if subjects had previously exercised in 35°C water, but this was not statistically significant.

Lymphocyte counts were significantly increased by cold-air exposure if this was preceded by the control pretreatment. Lymphocyte counts also tended to be elevated in response to cold exposure in the other three pretreatment conditions, but the changes were no longer statistically significant. After pretreatment with exercise (with or without a thermal clamp), lymphocyte counts reached peak levels within 1 h of cold air exposure. Monocyte counts were not significantly altered by any of the pretreatment conditions. Within 1 h of cold air exposure, the monocyte count was significantly increased when subjects had previously sat for 1 h in a warm water bath. Recruitment of monocytes during cold exposure was also significantly greater

with pretreatment by exercise with a thermal clamp than with the control pretreatment.

Forward stepwise multiple-regression analyses were performed to gain an understanding of the possible mechanisms contributing to the cellular changes observed during cold exposure. The variables initially included in the model were heart rate, rectal temperature, cortisol, norepinephrine, and epinephrine; the analyses indicated that both leukocyte and granulocyte counts were most closely related to heart rate and norepinephrine concentrations (Table 1). Lymphocyte counts were most closely related to norepinephrine. With the exception of monocytes, rectal temperature had no influence on circulating leukocyte subsets.

Lymphocyte Subsets

None of the four pretreatments had any effect on the circulating pan T (CD3$^+$)-, T-helper (CD4$^+$)-, and B (CD19$^+$)-cell counts (data not shown). In contrast, exercise with and without a thermal clamp recruited significant numbers of cytotoxic/suppressor T cells (CD8$^+$) (exercise in water at 18°C: pre = 0.475 ± 0.062 cells × 10^9/l cells vs. post = 0.711 ± 0.038 cells × 10^9/l; exercise in water at 35°C: pre = 0.460 ± 0.070 cells × 10^9/l vs. post = 0.765 ± 0.082 cells × 10^9/l) and NK cells (CD3$^-$/CD16$^+$56$^+$) into the circulation (Fig. 3).

After the control pretreatment, 2 h of cold exposure significantly increased levels of circulating T (CD3$^+$) and B (CD19$^+$) cells (pre-cold exposure CD3$^-$ cell counts, *time 0* = 1.18 ± 0.10 cells × 10^9/l blood vs. post-cold exposure CD 3$^+$ cell counts, *time 120* = 1.36 ± 0.08 cells × 10^9/l; pre-cold exposure CD19$^+$ cell counts, *time 0* = 0.17 ± 0.03 cells × 10^9/l vs. post-cold exposure CD 19$^+$ cell counts, *time 120* = 0.22 ± 0.03 cells × 10^9/l). NK (CD3$^-$/CD16$^+$56$^+$) cell counts increased significantly in response to cold exposure after both the control pretreatment and exercise in 35°C water. Pretreatment with either passive heating or exercise with a thermal clamp resulted in slightly higher baseline NK cell counts; when cold exposure followed either of these two conditions, NK cell counts again tended to increase, but the change no longer reached statistical significance.

Stepwise multiple-regression analyses explained from 2 to 42% of total variance, depending on which lymphocyte subset was examined (Table 1). With the exception

of CD3$^+$ cells, rectal temperature had no influence on circulating lymphocyte subsets. Epinephrine was an important determinant of CD19$^+$ cells, but norepinephrine accounted for much of the variance in CD3$^+$, CD8$^+$, and CD19$^+$ cell counts. None of the variables examined could significantly account for changes in NK cell counts.

NK Cell Activity

Total NK cell activity measured in lytic units (lytic units/10^6 PBMC) increased significantly in response to exercise with and without a thermal clamp (Fig. 3). Passive heating and sitting in 35°C water did not significantly alter total NK cell activity. Pretreatment with the control condition as well as with exercise with a thermal clamp resulted in significantly higher levels of total NK cell activity when subjects entered the cold chamber.

Total NK cell activity increased significantly within 60 min of cold exposure for the control pretreatment and in response to prior exercise without a thermal clamp. Passive heating followed by 2 h of cold exposure also induced a significant rise in total NK cell activity. Cold exposure after exercise with a thermal clamp tended to increase total NK cell activity, although levels did not reach statistical significance. A stepwise regression analysis (which began by correlating NK cell number, rectal temperature, cortisol, epinephrine, and norepinephrine with NK cell activity) indicated that NK cell activity was most closely related to changes in circulating NK cell counts (multiple R^2 = 0.337, $P <$ 0.0001).

When total NK cell activity was adjusted on a per-cell basis relative to NK cell counts, lytic activity per cell was significantly increased in response to exercise with a thermal clamp and remained elevated at entry into the climatic chamber. However, there were no longer any significant differences between pretreatment conditions in response to cold exposure.

Plasma Hormone Levels

The responses of the different stress hormones (free norepinephrine, free epinephrine, and cortisol) to the various treatments are presented in Fig. 4. Cold exposure preceded by either the control pretreatment or

Table 1. *Multiple-regression analysis for prediction of leukocyte subset and lymphocyte counts by heart rate, rectal temperature, and hormone concentrations*

Variable	R^2	Heart Rate	Rectal Temperature	Cortisol	Norepinephrine	Epinephrine
Total leukocytes	0.446	0.0018	NS	NS	0.0012	NS
Granulocytes	0.397	0.0042	NS	NS	0.0078	NS
Monocytes	0.161	NS	0.0372	NS	NS	NS
Lymphocytes	0.273	NS	NS	NS	0.0005	NS
CD3$^+$	0.201	NS	0.0348	NS	0.0057	NS
CD4$^+$	0.015	NS	NS	NS	NS	NS
CD8$^+$	0.216	NS	NS	NS	0.0071	NS
CD19$^+$	0.423	0.0483	NS	NS	<0.0001	0.0139
CD3$^-$/CD16$^+$56$^+$	0.192	NS	NS	NS	NS	NS

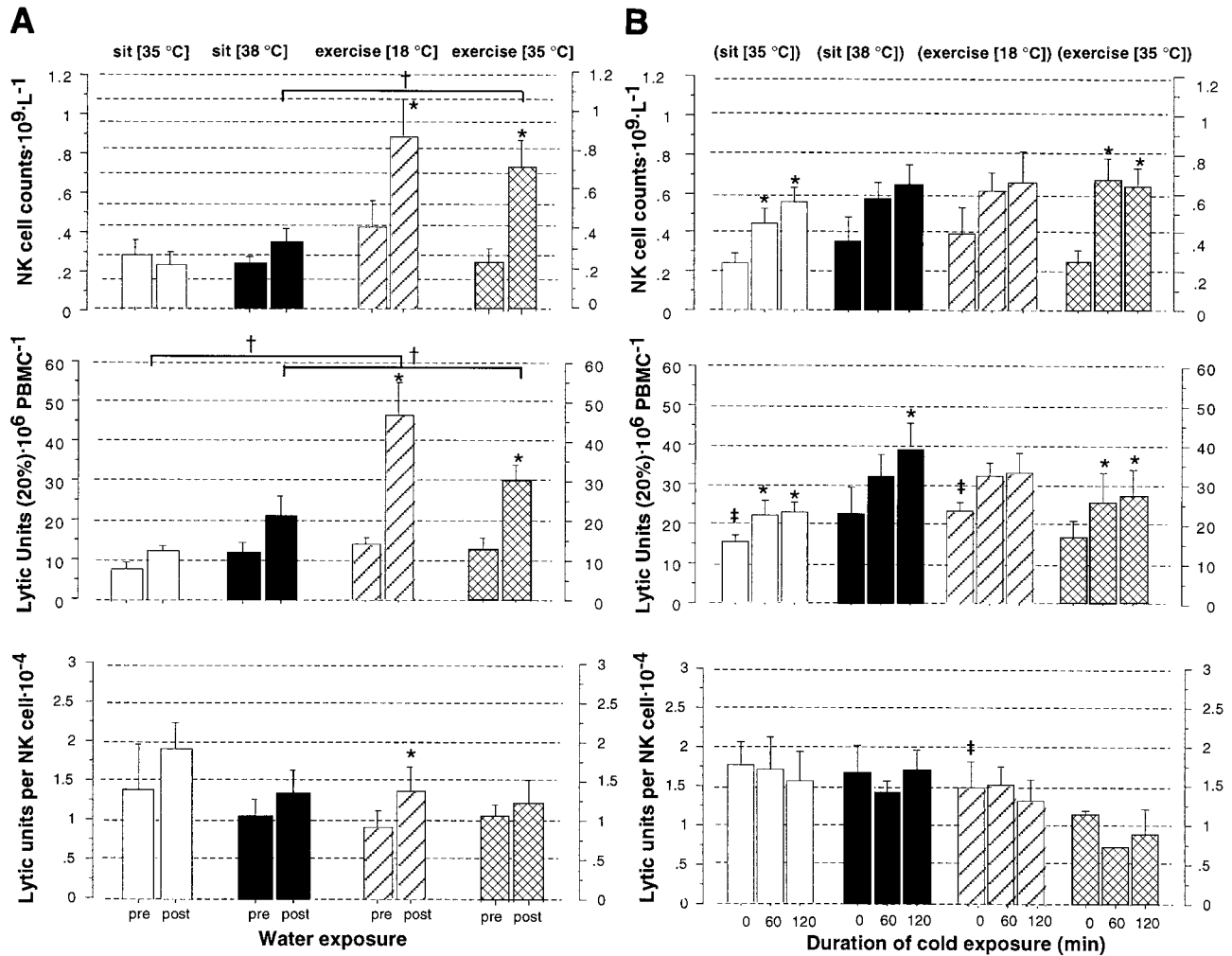Values are shown in terms of individual probability. NS, not significant.

Fig. 3. Impact of pretreatment and subsequent cold exposure on natural killer (NK) cell number, total NK cell activity [lytic units (20%)/10^6 peripheral blood mononuclear cells (PBMC)], and lytic units per NK cell. *A*: water-exposure treatment. *B*: cold exposure after 1-h pretreatment (water exposure). Values are means ± SE of data for 7 subjects. Open bars, pretreatment with 1 h sitting in water at 35°C before cold (5°C) exposure; solid bars, pretreatment with 1 h sitting in warm water (38°C) before cold exposure; hatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in cold water (18°C) before cold exposure; crosshatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in water at 35°C before cold exposure. *Significant difference from initial measurement (pre vs. post; *time 0* vs. *time 60* or *time 0* vs. *time 120*), $P < 0.05$. †Significant difference from condition marked by bracket, $P < 0.05$. ‡Significant difference from initial baseline values under same condition, shown in *A*, $P < 0.05$.

passive heating induced a significant rise in plasma norepinephrine concentration within 60 min (Fig. 4). When subjects exercised with a thermal clamp before cold exposure, plasma norepinephrine levels increased significantly only after 120 min. A similar pattern of response occurred when subjects exercised in 35°C water before entering the cold chamber; however, in part because of greater variability in the data, the response was no longer statistically significant. Cold exposure after the control pretreatment did not significantly alter levels of circulating epinephrine. When preceded by passive heating, plasma epinephrine concentration was significantly higher after 2 h of cold exposure.

With the exception of the condition involving pretreatment with passive heating, cold exposure had no significant effect on plasma cortisol levels. For this condition

only, 1 h of cold exposure led to significant reductions in plasma cortisol levels.

IL-6 Levels

Passive heating and exercise with a thermal clamp did not significantly alter plasma levels of IL-6 (Fig. 5). However, exercise without a thermal clamp induced a significant elevation in IL-6. Before entry into the cold chamber, levels of IL-6 were significantly higher compared with baseline in each of the exercise pretreatments.

In the control condition, 1 h of cold exposure induced a significant rise in plasma IL-6 concentration. When cold exposure was preceded by either passive heating or exercise with a thermal clamp, plasma IL-6 levels were significantly elevated after 2 h. Prior exercise

without a thermal clamp also tended to induce an elevation in response to cold exposure, but these results did not reach levels of significance. A stepwise multiple regression analysis began by correlating IL-6 with rectal temperature, cortisol, norepinephrine, and epinephrine concentrations. Two variables (cortisol and norepinephrine) were retained in the equation with a multiple R^2 of 0.191 and $P = 0.0015$ for the equation as a whole.

DISCUSSION

The present study examined the impact of cold exposure on selected aspects of immune function, to-
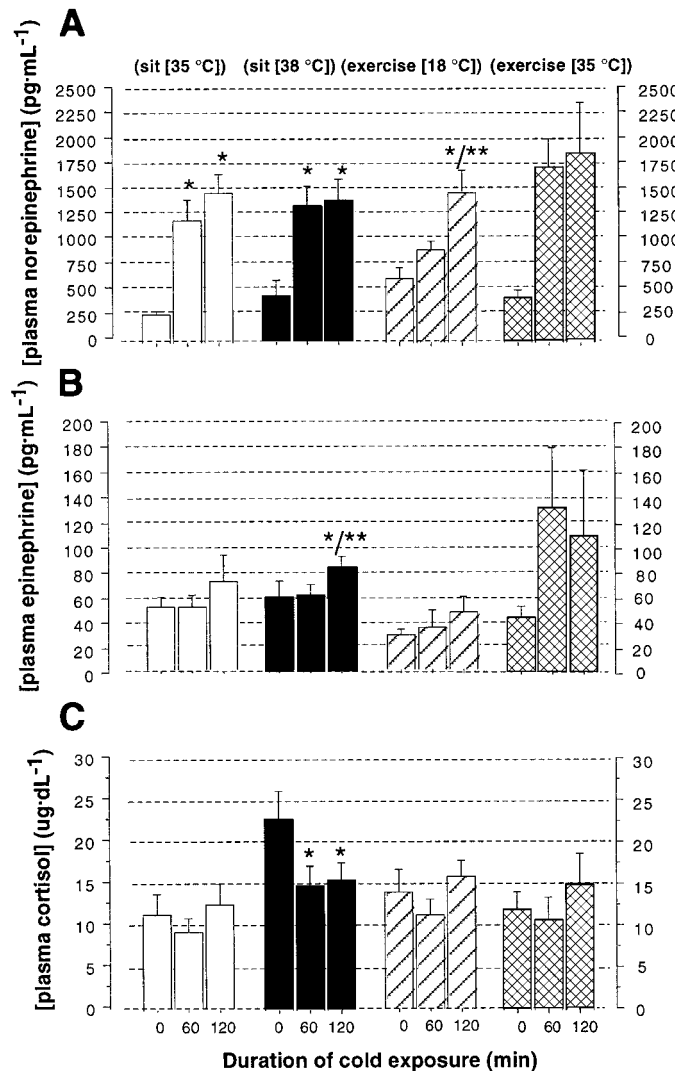

Fig. 4. Hormonal response to cold exposure after 1-h pretreatment with 4 different water exposure treatments: plasma norepinephrine (*A*), epinephrine (*B*), and cortisol (*C*). Values are means ± SE of data for 7 subjects. Open bars, pretreatment with 1 h sitting in water at 35°C before cold (5°C) exposure; solid bars, pretreatment with 1 h sitting in warm water (38°C) before cold exposure; hatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in cold water (18°C) before cold exposure; crosshatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in water at 35°C before cold exposure. *Significant difference from initial measurement (pre vs. post; *time 0* vs. *time 60* or *time 0* vs. *time 120*), $P < 0.05$. **Significant difference from 60 min (*time* 60 vs. *time 120*), $P < 0.05$.
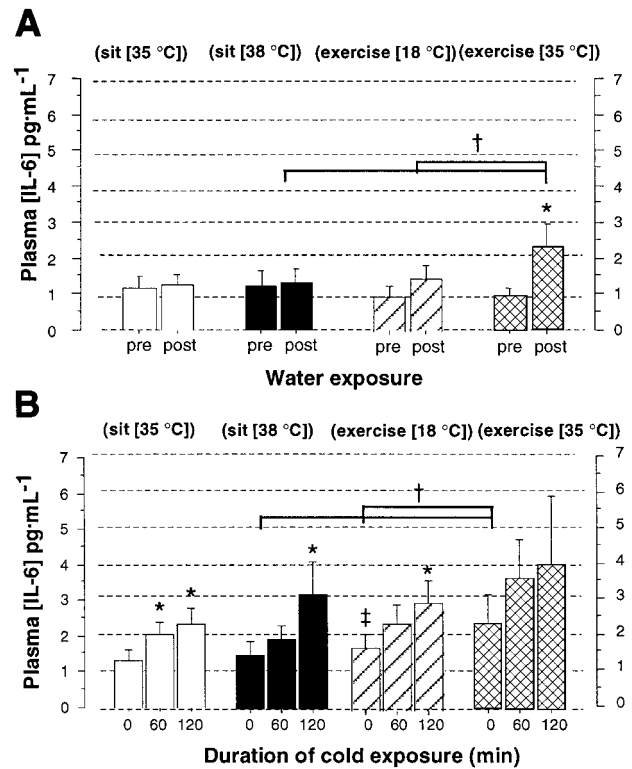

Fig. 5. Impact of pretreatment and subsequent cold exposure on plasma interleukin-6 (IL-6) concentrations. *A*: water exposure treatment; *B*: cold exposure after 1-h pretreatment (water exposure). Values are means ± SE of data for 7 subjects. Open bars, pretreatment with 1 h sitting in water at 35°C before cold (5°C) exposure; solid bars, pretreatment with 1 h sitting in warm water (38°C) before cold exposure; hatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in cold water (18°C) before cold exposure; crosshatched bars, pretreatment with 1 h exercise at 55% peak oxygen consumption in water at 35°C before cold exposure. *Significant difference from initial measurement (pre vs. post; *time 0* vs. *time 60* or *time 0* vs. *time 120*), $P < 0.05$. **Significant difference from 60 min (*time 60* vs. *time 120*), $P < 0.05$. †Significant difference from condition marked by bracket, $P < 0.05$. ‡Significant difference from initial baseline values under same condition, shown in *A*, $P < 0.05$.

gether with the possible modulating effects of prior passive heating and moderate exercise (with or without a thermal clamp). During cold exposure, the body attempts to maintain a normal body temperature by increasing heat production and minimizing heat loss (37). This is accomplished through involuntary tonic muscular activity, rhythmic muscular activity (shivering), peripheral vasoconstriction, and the suppression of sweat secretion. Although primarily thermoregulatory, these mechanisms can also induce metabolic and hormonal changes, which in turn alter immune status.

Anecdotal and experimental reports have suggested that cold exposure may be associated with an increased risk of infection (42). Laboratory animal research involving prolonged cold exposure or cold-water immersion demonstrated a reduction in the function of certain immune parameters (i.e., lymphocyte proliferation, NK cell activity) (1, 18, 43, 48). Such extreme physiological conditions do not represent the typical conditions that humans may encounter during normal daily athletic activities or military operations. Therefore, this study

was designed to examine the acute effects of a brief cold exposure. The parameters evaluated were those components of the immune system that we hypothesized would be most responsive to such a challenge. NK cell activity was included because NK cells represent one of the first lines of defense against virally infected cells, tumor cells, and certain microorganisms. Our findings that the selected aspects of immune function measured in this study were not adversely affected by a moderate exposure to cold air are reassuring. Indeed, we have demonstrated that exposing subjects to a cold environmental chamber (5°C) for 2 h can be immunoenhancing and that moderate exercise (with a thermal clamp) can further augment the response of certain parameters to subsequent cold exposure.

Effects of Passive Heating and Moderate Exercise (With and Without a Thermal Clamp)

Cellular responses. In confirmation of our previous work (40), we did not observe any increase in either total or differential leukocyte cell counts when our subjects were passively heated. Passive heating increased core body temperature by only 1°C, and a greater rise in body temperature (at least 2°C) seems necessary to induce significant changes in circulating cell counts. Both Kappel et al. (25) and Downing et al. (13) immersed their subjects in a hot-water bath (39.5–40°C) for at least 2 h to raise core temperature (2.0–2.5°C); significant increases in total and differential leukocyte counts were then observed.

Our observation that total leukocyte, granulocyte, and lymphocyte counts rise in response to underwater cycle ergometer exercise is consistent with the findings reported by other investigators in our laboratory (9, 37a). Similarly, thermal clamping attenuated this leukocyte and granulocyte response. A combination of a relatively small sample size, intersubject variability, a moderate exercise intensity (55% $\dot{V}O_{2 peak}$) and a mild 1°C rise in rectal temperature may explain why we did not observe any differences in lymphocyte and monocyte responses between the different exercise conditions. Increases in cardiac output (via shear stress) and/or changes in levels of circulating hormones (leading to alterations in adhesion molecules and recruitment of cells from reservoirs) account for the exercise-induced changes in cell counts (31). Thermal clamping by cold water immersion not only attenuates the hormonal responses but also blocks the additional increase in cardiac output that would otherwise have been induced by cutaneous dilatation. Thus we had expected that the leukocyte response to exercise would be attenuated by the thermal clamp.

In the present study, none of the four water pretreatments significantly altered CD3$^+$, CD4$^+$ or CD19$^+$ cell counts during subsequent exercise. In contrast, NK cells and cytotoxic T-lymphocytes (CD8$^+$) were significantly increased in response to exercise (with or without a thermal clamp). Cytotoxic cells (primarily NK cells) have the greatest number of β_2-adrenergic

receptors (26, 30) and tend to be more responsive to the release of catecholamines than are other lymphocyte subsets (16). In general, the rise in circulating NK cell count was adequate to explain the parallel rise in NK cell activity observed in response to exercise. However, NK cell activity per cell was significantly increased in response to exercise in cold water, indicating that other humoral factors may have contributed to the enhanced activity observed in this condition.

IL-6 response. Of the various proinflammatory cytokines (IL-1, tumor necrosis factor, and IL-6), we chose to analyze plasma IL-6. IL-6 is one of the few cytokines that is consistently responsive to exercise. The response of other proinflammatory cytokines is less consistent and indeed these cytokines are difficult to detect in plasma by currently available techniques. The finding that plasma IL-6 concentration was significantly increased in response to exercise without a thermal clamp is consistent with other studies of prolonged and strenuous exercise (2, 14, 15, 32).

Immune Changes During Cold Exposure: Effects of Prior Heating and Exercise

Cellular responses. In accord with the results of both animal (46) and human (22) studies, acute cold stress increased circulating leukocyte and neutrophil counts. Jansky et al. (22) reported increases in both red cell and leukocyte counts when their subjects were initially immersed in cold (14°C) water for 1 h. However, this response seems only transient, because chronic cold-water immersion (3 times a week for 6 wk) did not alter the resting leukocyte or granulocyte counts. The cold-induced leukocytosis was significantly augmented by prior exercise with a thermal clamp. Perhaps this is because increased leukocyte counts tend to persist after exercise, giving an additive cellular response to cold plus prior exercise.

When preceded by the control condition, cold exposure significantly increased levels of circulating T (CD3$^+$) and B (CD19$^+$) cells. This cellular response was no longer significant in the face of pretreatment with passive heating or exercise (with or without a thermal clamp). These results may partly be explained by the increase in subject variability that occurred in response to pretreatment. However, a lymphocytopenia has also been reported after exercise (35), and this may have attenuated the rise in T and B cells that would otherwise have occurred in the cold. Without assessment of the proliferative response of these cell subtypes to mitogens, the biological significance of such minor changes remains questionable.

T-helper (CD4$^+$)- and T-cytotoxic/suppressor (CD8$^+$)-cell counts were not significantly altered by 2 h of cold exposure (5°C). Similarly, 1 h of cold (14°C)-water immersion did not significantly alter circulating concentrations of these cells (22). In contrast, Hennig and associates (20) reported a decrease in peripheral CD4$^+$ counts when body temperature was reduced (0.25°C) by cold exposure (5°C for 20 min) as well as by administration of a serotonin-receptor (5-HT$_{1A}$) agonist. For the group receiving the 5-HT$_{1A}$ agonist, cortisol was demon-

strated to be the mediator of changes in CD4$^+$ cells. However, for the group exposed to cold air, the reduction of CD4$^+$ cells showed no relationship to changes in cortisol concentration. The discrepant results may be due to the timing of samples. Because our first data were collected 1 h after cold exposure, there could have been an undetected reduction in cell counts during the first 20 min of cold exposure, associated with an early "stress" response, with a return to baseline levels after 1 h of cold exposure. Nevertheless, this is not very likely, because the stress of cold exposure tends to be cumulative and related to the fall in core temperature.

Animal studies have demonstrated either a decrease (1, 23, 48) or no change (43) in NK cell number and activity in response to cold exposure. In these studies, mice and/or rats were subjected to repeated bouts (2–5 min) of very-cold-water (4°C) stress over several days (4–5 days) (1, 23, 43) as well as to prolonged cold-air exposure (4°C) for up to 16 days (48). Such conditions can be stressful for the animal. It remains unclear how much of this response is attributable to a generalized stress response rather than to cold exposure and how far reactions are affected by acclimatization to cold. Most of these studies have postulated that a stress-induced release of corticosteroids accounts for the reduction in NK cell number and activity that occurs with cold exposure.

In contrast, studies involving human subjects have demonstrated an increase in NK cell activity in response to local or generalized cold exposure (11, 27). Delahanty et al. (11) examined the effects of a cold-pressor task on NK cell function. In their study, 10 male subjects (age 20–45 yr) were required to place their hands intermittently in very cold (3°C) water for a total submersion time of 3 min (the durations of immersion were 30, 20, 45, 15, 40, and 30 s with a 30-s rest period in between each submersion). Here, there was a local stressful stimulus, with little change of core temperature. They demonstrated a trend toward an increase in NK cell activity 2 min into the cold-pressor task. Lackovic et al. (27) exposed 8 naked male volunteers (age 20–26 yr) to a cold (4°C) room for 30 min. Body temperatures showed only a moderate decrease (an average of 0.45°C), but NK cell activity was significantly increased. Similarly, our study has demonstrated that NK cell counts and activity increased in response to cold exposure that caused quite modest reductions in core temperatures (ranging between 0.6 and 1.6°C).

IL-6 response. Our finding that plasma concentrations of IL-6 rose in response to cold exposure is in contrast to the work of Jansky et al. (22). They reported a slight and nonsignificant trend to a reduction in the concentration of IL-6 when subjects were immersed for 1 h in cold (14°C) water. They also showed that repeated cold-water immersions over a period of 3–6 wk had no significant effect on resting levels of circulating IL-6. It is possible that their cold stimulus was not sufficiently prolonged or severe to induce any significant changes. We observed a significant rise in plasma levels of IL-6 when cold exposure was preceded by the

control condition, passive heating, or exercise with a thermal clamp but not in the condition when initial core body temperature was highest (exercise in water at 35°C).

Several physical stressors increase circulating levels of IL-6 (33, 35, 50); however, the mechanisms involved have not been clearly identified. Pedersen et al. (35) have proposed that muscle damage associated with eccentric muscular activity induces the release of proinflammatory cytokines. Zhu et al. (51) provided experimental data showing that the secretion of IL-6 from mouse peritoneal macrophages after cold-water stress (5-min swim tests in 10°C water) may be related to increased tissue levels of immunoreactive substance P. In our study, regression analyses indicated that the changes in levels of IL-6 were most closely related to plasma levels of norepinephrine and cortisol. Animal studies have shown that catecholamines stimulate endogenous IL-6 secretion, whereas glucocorticoids inhibit it (34). It is possible that norepinephrine exerts its effects by a cAMP-dependent mechanism, because cAMP can modulate cytokine production. Szabo et al. (47) have demonstrated that pretreatment with isoproterenol increased the lipopolysaccharide-induced production of IL-6 in rats.

Hormonal responses. Catecholamines and cortisol play a major role in the physiological responses to cold exposure. Norepinephrine mediates "futile" metabolic cycling in white and brown adipose tissue, together with the acute, general cutaneous vasoconstriction that occurs in the extremities (28). Epinephrine and cortisol facilitate the metabolism of glucose and triglycerides, also contributing to increased heat production.

Levels of plasma norepinephrine were significantly increased within 1 h of cold-air exposure in the control condition as well as after passive heating and exercise with a thermal clamp. Presumably because of higher initial core temperatures, changes were not statistically significant after pretreatment with exercise at 35°C. Peripheral vasoconstriction, induced by sympathetic release of norepinephrine, is one of the first responses to cold-air exposure (28). Thus it is not surprising that a significant rise in plasma norepinephrine concentration was observed. In contrast [as reported Juránková et al. (24)], cold exposure did little to alter levels of circulating epinephrine and cortisol. Although cortisol and the catecholamines (epinephrine and norepinephrine) have typically been referred to as stress hormones, the release of cortisol tends to lag behind that of catecholamines. This may explain why we did not observe a cortisol response to cold exposure. A 24-h urine sample would have provided a more complete picture of cortisol responses.

Norepinephrine can mobilize cells through adrenergic receptor stimulation (16) as well as by its action on sympathetic nerve terminals within the lymph nodes and spleen (3, 7). Cortisol stimulates the release of granulocytes from the bone marrow and reduces lymphocyte counts by inhibiting their entry into, and facilitating their egress from, the circulation (10). It is unlikely that changes in the levels of circulating corti-

sol contributed to a delayed neutrophilia or lymphopenia in this particular design, because circulating cortisol levels tended to remain the same during cold exposure. Our multiple regression analyses suggest rather that the rise in circulating leukocytes during cold exposure may be attributed to a norepinephrine-mediated mobilization of demarginated cells. These results should be interpreted with caution, because correlation coefficients were relatively low. Nevertheless, our results support the hypothesis that the observed changes in cell counts may be due to a change in levels of circulating hormones induced by a combination of exercise and changes in core body temperature.

Further work should examine whether other components of the innate immune system (neutrophil oxidative burst) and components of the adaptive immune system (lymphocyte proliferative responses and immunoglobulin levels) respond in a similar manner. Research should also be completed to examine the influence of more prolonged and repeated bouts of cold exposure on immune function. Adverse effects might possibly be observed if the combination of cold exposure and prior exercise were sufficient to deplete key nutrients important to cellular immune responses. Finally, investigators should explore whether the rate of any adaptations to a cold environment is modified when bouts of physical activity are performed immediately before or during cold exposure.

Conclusions

This study suggests that, despite popular beliefs that cold exposure can precipitate a viral infection, the innate component of the immune system is not adversely affected by a brief period of cold exposure. Indeed, the opposite seems the case. The fall in core body temperature resulting from cold exposure led to a consistent and statistically significant mobilization of circulating cells, an increase in NK cell activity, and elevations in circulating IL-6 concentrations. Moreover, in agreement with one of our hypotheses, prior exercise with a thermal clamp significantly augmented the leukocyte, granulocyte, and monocyte response to cold exposure. Prior passive heating and exercise without a thermal clamp also tended to augment the effect of cold exposure alone, but, because of the small sample size and intersubject variability, these changes were not statistically significant.

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