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Y. Severs · I. Brenner · P.N. Shek · R.J. Shephard

Effects of heat and intermittent exercise on leukocyte and sub-population cell counts

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Abstract This study examined the *combined* effects of heat stress and *intermittent* exercise on circulating leukocyte and sub-population cell counts. Using a randomized-block design, 11 healthy male subjects [mean (SD) age = 29.1 (3.0) years maximal oxygen consumption ($\dot{V}O_{2max}$) = 47.6 (6.1) ml/(kg·min)] were assigned to four conditions. Each subject exercised on a cycle ergometer at 50% $\dot{V}O_{2max}$ (two 30-min bouts, with 45 min rest between), or acted as his own control by sitting at 23°C, or at 40°C, 30% relative humidity, for 3 h. Blood samples taken prior to, during, and after each rest and exercise bout, and at corresponding times when sitting were used for Coulter cell counter and flow cytometric analysis. Sitting conditions did not produce any significant immunological changes. Intermittent exercise induced a biphasic response of granulocytosis, monocytosis and lymphocytosis, with a return to baseline between exercise bouts. One hour following the second exercise bout, samples showed a consistent granulocytosis, monocytosis and lymphocytosis ($CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cell counts). The second exercise bout produced a larger response than the first, further exacerbated when exercising at 40°C. We conclude there is a synergism between heat and exercise exposure. An increase in core temperature and exercise stress recruit leukocytes into the peripheral circulation, with potentiation of the response during a second bout of exercise. However, while the increase of core temperature remains moderate, the disturbance of immune function does not appear to have great clinical significance.

Key words Cell proliferation · Granulocytes · Immune function · Immunoglobulin production · Lymphocytes

Y. Severs · I. Brenner · P.N. Shek · R.J. Shephard
Defence and Civil Institute of Environmental Medicine, Toronto, Ontario, Canada

I. Brenner · P.N. Shek · R.J. Shephard
Programme in Exercise Sciences, Graduate Department of Community Health, University of Toronto, Ontario, Canada

P.N. Shek
Department of Clinical Biochemistry, University of Toronto, Ontario, Canada

R.J. Shephard
Health Studies Programme, Brock University, St. Catherines, Ontario, Canada

R.J. Shephard (✉)
School of Physical and Health Education, 320 Huron St. Toronto, Ontario, Canada, M5S 1A1

Introduction

Whenever homeostasis is upset, the human body attempts to adjust biological functions in such a way as to reduce the resulting stress (Fry et al. 1991). Physical exercise and exposure to a hot environment are two relatively independent stressors that threaten homeostasis, with resulting alterations in the numbers of circulating immune cells (Jampel et al. 1983; Keast et al. 1988; Khansari et al. 1990; Olkowski 1990; Peatfield et al. 1985; Shinkai et al. 1992; Tvede et al. 1994; Uhlenbruck and Order 1991; Winther and Trap-Jensen 1988). The extent of the changes observed during and following exercise depends upon the intensity and duration of the exercise bout relative to the fitness level of the individual (Bourey and Santoro 1988; Katz 1994; Kuipers and Keizer 1988; MacKinnon 1991; McCarthy and Dale 1988; Resina et al. 1986; Rhind et al. 1994, 1995; Ricci and Masotti 1989; Shephard et al. 1991; Shinkai et al. 1992; Tvede et al. 1993; Weicker and Werle 1991).

Normal occupational activity may demand repeated bouts of moderate activity in a warm environment, rather than the single controlled-climate exercise bout typical of laboratory exercise testing. However, changes in circulating immune counts during repeated bouts of high-intensity short-interval exercise (Cameron et al.

1989; Gray et al. 1993) parallel reported responses to a single bout of high-intensity exercise (Nieman and Nehlsen-Cannarella 1994; Rhind et al. 1995; Shephard et al. 1991; Shinkai et al. 1992); immediately post-exercise there is a leukocytosis, granulocytosis, lymphocytosis and monocytosis. Gray et al. (1993) reported a significant increase in circulating $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ counts, with a decrease in the $CD4^+/CD8^+$ ratio (to 0.71) immediately following their selected pattern of interval exercise (15 1-min bouts at 100% of maximal oxygen consumption or $\dot{V}O_{2max}$). An overall reduction in $CD3^+$ count was noted 1 h post-exercise, with increases in the numbers of circulating leukocytes, granulocytes and monocytes 6 h post-exercise. Inter-trial differences in the response to interval exercise seem dependent on the intensity and duration of exercise bouts. Thus Frisina et al. (1994) reported a significant decrease in the percentages of circulating T and B cells 3 min after a protocol of 25 1-min bouts at a higher work-rate (112% of $\dot{V}O_{2max}$).

No previous investigators have studied the changes associated with two rapidly successive but sustained bouts of exercise. The changes induced by a hot environment have also had only limited study. Heat exposure alone (water immersion, irradiation or sauna bathing) seems to induce a leukocytosis, a granulocytosis, a minor monocytosis and a lymphocytosis (Bouchama et al. 1992; Kappell et al. 1991a, 1994; Pedersen et al. 1994; Cross, Radomski, Van Helder and Shephard in press). Increases in total lymphocyte count have been attributed to an increase in T suppressor cells with a concomitant decrease in T helper cells.

There have been no prior studies examining the combined influence of intermittent exercise and heat upon circulating lymphocyte numbers and function. The present study was undertaken to provide such information. Individuals performed two 30-min bouts of moderate work (50% $\dot{V}O_{2max}$) under thermoneutral (23°C) and hot (40°C) conditions. Multi-point sampling explored not only the cytological response, but also the functional capacity of the circulating mononuclear cells (proliferative response and serum immunoglobulin levels). Reactions to heat and to repeated bouts of moderate exercise were studied singly and in combination.

Materials and methods

Subjects

Eleven healthy, non-smoking, male subjects [age: 29.1 (3.0) years, $\dot{V}O_{2max}$: 47.6 (6.1) ml/(kg·min)] were recruited under conditions approved by the University of Toronto and Defence and Civil Institute of Environmental Medicine (DCIEM) Human Experimentation committees. Pre-entry requirements included a medical examination to exclude conditions influencing immune function and to ensure fitness to participate, and the measurement of maximal aerobic power, using an electrically braked cycle ergometer. The minimal $\dot{V}O_2$ for acceptance into the study was 40 ml/(kg·min). Subjects were informed of the potential risks involved and gave their written informed consent.

Experimental protocol

Each subject visited the laboratory on five occasions. Attendance for each session occurred on the same day of the week, at the same time of day, with a 3-week interval between sessions. Subjects refrained from all moderate or heavy exercise for 48 h prior to each visit and avoided eating or drinking caffeinated beverages for 8 h prior to each session. An initial 2-h habituation session allowed participants to become familiar with the testing protocol and the climatic chamber. This visit was followed by four 3-h sessions in the environmental chamber. Experimental conditions were assigned according to a randomized-block design: sitting at thermoneutral temperature (N) (23°C) or in a heated environment (H) (40°C, 30% relative humidity or r.h.) and exercising at a thermoneutral temperature (NE) or in the heat (HE). On exercise days (NE and HE), subjects entered the environmental chamber 15 min prior to exercise, performing two 30-min bouts of cycle ergometer exercise at 50% of their individually determined $\dot{V}O_{2max}$, with 45 min of seated rest between exercise bouts. Subjects remained seated in the environmental chamber for 1 h following the second exercise bout. Participants were encouraged to maintain their plasma volumes. Prior to the exercise sessions, they consumed 250 ml (NE) or 400 ml (HE) of water. Water was also provided ad libitum throughout each test. The remaining sessions (N and H) followed a similar routine to NE and HE, except that (1) there was no preliminary fluid loading, and (2) no exercise was performed. The heart rate and rectal temperature were monitored continuously during all sessions.

Blood sampling

Blood samples were collected from an in-dwelling heparin-locked catheter (Deseret Medical, Sandy, Utah, USA) that was inserted into the median antecubital vein 30 min prior to collection of the first blood sample. In NE and HE, samples were taken prior to, during, and following each rest and exercise bout, with a final recovery sample collected 1 h post-exercise (i.e. at times of 0, 15, 30, 45, 75, 90, 105, 120 and 165 min). Blood samples were taken at corresponding times during the seated rest sessions (N and H): the total blood volume collected over the nine samples on any one day was 305 ml. Aliquots of blood were drawn into non-additive, tripotassium ethylenediamine tetra-acetate (K_3EDTA) and heparinized (500 U 10 ml^{-1}) sterile gas vacutainers (Becton-Dickinson, Oakville, Ontario, Canada). Hemoglobin, red blood cell and reticulocyte counts were checked prior to each session; the cell values proved normal, but the study design provided for more detailed hematological evaluation if any person had shown a residual hematological deficit from a previous experiment.

Cell sub-set counts

The total numbers of circulating erythrocytes, thrombocytes, and leukocytes, hemoglobin concentration and hematocrit were determined from K_3EDTA-treated samples of peripheral venous blood, using a Coulter JT Automatic Hematology System (Coulter Electronics, Hialeah, Fla., USA). All leukocyte and thrombocyte parameters were adjusted for blood volume changes, using the method of Dill and Costill (1974).

Preparation of mononuclear cells

Of heparinized peripheral venous blood, 7 ml was mixed with an equal volume of phosphate-buffered saline (PBS), layered over 5 ml of Ficoll-Paque (Baxter, Missisauga, Ontario, Canada) and centrifuged at 400 *g* for 30 min at 20°C. The mononuclear cell layer was removed and washed twice, firstly with 10 ml of PBS and secondly with 10 ml of 10% fetal calf serum (FCS)-RPMI 1640 culture medium (Gibco, Burlington, Ontario) supplemented with 10% FCS. Tubes were then centrifuged at 330 *g* for 10 min at 20°C. The washed peripheral blood mononuclear cells (PBMC) were re-suspended in 10% FCS-RPMI 1640 and the cell concentration was determined using a Royco Cell-Crit 920A electronic cell counter. Cell concentrations were adjusted to 1×10^6 cells/ml, using 10% FCS-RPMI 1640 as a diluent.

Determination of lymphocyte sub-sets

A 100 µl volume of whole blood was mixed with 10 µl of selected monoclonal antibodies (mAb, Becton-Dickinson) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) in the following double staining combinations: anti-CD3 mAb (FITC)/anti-CD19 mAb (PE), anti-CD3 mAb (FITC)/anti-CD8 mAb (PE), and anti-CD4 mAb (FITC)/anti-CD8 mAb (PE). Following a 30-min incubation on ice in the dark, 2 ml of 10% diluted FACS lysing solution (Becton-Dickinson) was added and vortexed. The vials were left in the dark at room temperature for a further 10 min to lyse the red cells. Non-lysed cells were separated by centrifuging at 300 *g*, 4°C for 5 min. They were washed twice with 2 ml of PBS containing 0.1% sodium azide and were then centrifuged for a further 5 min at 300 *g*; 4°C. The resulting pellet was re-suspended with 0.3 ml of PBS containing 0.1% sodium azide; it was then placed on ice and stored in the dark until analysis.

Flow cytometry

Stained cell samples were analyzed on the same day as the experimental session, using a FACScan flow-cytometer (Becton-Dickinson) set at a wavelength of 488 nm. The FACScan system was calibrated daily, using in turn CaliBrite beads (Becton-Dickinson), an isotype negative control and a $CD4^+/CD8^+$ double-stained sample. The data were acquired and analyzed using Consort 30 and LYSIS software. Usually, 10000 cells were scanned per sample. Findings were expressed as the percentage of cells yielding a specific fluorescence in a gated lymphocyte region. The absolute count for a given lymphocyte sub-set was derived by multiplying the observed percentage by the total number of lymphocytes in peripheral blood, the latter value having been adjusted for any blood volume changes. An unstained cell sample was used to determine the relative proportions of granulocytes, lymphocytes and monocytes, based on cell size and granularity, on a dot plot of forward versus side light scattering.

Proliferative response to mitogens

Data were obtained from 9 of the 11 subjects. Triplicate samples of PBMC (1×10^6 cells/ml) were cultured in a sterile 96 U-shaped well plate (Costar, Toronto, Ontario, Canada), using RPMI 1640 medium that contained 10% heat-inactivated FCS and 100 U each of penicillin and streptomycin (Gibco). Each culture was stimulated with 100 µl of serially diluted phytohemagglutinin (PHA, Sigma, St. Louis, Mo., USA) (200 µg/ml, 100 µg/ml, 75 µg/ml, 50 mg/ml and 25 µg/ml) or pokeweed mitogen (PW, Gibco) (200 µg/ml, 100 µg/ml, 50 mg/ml, 25 µg/ml and 10 µg/ml). The plates were incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. The optimal mitogen dose was selected, based upon a maximized uptake of [^{3}H]thymidine. The control culture was incubated under identical conditions, but in the absence of mitogen. [^{3}H]Thymidine (18.5 kBq, specific activity 37 MBq/ml; Amersham, Arlington, Ill., USA) in 20 µl of FCS-RPMI 1640 was added to each well. The plates were then incubated for an additional 24 h. The cells were collected with an automated PHD Cell Harvester (Cambridge Technology, Mass., USA), and their radioactivity was counted using a Beckman scintillation counter (Model LS5801).

Determination of immunoglobin concentrations

Data were obtained from 9 of the 11 subjects. The concentrations of immunoglobulins IgG and IgM in tissue culture were quantified using a double-antibody enzyme-linked immuno-sorbent assay (ELISA). All wells of a 96-well Immulon 2U plate (Dynatech, Toronto, Ontario, Canada) were initially coated with 100 µl of goat anti-human IgG or IgM antibody (AKZO, Organon Tenika, Durham, N.C., USA) at a concentration of 400 ng/ml. The IgG antibody dilution was prepared with 0.02 M PBS at a pH of 7.3, whereas the IgM antibody was diluted with 0.05 M bicarbonate buffer at a pH of 9.6. The antibody-coated plates were covered and incubated overnight in a humid chamber at 4°C. They were then washed 3 times with 0.15 M saline containing 0.05% Tween 20 (Sigma). Tissue culture supernatants and specific immunoglobulin standards were appropriately diluted in PBS containing 0.5% bovine serum albumin at a pH of 7.4 (Sigma). Standard and supernatant solutions (100 µl) were each added to antibody-coated wells in quadruplicate. The reaction plates were covered and incubated for 1 h in a humidified chamber at 37°C. They were then washed as described above, individual wells being treated with 100 µl of the corresponding peroxidase-conjugated antibody (Organon Teknika), at the appropriate dilution (IgG 1:6000 and IgM 1:10000). The plates were covered, incubated in a humid chamber for 1 h at 37°C, and washed as described above. The substrate, *o*-phenylenediamine dihydrochloride (OPD), was freshly prepared by dissolving a 30-mg OPD tablet (Sigma) in 60 ml of distilled water containing 120 µl of 30% hydrogen peroxide. A 100-µl volume of substrate solution was added to each well, and plates were covered and incubated in the dark for 30 min at room temperature. The reaction was halted by the addition of 50 µl of 1N sulfuric acid to each well. The optical density of the well contents was read at 492 nm and the concentration of immunoglobulin was determined (Bio-Kin plate reader EL340, Bio-Tek Instruments, Winooski, Vt., USA). The Behring ELISA II Processor (Behring Diagnostics, Montreal, Quebec, Canada) was used to add reagents and wash plates in this assay procedure.

Statistical analysis

Data are shown as mean ± SD throughout. In order to reduce the number of statistical comparisons, the "area under the curve" was computed for each exercise and rest period, and for corresponding times during rest sessions (see Fig. 1). For ease of subsequent discussion, each experimental session has been categorized as four segments: A, B, C and D. An analysis of variance (ANOVA) for repeated measures was then applied to determine significant differences between and within experimental conditions. When the *F* value showed significant main effects, specific parametric post hoc contrasts tested differences among exercise and rest period values. Probability values were adjusted using the Geisser-Greenhouse estimate of epsilon (Geisser and Greenhouse 1959). An adjusted *P* value of < 0.05 was accepted as statistically significant.

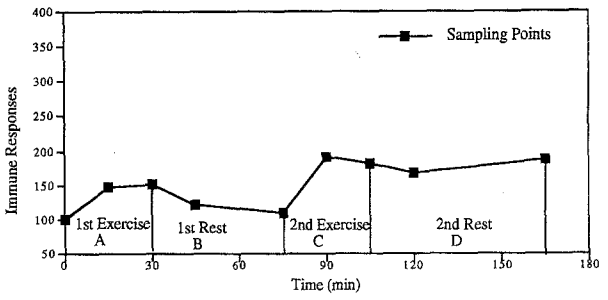

Fig. 1 Illustration of "area under curve" procedure used in statistical analysis

Results

Physical characteristics and experimental responses

The physical characteristics were typical of healthy moderately active male subjects [mean (SD), age = 29.1 (3.0) years, height = 178 (0.06) m, body mass = 77.2 (4) kg]. The $\dot{V}O_{2max}$ prior to participation in the study averaged 47.6 (6.1) ml/(kg · min).

Heart rate did not change significantly during the seated conditions; the mean heart rate was 60.5 (0.9) beats/min during N and 70.5 (3.6) beats/min during H. During NE, the average heart rate for the entire session (nine sampling points) was 102.5 (38.4) beats/min; the peak heart rates reached at the end of each 30-min exercise period were 139.7 (21.5) beats/min during the first bout, and 148.6 (22.9) beats/min during the second bout. During HE a similar pattern occurred; the peak heart rate was seen after 30 min of exercise [first bout 164.5 (16.6) beats/min, second bout 175.8 (10.9) beats/min]. Values declined during rest periods, but did not return completely to baseline; the average working heart rate was 124.9 (41.0) beats/min. The peak increase in rectal temperature was 0.7 (0.3)°C for the H condition, 0.9 (0.3)°C in NE and 1.6 (0.3)°C in HE; the increase in rectal temperature was significantly larger in HE than in NE ($P < 0.0001$). During normothermia, the rectal temperature showed a small but statistically significant decrease [0.3 (0.03)°C, $P < 0.0004$]. The body mass of the subjects was recorded prior to and upon completion of each session: small but significant decreases were seen during all sessions [0.33 (0.23) kg, $P < 0.0009$ for N; 0.42 (0.22) kg, $P < 0.00009$ for H; 0.59 (0.30) kg, $P < 0.00007$ for NE; and 0.88 (0.72) kg, $P < 0.004$, for HE]. The blood volume remained unchanged in N [− 0.30% (0.91)] and H [0.10% (0.85)], but showed small decreases in NE [− 2.89% (3.34), $P < 0.03$] and HE [− 1.58% (2.44), $P = < 0.03$].

Circulating cell counts

There were no significant changes in circulating counts (Figs. 2, 3) for leukocytes, leukocyte subsets, or platelets under conditions of seated rest (N or H). The percentage of circulating platelets tended to increase during both bouts of exercise (NS), but returned to near baseline during rest periods under both the heated and normothermic conditions (Figs. 2, 3).

Both first and second bouts of exercise (NE and HE) induced a leukocytosis, with increases in circulating granulocyte, monocyte and lymphocyte counts (Figs. 2, 3). In NE, the increase in all cell counts peaked after 15 min during the first bout of exercise, with a return to near baseline levels [99.9% (3.0%)] after 15 min of subsequent rest. During the second bout of exercise, peak increases were not seen until 30 min of exercise. Comparing the first and second bouts of exercise, there was a significant difference in total leukocyte count (C > A, $P > 0.01$), but no significant inter-bout difference for the various leukocyte subsets (Table 1).

In the first bout of HE, granulocyte and monocyte counts had peaked after 15 min of exercise, but the total leukocyte and lymphocyte count continued to rise to 30 min, with a significantly greater total leukocyte response during HE than NE (Table 2, $HE_A > NE_A$, $P < 0.05$). Recovery during the initial post-exercise period was also slower in HE than in NE; lymphocyte, granulocyte and monocyte counts remained respectively 9%, 19% and 21% above baseline 45 min following this bout of exercise. A comparison of rest periods revealed significant differences (all $HE_B > NE_B$) for leukocyte count ($P < 0.003$), granulocyte count ($P < 0.04$) and monocyte count ($P < 0.04$), but no significant inter-trial difference of lymphocyte count (Table 2).

During the second bout of HE, there was a pronounced increase of both total leukocytes and each of the subsets relative to the initial bout of HE ($HE_C > HE_A$). At 15 min of exercise, the leukocyte count had increased by 91% ($P > 0.0001$) and granulocyte, monocyte and lymphocyte counts had increased by 96% ($P > 0.003$), 127% (not significant, n.s.) and 107% ($P > 0.02$) respectively (Table 1). With the exception of the monocyte count, these responses were all significantly greater than during the second bout of exercise NE ($HE_C > NE_C$, Table 2).

During the final hour of rest, all cell counts initially decreased, but this was followed by a late increase in all counts except that of lymphocytes (Figs. 2, 3). Comparing the two post-exercise periods, there were statistically significant differences in leukocyte count (D > B, $P < 0.0001$) for both NE and HE conditions, with a significant difference for lymphocytes (D > B, $P < 0.007$) in the HE experiment only. Between-group comparisons indicated that the second post-exercise response was greater in HE than NE for all cell types except monocytes ($HE_D > NE_D$, $P < 0.005$). Finally, comparing exercise and post-exercise periods jointly (A + B + C + D), all cell counts rose significantly more in HE than in NE ($P < 0.02$) (Table 2).

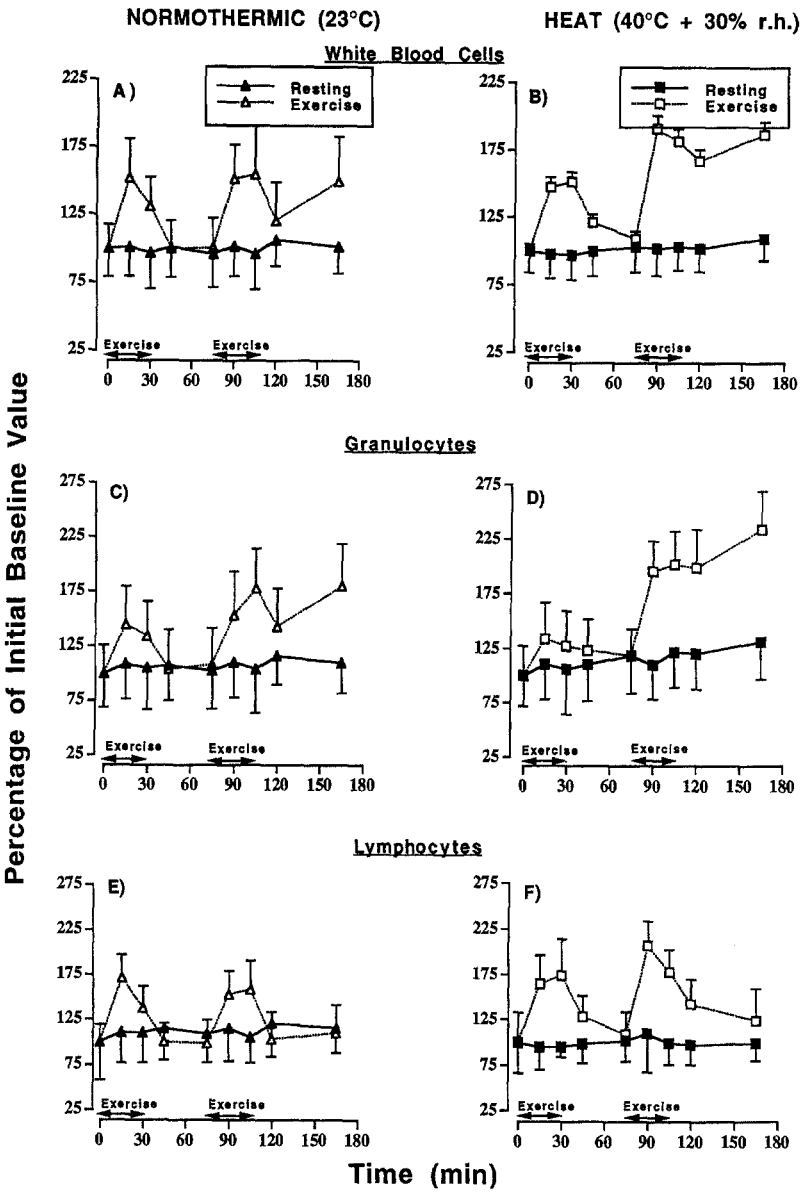

Fig. 2A–F Leukocyte, granulocyte and lymphocyte counts during seated rest conditions (N, 23°C and H, 40°C, 30% r.h.) and repeated exercise (NE, 23°C and HE, 40°C, 30% r.h.). All values are expressed as percentage of initial baseline readings after adjustment for changes in blood volume. (*N* Thermoneutral temperature, *H* heated environment, *NE* exercising at thermoneutral temperature, *HE* exercising in a heated environment)

Regression analysis indicated a significant overall relationship between core temperature increase and total leukocyte cell count ($r = 0.689$, $P = 0.0001$, Fig. 4).

Lymphocyte subsets

T cells

Under N and H conditions there were no significant changes in lymphocyte subsets over the 3 h of observation (Figs. 5, 6). During the first bout of exercise under NE and HE conditions the $CD3^+$ T cell count showed similar increases, with reversion to 14% above baseline for both NE and HE conditions during the first post-exercise period (Fig. 5). Responses to the second bout of exercise differed between NE and HE. During NE, $CD3^+$ cells rose during the entire exercise period, peaking at 54% above baseline after 30 min ($HE_C > NE_C$, $P = 0.02$). During the HE condition, the response peaked after 15 min of exercise (94% above baseline), and then began a decline that continued into the final rest period, 10% above baseline. Comparisons between the two exercise bouts were significant for HE only ($HE_C > HE_A$, $P < 0.03$). During the final hour post-exercise, $CD3^+$ counts showed an initial decrease, with a subsequent increase in NE only ($HE_D > NE_D$, $P = 0.03$) (Table 2).

B cells

During the initial bout of exercise, B cells tended to similar increases under NE and HE conditions. After 15 min of exercise, values had risen by 75% (NE) and

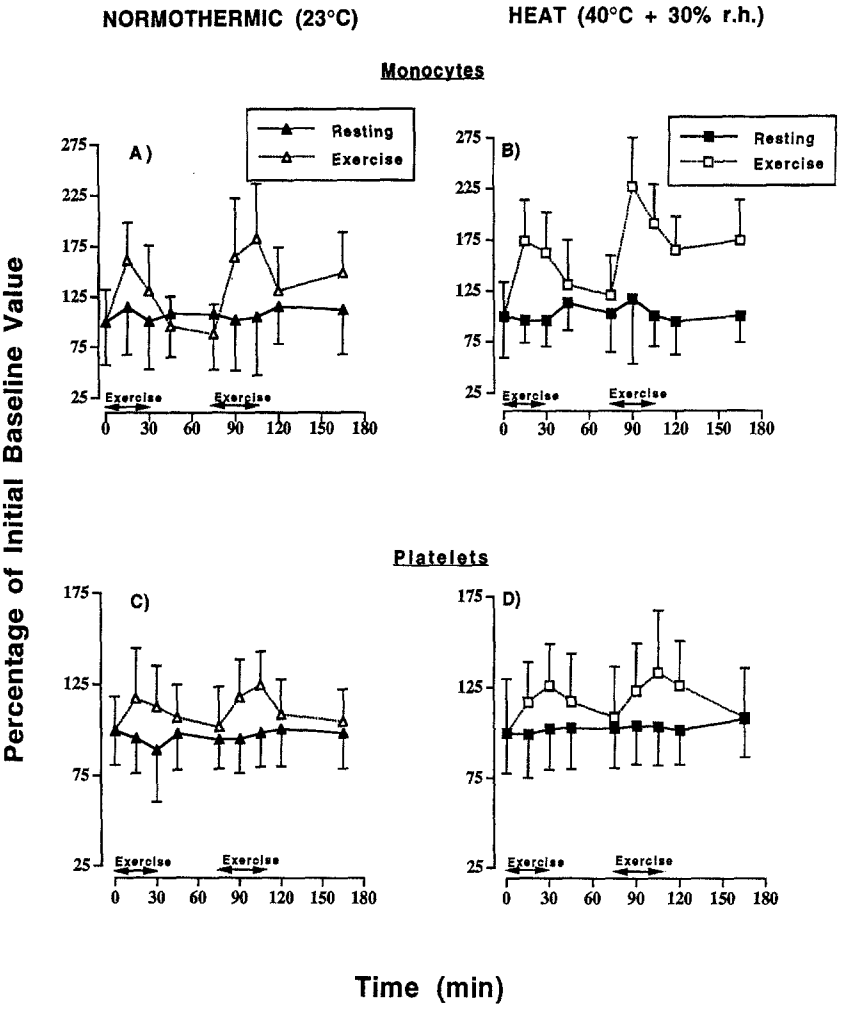

Fig. 3A–D Monocyte and platelet counts during seated rest conditions (N, 23°C and H, 40°C, 30% r.h.) and repeated exercise (NE, 23°C and HE, 40°C, 30% r.h.). All values are expressed as percentage of initial baseline readings after adjustment for changes in blood volume

Table 1 A statistical test of differences in absolute cell counts between the first and second bouts of exercise (A ≠ C) and between the first and second post-exercise periods (B ≠ D) for exercise at a thermoneutral temperature (NE) and under hot conditions (HE, 40°C, 30% relative humidity, r.h.). Only significant comparisons are shown (*WBC* White blood cells, *PHA* phytohemagglutinin)

Cell type and experimental condition	A ≠ C	B ≠ D
Thermoneutral (NE):		
WBC	0.01	0.0001
Monocyte	NS	0.03
Hot (HE):		
WBC	0.0001	0.0001
Granulocytes	0.003	0.0003
Lymphocytes	0.02	0.02
Total T cells ($CD3^+$)	0.03	NS
T helper ($CD4^+$)	0.03	NS
T suppresor ($CD8^+$)	0.02	NS
PHA	0.05	NS

58% (HE) (Fig. 5). However, during HE there was a steady decline to near baseline after the first bout of exercise, whereas during NE values decreased, although not significantly, to 19% above baseline and remained there during the entire rest interval. During the second bout, the $CD19^+$ response after 15 min of exercise in HE was almost twice that seen in NE (50% vs 85%), but contrasts of NE versus HE showed no significant differences for either exercise or for post-exercise periods (Table 2). Contrasts within conditions (A vs C and B vs D) also showed no significant intra-trial differences between the first and second bouts of exercise and rest (Fig. 5).

T helper and T suppressor cells

T helper and T suppressor cell counts did not differ between N and H conditions. The percentage increase of the $CD4^+$ cell count during the first bout of exercise and the values seen during the subsequent rest interval were comparable for NE and HE conditions (Fig. 6). By 45 min post-exercise, values were 6% below baseline in NE and 27% above baseline in HE, but NE_B did not differ significantly from HE_B. During the second bout of exercise, the increase of $CD4^+$ counts in HE continued throughout exercise, reaching 110% above baseline, whereas in NE the increase in $CD4^+$ count

Table 2 A statistical test of differences in cell counts, mitogen response and in vitro immunoglobulin production between exercise in thermoneutral conditions (NE) and under hot conditions (40°C, 30% r.h. = HE). All comparisons are based on "areas under the curve". For areas compared, see Fig. 1. Only significant comparisons are listed. (*PW* pokeweed mitogen, *Ig* immunoglobulin

Area	Cell type	NE ≠ HE	Area	Cell type	NE ≠ HE
A	WBC	0.05	A + B	WBC	0.007
	T suppressor ($CD8^+$)	0.02		Granulocytes	0.05
	PW IgM	0.0003		Monocytes	0.05
				T helper ($CD4^+$)	0.05
				T suppressor ($CD8^+$)	0.008
				PW IgM	0.0001
B	WBC	0.003	C + D	WBC	0.0001
	Granulocytes	0.04		Granulocytes	0.0002
	Monocytes	0.04		Lymphocytes	0.001
	T suppressor ($CD8^+$)	0.01		Monocytes	0.04
	PW IgM	0.0006		Total T cells ($CD3^+$)	0.01
				T helper ($CD4^+$)	0.02
				T suppressor ($CD8^+$)	0.002
				PHA	0.04
				PW IgM	0.0004
C	WBC	0.0005	A + B + C + D	WBC	0.0001
	Granulocytes	0.002		Granulocytes	0.0005
	Lymphocytes	0.006		Lymphocytes	0.008
	Total T cells ($CD3^+$)	0.02		Monocytes	0.02
	T helper ($CD4^+$)	0.02		Total T cells ($CD3^+$)	0.02
	T suppressor ($CD8^+$)	0.003		T helper ($CD4^+$)	0.01
	PW IgM	0.0004		T suppressor ($CD8^+$)	0.001
				PHA	0.03
				PW IgM	0.0001
D	WBC	0.0001			
	Granulocytes	0.0003			
	Lymphocytes	0.005			
	Total T cells ($CD3^+$)	0.03			
	T helper ($CD4^+$)	0.04			
	T suppressor ($CD8^+$)	0.005			
	PHA	0.03			
	PW IgM	0.02			

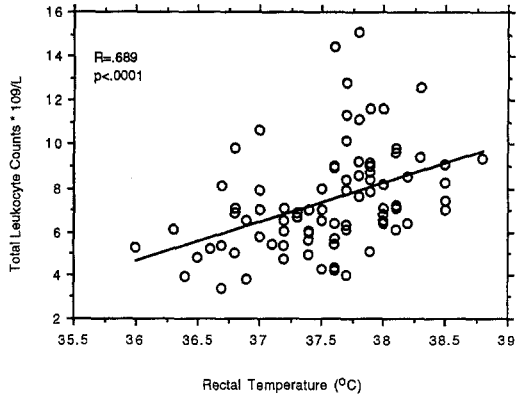

Fig. 4 Relationship between leukocyte and rectal temperature. Linear regression fitted by the method of least squares

plateaued at 39% after 15 min of exercise ($HE_C > NE_C$, $P < 0.02$). Within 15 min following the second bout of exercise, the $CD4^+$ cell count in NE had decreased significantly, to 8% above baseline; in HE the count declined further, to 20% above baseline ($NE_D > HE_D$, $P < 0.04$). Responses to the two bouts of exercise (A vs C) and to the two post-exercise periods (B vs D) did not differ significantly for NE. However, in HE the response to the second bout of exercise yielded a significantly greater response ($HE_C > HE_A$, $P = 0.03$). A comparison of counts for the entire trial showed significantly higher values for HE than for NE ($HE_{A+B+C+D} > NE_{A+B+C+D}$, $P < 0.01$).

The overall response of the $CD8^+$ count to exercise differed significantly between the normothermic and heated environment ($HE_{A+B+C+D} > NE_{A+B+C+D}$, $P < 0.001$) (Table 2). During the initial exercise period, the $CD8^+$ cells in NE peaked after 15 min at 65% above baseline, whereas in HE there was a continued rise throughout the 30 min of exercise to 65% ($HE_A > NE_A$, $P < 0.02$). During NE, recovery was complete by 45 min after the first exercise bout: in HE, the $CD8^+$ count had dropped 2% below baseline ($HE_B > NE_B$, $P < 0.01$) (Fig. 5). The NE and HE responses also differed significantly during the second exercise bout and the subsequent rest period ($HE_C > NE_C$, $P = 0.003$, $HE_D > NE_D$, $P < 0.005$). In both conditions, the $CD8^+$ count peaked after 30 min of exercise, 55% above baseline in NE and 91% in HE. Recovery differed between the two conditions, in that

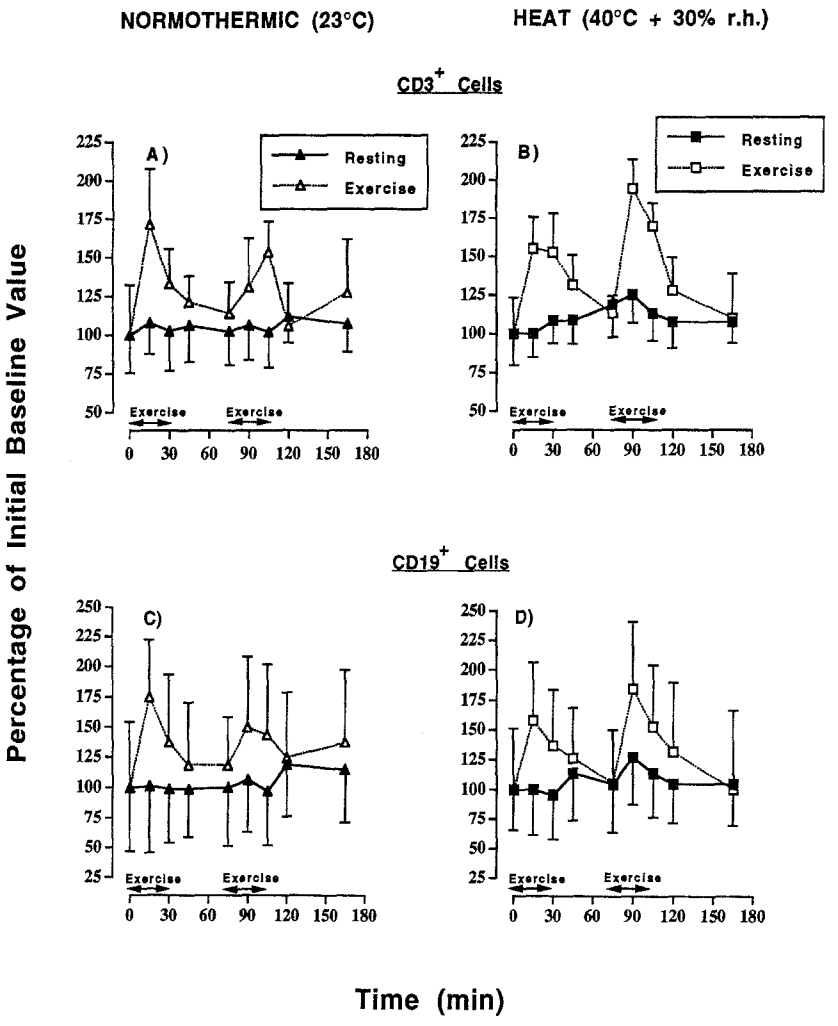

Fig. 5A–D $CD3^+$ (total T cell) and $CD19^+$ (B cell) cell counts during seated rest conditions (N, 23°C and H, 40°C, 30% r.h.) and repeated exercise (NE, 23°C and HE, 40°C, 30% r.h.). All values are expressed as percentage of initial baseline readings after adjustment for changes in blood volume

NE dropped to 12% above baseline at 15 min and then rose to 20% above, whereas HE continued to decline to reach baseline 45 min after ceasing exercise (Fig. 6). Differences in response between the first and second exercise bouts were significant for HE only ($HE_C > HE_A$, $P < 0.02$).

Lymphocyte proliferative response

Data on the proliferation of PBMC in general confirm the impression gained from cell counts. There was a cumulative response, most obvious when the two bouts of exercise had been performed in a warm environment. The proliferative response of blood samples taken during seated control conditions remained unchanged throughout the N and H experiments (data not shown). Cells from the NE and HE experiments showed a non-significant trend to a suppression of PHA-induced proliferation during the first exercise session (Table 3). In NE, the second bout of exercise yielded a similar response as to the first bout, but there was a greater 56% suppression of baseline proliferation in HE ($HE_A > HE_C$, $P < 0.05$). In NE, proliferation rebounded to exceed baseline by 12% 15 min post-exercise, whereas in HE recovery took 1 h ($NE_B > HE_B$, $P < 0.03$). There was also a non-significant trend to reduction of [^{3}H]-thymidine incorporation in both NE and HE, when cells were stimulated with PW (Table 3). After the second bout of NE, values rose to 54% above baseline. In HE, proliferation post-exercise was more variable (Table 3).

Immunoglobulin production

PW stimulated PBMC showed no changes in immunglobulin production over the course of either N or H experiments (data not shown). The response pattern for serum IgM differed significantly between NE and HE conditions (Table 3, $HE_A > NE_A$, $P < 0.0003$, $HE_B > NE_B$, $P < 0.0006$, $HE_C > NE_C$, $P = 0.0004$, $HE_D > NE_D$, $P < 0.02$), but, within-condition comparison showed no differences between first and second exercise bouts (A vs C and B vs D) (Table 1). IgM rose after 15 min of exercise in NE, to 89% above baseline during the first bout and to 42% in the second. A slower increase occurred in HE; in the first bout of

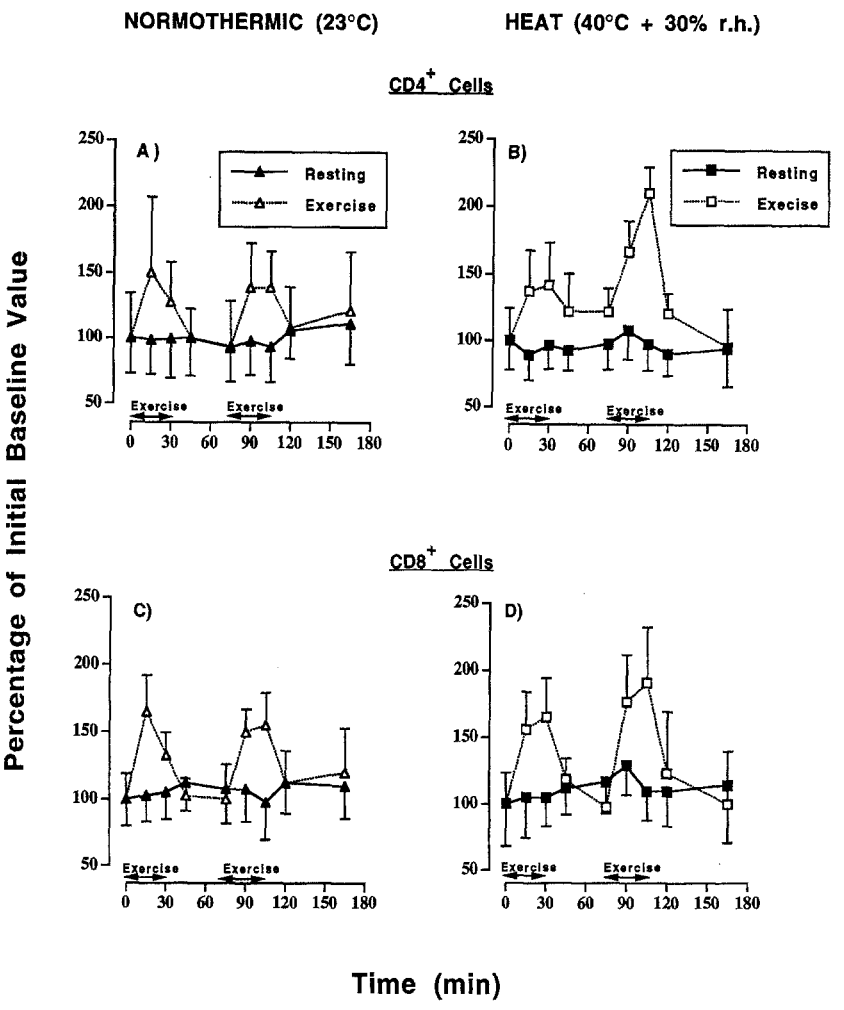

Fig. 6A–D $CD4^+$ (T helper cell) and $CD8^+$ (T suppressor cell) cell counts during seated rest conditions (N, 23°C and H, 40°C, 30% r.h.) and repeated exercise (NE, 23°C and HE, 40°C, 30% r.h.). All values are expressed as percentage of initial baseline readings after adjustment for changes in blood volume

Table 3 Peak change in PHA and PW mitogen-stimulated in vitro proliferation of peripheral blood mononuclear cells (PBMC) and in vitro production of IgM and IgG during exercise in normothermia (NE) and hot (HE, 40°C, 30% r.h.) conditions. Each value represents the mean (SD) of data from 9 subjects. For timing of exercise and rest periods, see Fig. 1

	A NE %	A HE %	B NE %	B HE %	C NE %	C HE %	D NE %	D HE %
PHA	71 (59)	77 (64)	82 (60)	100 (60)	79 (66)	56 (44)	131 (53)	104 (45)
PW	83 (55)	72 (36)	102 (64)	117 (53)	82 (68)	78 (56)	154 (59)	145 (41)
IgM PW	189 (135)	256 (116)	165 (137)	270 (42)	142 (141)	214 (100)	60 (118)	308 (95)
IgG PW	41 (120)	59 (131)	50 (127)	172 (124)	68 (173)	139 (124)	51 (207)	120 (106)

exercise, IgM production was 156% above baseline after 30 min and during the second bout it rose to 114% above baseline, with a continuing rise into the final post-exercise period. Under both NE and HE conditions, IgG production tended to be depressed during the first bout of exercise. In NE, serum IgG levels remained depressed over the entire study period, whereas in HE IgG rose to 72% above baseline 15 min after the first exercise bout, remaining above baseline for the remainder of the observed period (Table 3). Contrasts within and between exercise conditions were not statistically significant.

Discussion

Change in circulating cell counts

Most of the changes observed in this study represent the effects of cell-trafficking between reservoir sites (liver, lungs and bone marrow) and the peripheral blood. Given that only about 1% of the total cell population is found in the peripheral blood, the clinical significance of the exercise-induced changes remains uncertain.

Circulating cell counts were not significantly affected by sitting at either 23°C or 40°C. These results show that during the morning working hours (8–11 a.m.) there is no appreciable circadian effect, and counts are not affected by small changes in rectal temperature. We concur with Kappell et al. (1991a, b): during passive heating, body core temperature must be increased to at least 38°C in order to modify immune parameters. The "typical" changes in leukocyte counts during a single steady-state bout of exercise were unaltered when a second bout of exercise was performed after a 45-min rest interval. However, several new features were observed when the stress of moderate heat exposure was superimposed upon the stress of exercise. To the extent that the empirical blood volume correction was (surprisingly) greater for the temperate than for the warm condition, our differences in cell counts are a conservative estimate of true differences. The new features associated with HE were particularly apparent during the second bout of exercise, and continued into the final post-exercise period.

A single bout of steady-state aerobic exercise leads to a leukocytosis, granulocytosis and lymphocytosis (Hedfors et al. 1983; Landmann et al. 1984; Lewicki et al. 1987; Moorthy and Zimmerman 1978; Oshida et al. 1988; Pedersen 1991; Robertson et al. 1981; Shinkai et al. 1992; Tvede et al. 1993, 1994). In the first 2 h post-exercise, there is a granulocytosis and monocytosis, with an associated lymphopenia. Interval exercise (1 min activity/1 min rest) and heat exposure each produce similar changes (Bouchama et al. 1992; Cameron et al. 1989; Gray et al. 1993; Kappell et al. 1994).

Our results demonstrate a similar biphasic response. Between exercise bouts, most values returned to baseline, although recovery was delayed when exercising in a warm environment. Under normothermic conditions, the second bout of exercise induced a matching cellular response, but in the heat, the core temperature differential was larger for the second bout, and the cellular response was also greater, continuing into the post-exercise period. This suggests some synergism between heat and exercise exposure, possibly due to larger increments in catecholamine and cortisol levels under the hot conditions (I. Brenner, Y. Severs, P.N. Shek et al. in preparation). The release of "stress" hormones mediates both a mobilization of cells into the peripheral circulation and their subsequent escape into the tissues (Bourey and Santoro 1988; McCarthy and Dale 1988). Catecholamine levels may have fallen more slowly during the second post-exercise period, thus modulating the cortisol-related decrease of circulating lymphocyte counts (Weicker and Werle 1991). A similar potentiation of leukocyte and sub-set responses is seen during prolonged exercise (Mackinnon 1991; McCarthy and Dale 1988), where again there is a substantial elevation of core temperature. The differences in rectal temperature between NE and HE conditions were fairly small in our study (an additional 0.26°C for the first exercise bout and 0.51°C for the second bout), but during the second bout they were presumably sufficient to carry subjects above any threshold for a thermal response.

The increase of platelet count (Fig. 3) typifies that observed during 20 min of incremental cycling (Peatfield et al. 1985) or cycle ergometer exercise to exhaustion (Gimenez et al. 1986; Resina et al. 1986). The response reflects a combination of hemodynamic and adrenergic effects, mobilizing stored platelets into the circulation (Resina et al. 1986). In combination with other mediating factors, platelet number may be controlled by proaggregatory factors (Bourey and Santoro 1988; Rudmann 1991).

Changes in lymphocyte subsets

Most authors have found an increase in all lymphocyte subsets at the immediate end of a bout of steady-state exercise, with a fall in counts for all subsets except B cells for at least 2 h following exercise (Kendall et al. 1990; Pedersen 1991; R. Shephard and P.N. Shek in preparation, Shinkai et al. 1992; Tvede et al. 1993, 1994). Gray et al. (1993) had similar findings following rapidly repeated interval exercise (1 min exercise/1 min rest for an average of 15.1 min). Frisina et al. (1994) found that after 25 1-min bouts of exercise, with a 2-min rest between each bout, all cell counts had decreased as early as 3 min post-exercise. Hyperthermia has also resulted in a decrease in circulating $CD3^+$ and $CD4^+$ counts: however, either no change in $CD19^+$ or $CD8^+$ counts (Kappell et al. 1991a–c), or an increase in the $CD8^+$ count 2 h post-exposure (Bouchama et al. 1992) have been demonstrated.

Our results for the first bout of exercise reflect the commonly observed pattern. After the first exercise bout, all cell counts declined, but, with the exception of the $CD4^+$ count (NE and HE) and $CD8^+$ count (HE only), values remained above baseline. In the HE condition all cell counts also decreased to near baseline over the second post-exercise period. However, in the NE experiment, all cell types showed a rebound following the second period of exercise.

Differences in response between the NE and HE experiment can apparently be explained by the greater increase of rectal temperature in HE, with resulting larger increments in catecholamine and cortisol levels (I. Brenner, Y. Severs, P.N. Shek et al. in preparation). It might be argued that catecholamine levels fell more slowly during the second post-exercise period and thus may have modulated the commonly observed cortisol-related decrease of lymphocyte counts post-exercise (Weicker and Werle 1991), but this seems a minor factor, given the short half-life of catecholamines.

Mitogen proliferative response

Mitogen-stimulated proliferation of PBMC is decreased during a single bout of aerobic exercise (Eskola et al. 1978; Fitzgerald 1988; Gmünder et al. 1986; Hedfors et al. 1983; Landmann et al. 1984; Nehlsen-Cannarella et al. 1991b; Oshida et al. 1988; Robertson et al. 1981; Shinkai et al. 192; Tvede et al. 1993). Likewise, intense interval exercise (25 repetitions of 1 min at 112% of maximal aerobic power) reduced the responsiveness of peripheral blood lymphocytes to the lectin Concavalin A (CON A) (Frisina et al. 1994). However, Cameron et al. (1989) found no consistent changes in [^{3}H]DNA response to CON A, PHA or PW following more moderate interval exercise. Likewise when cells were incubated in vitro at 39°C, the proliferative response of PBMC to PHA was unchanged by sauna exposure (Kappel et al. 1991). Animal studies in which animals were exposed for 5 days to a heated atmosphere (35°C or 36°C) revealed either a depressed mitogen response (Regnier and Kelly 1980) or no effect (Kelly et al. 1982).

We used both PHA (a non-specific mitogen) and PW. The latter is mainly a B cell mitogen, although it also stimulates T cell proliferation (Keast et al. 1988; Verde 1992). Our results concur with the observations made during single bouts of endurance exercise: the proliferative response of circulating cells is decreased during exercise, the decrease in PHA response being exacerbated when a second bout of exercise was performed in the heat. A constant number of PBMC was used in the assay, so that the decreased mitogen response could reflect either a change in responsiveness of a given cell population or a shift in the relative proportions of the various lymphocyte subsets, (for example, an increase in natural killer cells or B cells and a decrease in the T cell or the T helper/T suppressor ratio; Nieman 1994). The cell counts seem to confirm the second hypothesis: as counts for the various subsets returned to baseline levels, resting PW and PHA responses were restored.

In vitro immunoglobulin synthesis

Several studies have examined immunoglobulin levels in serum and other body fluids during and following single bouts of aerobic exercise, but no previous studies have looked at the response to repeated bouts of exercise with and without heat exposure. Changes of serum immunoglobulins following a single bout of aerobic exercise are generally small and statistically insignificant (Asgeirsson and Bellanti 1987; MacKinnon 1991; Nehlsen-Cannarella 1991a; Stephenson et al. 1985). Pedersen et al. (1994) noted that M. Kayal, B.K. Pederson, N.H. Secher et al. (unpublished) had found significant increases of serum IgM but not of IgA serum 2 h after a sauna exposure.

Our results showed that the capacity of the blood specimens to synthesize immunoglobulin was unaffected by exercise. However, when the additional stressor of heat was imposed, immunoglobulin synthesis (particularly IgM) was increased. Antibody production depends on B and T cell counts; T helper cells aid and T suppressor cells inhibit or control the process (Stobo 1987). Specific concentrations of leukocytes were stimulated at each time interval, and increases in the proportion of T helper and B cells within the specimen could explain the increase of immunoglobulin synthesis seen with the double stress of heat plus exercise (Nieman and Nehlsen-Cannarella 1994).

In conclusion the present results show that circulating leukocyte counts, mitogen response and in vitro immunoglobulin production are all highly influenced by exercise, but are little affected by moderate heat stress. A combination of repeated bouts of exercise with heat stress has a cumulative effect, attenuating the immune responses of circulating leukocytes and creating a disturbance that persists post-exercise. Nevertheless, moderate heat stress adds only a small stimulus to the basic response induced by moderate exercise, and normal function is restored relatively quickly post-exercise. We conclude that unless the combined stresses of exercise and heat exposure cause a major increase of core temperature, clinically significant disturbances of immune function are unlikely. Although regression analysis indicates an overall relationship between rectal temperature and cell counts, the rise of core temperature may be serving mainly as a trigger to release of mediating hormones.

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