Leukocyte counts and lymphocyte responsiveness associated with repeated bouts of strenuous endurance exercise

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Ronen, Ola, Bente Klarlund Pedersen, Tone Rasmussen Ørtsland, Roald Bahr, and Jens Kjeldsen-Kragh. Leukocyte counts and lymphocyte responsiveness associated with repeated bouts of strenuous endurance exercise. J Appl Physiol 91: 425–434, 2001.—This study compared leukocyte counts and lymphocyte responsiveness during and after a second bout of high-intensity endurance exercise on the same day with the response to a single bout of exercise. Nine athletes participated in three 24-h trials: 1) rest in bed (Rest); 2) one bout of exercise (One); and 3) two bouts of exercise (Two). All bouts consisted of 75 min at ~75% of maximal O2 uptake on a cycle ergometer. Lymphocytes in whole blood were stimulated with monoclonal antibodies against CD2 and assessed by flow cytometry for expression of the early activation molecule CD69. The second bout of exercise in the Two trial was associated with significantly increased concentrations of total leukocytes, neutrophils, lymphocytes, CD4+, CD8+, and CD56+ cells and a significantly decreased percentage of CD56+ cells expressing CD69 compared with a single bout. Additionally, there was a significantly decreased CD69 fluorescence in CD56+ cells postexercise. These differences suggest a “carry-over” effect in the immune system from a first to a second bout of exercise on the same day.

CD69 expression; lymphocyte activation; natural killer cells; elite athletes; immunity

A single bout of high-intensity endurance exercise leads to highly stereotyped changes within leukocyte subsets in peripheral blood in which both neutrophil and lymphocyte concentrations increase markedly during intensive exercise. However, during subsequent recovery, lymphocyte counts fall below basal values, whereas the neutrophils remain elevated for several hours (22, 30). Although recruitment of all lymphocyte subpopulations to the vascular compartment has been reported, natural killer (NK) cells show the largest increase during strenuous exercise (16, 39, 42, 49). Compared with the resting state, mobilized cells from the endothelial wall, lungs, bone marrow, lymphatic organs, etc. (36, 52) differ with regard to maturity and functional capacities, such as adhesion, activation, proliferation, migration, as well as cytokine and antibody production (16, 18, 20, 34). Because changes in both concentrations and function of leukocytes are linked to the intensity and duration of exercise (14, 39), measurements of such changes become interesting when the immune response to a new protocol with repeated bouts of exercise is assessed.

In contrast to a vast number of investigations on immune cell responses to a single bout of exercise, there is limited information on how repeated bouts of exercise on the same day affect the immune system. The few studies that have measured changes in concentration and function of leukocytes associated with repeated bouts of exercise have all used different exercise and recovery protocols, as well as subjects with varying training status (11, 19, 31, 35, 46, 48, 50). A methodological limitation that applies to all of the previous investigations is the lack of control for diurnal variations in various blood constituents, as the response to a first bout of exercise in the morning has been compared with a second or third bout later on the same day. Besides, the exercise and recovery protocols used in the aforementioned studies hardly reflect the daily exercise and recovery regime practiced by most elite endurance athletes.

Athletes in endurance sports are reported to experience increased susceptibility to respiratory tract infections after periods of heavy training and strenuous competitions (24, 26, 38, 44). Based on the temporary immunosuppressive changes that have been observed a few hours after prolonged heavy exertion (17, 25, 39), the “open window” theory has been proposed. This suggests that repeated bouts of strenuous exercise with incomplete immunological recovery could leave an athlete with increased risk of infections (40, 43). It is, therefore, important to determine whether or not a second bout of high-intensity exercise on the same day is associated with increased perturbations in the immune system.

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A commonly used method for evaluating the functional capacity of lymphocytes is the assessment of mitogen- or antigen-induced proliferative responses by measuring incorporation of \(^{3}H\)thymidine in DNA. Another method frequently applied to measure NK cell activity is the \(^{51}Cr\) release assay, for which the percentage of lysed target cells is the end point. The results reported on exercise-induced changes in proliferative responses of lymphocytes have, for the most part, been inconsistent (34). However, a decreased NK-cell activity after prolonged, strenuous exercise has been demonstrated by several investigators (37, 42). More recently, flow cytometry has been used to assess lymphocyte responsiveness to mitogens by measuring the expression of CD69 on lymphocyte subsets in whole blood (12, 23). Because the CD69 molecules appear on the surface of lymphocytes only a few hours after stimulation (29, 54), this is a rapid method that provides an accurate measurement of lymphocyte responsiveness compared with the \(^{3}H\)thymidine assay (28, 53). Furthermore, it does not require isolation of mononuclear cells from the blood, thus avoiding the separation of soluble factors in serum known to influence lymphocyte responsiveness (1). For these reasons, a flow cytometric determination of CD69 expression was chosen to assess lymphocyte responsiveness in the present investigation.

The aim of this investigation was to study the changes in concentrations of leukocyte subsets and mitogen-induced lymphocyte activation during and after one and two bouts of high-intensity endurance exercise. Moreover, we wanted to compare the changes induced by a single bout of exercise with the changes induced by a second bout of similar exercise on the same day.

METHODS

Subjects

Nine male, elite endurance athletes [age 21–27 yr, weight 74.7 ± 5.4 kg (mean ± SE), maximal \(O_2\) uptake \((V_{O2 max})\) 69.1 ± 3.7 ml·min\(^{-1}\)·kg\(^{-1}\); 4 triathletes and 5 speed skaters from the national teams] participated. All subjects were accustomed to two daily training sessions as part of their exercise schedule, including cycling as one of the training modalities. A medical examination of each subject was performed before the subjects entered the study, and they were thoroughly informed about the purposes and procedures of the study before a written consent was obtained. The protocol was approved by the Regional Ethics Committee for Medical Research in Norway.

Design

Each subject participated in three trials, each lasting from 0700 to 0800 the next day: 1) complete bed rest (Rest); 2) one bout of exercise from 1515 to 1630 (One); and 3) two bouts of exercise, one from 1100 to 1215 and the other from 1515 to 1630 (Two) (Fig. 1). Trials were separated by 12–17 days to ensure complete recovery between trials and were randomized in a counterbalanced order, with each subject serving as his own control. The triathletes were tested between January and March, and the speed skaters between April and June, i.e., outside the competitive season for both groups. Except for the last 2 days before each trial, when exercise was regulated by the study protocol, the subjects completed their regular training program without any interruption during the study period.

Pretrial Procedures

Approximately 2 wk before the start of the study, the subjects performed an incremental exercise test on a cycle ergometer (Lode, Groningen, the Netherlands), starting at a workload of 175 W with a subsequent increase of 25 W every 5 min until they had reached a workload of 275 W. The subjects then rested for 10 min before a continuous ramp test was used to estimate \(V_{O2 max}\), starting at 275 W with a subsequent increase of 25 W every 30 s until volitional exhaustion (i.e., the subject could not sustain the workload for a period of >30 s). A respiratory quotient > 1.1 was used as an additional criterion that \(V_{O2 max}\) had been reached. The results were used to estimate a workload corresponding to 70% of \(V_{O2 max}\) for each subject based on the regression line of \(O_2\) uptake vs. workload from the incremental exercise test.

Serum...
ferritin and whole blood hemoglobin concentrations were measured 2 wk before the first trial and at the end of each trial. Iron supplementation was given if serum ferritin concentration was <30 μg/l but discontinued 7 days before each trial. Hemoglobin concentration was measured again in the morning before each trial, and the trial was postponed for 1 wk if the concentration was reduced by >1.0 g/dl from the previous trial. If a subject had an episode of illness with fever or malaise, the trial was postponed until he was without symptoms or medication for at least 5 days.

High-intensity exercise was not allowed during the last 2 days before trials, and no exercise was permitted the last day before each trial. A dietary record was obtained for the last 24 h before the first trial, and the subjects were instructed to consume an identical diet the day before each subsequent trial. A standardized meal of cereal and milk was served at 2100 the evening before each trial, and the subjects spent the night at the National Sports Centre next to the laboratory.

**Trial Procedures**

The subjects arrived in the laboratory at 0700, emptied their bladder, had their body weight measured, and were subsequently put to bed. A flexible temperature probe was inserted in the rectum, and the subjects were connected to a temperature, electrocardiogram, and heart rate monitor (Siemens SC 6000 P, Siemens Medical Systems). A flexible intravenous catheter (Venflon 1.2; 32 mm; BOC Health Care, Helsingborg, Sweden) was inserted into an antecubital vein and kept there for the whole trial.

The exercise bouts were performed in the morning between 1100 and 1215 (Ex-M; only in the Two trial) and in the afternoon from 1515 to 1630 (Ex-A; One and Two trials). The Ex-M and Ex-A bouts were equal in both intensity and duration and consisted of a 10-min warm-up period at 50% of VO2 max, immediately followed by 65 min at the subject’s predetermined workload and a cadence of 90–100 rpm. The O2 uptake was measured for 60 s after 15, 30, 45, 60, and 70 min of exercise. The ambient room temperature was kept at 20 ± 2°C and humidity at 40 ± 10%.

The subjects rested in bed at all times when they were not exercising and spent the following night sleeping in the lab until 0700 the next morning. The subjects were allowed to read and thus had a 45° angle headrest except for the last 15 min before each blood sampling. They were allowed to listen to music during exercise and rest, but TV watching was restricted to a maximum of 3 h in the evening between 1800 and 2200. No sleep was allowed during the day.

During each trial, the subjects were served four standardized meals at 0830, 1330, 1740, and 2145 (Fig. 1). The meals consisted of double sandwiches with butter, ham, cheese, jam, and honey: three for breakfast, four for lunch, three for dinner, and four for supper, (4,000 kcal/trial). The same type and number of sandwiches were served during each trial. Water was consumed ad libitum during exercise and recovery except for the first 60 min postexercise, when O2 uptake was measured continuously. Body weight was measured using a scale (Seca) at 0710, 1510, 2140, and again at 0710 the next morning.

**Sampling Protocol**

The first blood sample was drawn at 0730, and subsequent samples were obtained according to the schedule outlined in Fig. 1. To avoid coagulation, 0.3 ml heparin (100 IE) were injected into the catheter after each sampling. All samples were collected during bed rest, except for the sample taken during the last minute of the exercise, which was obtained with the subjects seated on the cycle ergometer. At each sampling, blood was collected in a 3-ml 21% K3 EDTA tube for hemoglobin, hematocrit (Hct), neutrophil, and lymphocyte count. Blood for CD4+, CD8+, and CD56+ cell analysis was collected in a 3-ml heparinized tube (Becton-Dickinson vacutainer system). The EDTA and heparin tubes were kept on a Mixer 440 (Swelab Instrument, Stockholm, Sweden) until they were analyzed later the same day.

**Measurements**

During the pretrial test, the O2 uptake was measured during the last 90 s at each increment using an automated Oxycon Champion System (Erich Jaeger) with a nose clip and a Rudolph mouthpiece, and gas exchange was recorded for every expiration. In the three study trials, the O2 uptake was measured by collection of expired air in Douglas bags (Hans Rudolph, Kansas City, MO). The bags were emptied through a flow controller (flow transducer K 520; K. L. Engineering) and volume counter (spirometric module; K. L. Engineering). The CO2 and O2 content were measured on an O2 analyzer (oxygen analyzer, model S-3A, and oxygen sensor, model N-22M; Ametek, Pittsburgh, PA), and a CO2 analyzer (carbon dioxide analyzer, model CD-3A, and carbon dioxide sensor, model P-61B, Ametek). Additional measurements of air pressure and temperature were performed on an EOS-sprint system (Erich Jaeger).

The total number of leukocytes, neutrophils, lymphocytes, hemoglobin, and Hct were analyzed on a Sysmex K 1000 cell counter (Toa Medical Electronics, Kobe, Japan). All cell counts were corrected for plasma volume changes relative to the values from the first morning sample, according to the method of Dill and Costill (10).

**Assessment of Lymphocyte Subsets and Activation**

T and NK cells were stimulated by a mixture of two monoclonal antibodies (MAbs) against CD2, clone L303.1, IgG2a; and CD2R, clone L304.1, IgG1 (Becton Dickinson, San Jose, CA). Five and zero micrograms of each of these antibodies were added to 300-μl aliquots of fresh samples of heparinized blood, and the mixtures were incubated for 4 h at 37°C. After the incubation, each of these mixtures was further aliquoted into three 90-μl aliquots. The following fluorochrome-conjugated MAbs were added to the three tubes of each set of aliquots corresponding to 10 and 0 μg of the stimulating MAbs: 1) anti-CD69, clone L78, IgG1, FITC conjugated (Becton Dickinson) plus anti-CD4, clone RPA-T4, IgG1, phycoerythrin (PE) conjugated (Serotec, Oxford, UK) plus anti-CD8, clone SK1, IgG1, peridinin chlorophyll protein conjugated (Becton Dickinson); 2) anti-CD69, FITC (Becton Dickinson) plus anti-CD56, clone B-A19, IgG1, PE conjugated (Serotec); 3) mouse IgG1, clone X40, FITC conjugated (Becton Dickinson) plus mouse IgG1, clone W3/25, PE conjugated (Serotec) plus mouse IgG1, clone X40, peridinin chlorophyll protein conjugated (Becton Dickinson). After 30-min incubation at room temperature, the erythrocytes were lysed by a Multi-Q-Prep (Coulter, Miami, FL). After the addition of 50 μl of Flow-Count (Coulter), the samples were analyzed by a FACSScan or FACSCalibur (Becton Dickinson) flow cytometer.

The lymphocytes were identified on the forward-light vs. side-light scatterplot and the Flow-Count fluorospheres on the plot of green vs. orange light. The degree of activation was assessed in two ways: 1) as the percentage of the total number of CD4+, CD8+, and CD56+ cells that expressed the CD69 molecule; and 2) as the degree of fluorescence from CD69 molecules on the CD4+, CD8+, and CD56+ cells. The
latter was expressed as a ratio of the geometric mean of the CD69 fluorescence of stimulated cells (10 μg of anti-CD2/CD2R) divided by nonstimulated cells (0 μg of anti-CD2/CD2R). Estimation of absolute cell numbers was based on the acquired numbers of Flow-Count fluorospheres.

Statistical Analyses

The analysis of changes in total leukocyte, neutrophil, and lymphocyte counts was done with an ANOVA procedure for repeated measures to estimate main effects (trial or time) and interaction effect (trial × time), including all three trials (Rest, One, and Two). Nine measurements from 1500 until 0730 the next morning were included, and the Huynh-Feldt method for adjustment of degrees of freedom for the P-tests was applied. Where significant effects were found, separate tests were performed for effects of exercise (One and Two vs. Rest trials) and for the effect of a previous bout (Two vs. One trial). Because changes in the CD4<sup>+</sup>, CD8<sup>+</sup>, and CD56<sup>+</sup> cells and their respective CD69 expression occurred mostly during exercise, we analyzed the lymphocyte subset concentrations with t-tests. When we tested for the effect of exercise, pre- and postexercise concentrations in each trial were compared using a paired t-test. When we tested for the effect of a previous bout, changes in pre- to postexercise concentration (delta values) in the Two vs. One trial were performed using an unpaired t-test. Additionally, t-tests were used for pre- and posttrial comparisons and for comparisons at the same time point in the different trials.

Results are presented as means ± SE unless otherwise noted. When the ANOVA results are reported, time effects are given when testing was done for the effect of exercise, and trial by time effects are given when testing was done for the effect of a previous bout of exercise. P and adjusted F values are presented, and P values < 0.05 were considered significant.

RESULTS

O<sub>2</sub> Uptake and Plasma Volume Changes

Mean O<sub>2</sub> uptake during Ex-A (5 measurements) was higher in the Two (52.2 ± 0.6 ml·min<sup>-1</sup>·kg<sup>-1</sup>) than One trial (49.4 ± 0.4 ml·min<sup>-1</sup>·kg<sup>-1</sup>; P = 0.002). There was no difference in rectal temperature during exercise between the One and Two trials (38.8 ± 0.1 and 39.0 ± 0.1°C, respectively; P = 0.1). The 24-h water intake during the Rest, One, and Two trials was 2.5 ± 0.2, 3.1 ± 0.3, and 3.9 ± 0.3 liters, respectively. Based on the Hct values, we estimated a plasma volume decrease of 13% (Hct 37.1 ± 0.7 to 41.9 ± 0.7; P = 0.001) in the One trial and 12% (Hct 37.2 ± 1.0 to 41.5 ± 1.0; P = 0.001) in the Two trial from pre- to postexercise. However, after 15 min of recovery, Hct had increased to preexercise values (Hct 37.2 ± 0.8 and 36.8 ± 0.9 in One and Two, respectively).

Leukocyte Concentrations

Effect of exercise. During exercise, there was an increase in concentration of total leukocytes, neutrophils, and lymphocytes (all P < 0.001; Fig. 2), as well as for CD4<sup>+</sup>, CD8<sup>+</sup>, and CD56<sup>+</sup> cells (all P < 0.01; Fig. 3) in both the One and Two but not Rest trials.

Effect of previous bout. Before the start of Ex-A (1500), changes in cell concentrations from Ex-M in the Two trial had normalized for all leukocyte subpopulations, except for the neutrophils, which were elevated (Two: 6.9 ± 0.5 vs. Rest: 3.0 ± 0.2 × 10<sup>9</sup>/l; P < 0.001; Fig. 2B).

During the time period from 1500 to 0730, we observed increased concentrations of total leukocytes (F<sub>1,64</sub> = 8.6, P < 0.001), neutrophils (F<sub>1,64</sub> = 6.6, P < 0.001), and lymphocytes (F<sub>1,64</sub> = 8.3, P < 0.001) in the Two compared with the One trial (Fig. 2). During the recovery period, lymphocyte counts had normalized after 1 h of rest (Fig. 2C), whereas the neutrophil counts were elevated until 4 h postexercise (Fig. 2B). At the end of the three trials (0730), there was no difference between the concentrations of the leukocyte subsets, and no differences in cell counts were observed from the beginning to the end of each trial.

The increase in concentration from pre- to postexercise was larger in the Two compared with the One trial for CD4<sup>+</sup> cells (t = 3.36, P = 0.01), CD8<sup>+</sup> cells (t = 3.26, P = 0.01), and CD56<sup>+</sup> cells (t = 2.49, P = 0.04). However, for all subsets, the concentrations were normalized after 4 h of recovery. Again, no differences in cell counts were observed from the beginning to the end of each trial.

Lymphocyte Activation

Effect of exercise. There was no effect of exercise on the expression of the CD69 activation marker on CD4<sup>+</sup> and CD8<sup>+</sup> cells, neither in mitogen-stimulated cells (Fig. 4, A and B) nor in unstimulated cells (data not shown). However, in the CD56<sup>+</sup> cells, a decrease in the percentage of cells expressing the CD69 molecule was observed in the Two but not the One trial, both for stimulated (t = 3.05, P = 0.02; Fig. 4C) and unstimulated CD56<sup>+</sup> cells (t = 2.31, P = 0.05, data not shown). Furthermore, we observed a decrease in the CD69 fluorescence intensity ratio on CD56<sup>+</sup> cells (t = 2.76, P = 0.02; Fig. 5C) in the Two trial but not in the One trial (t = 1.46, P = 0.1). No significant change in CD69 fluorescence intensity ratio was observed for CD4<sup>+</sup> or CD8<sup>+</sup> cells during exercise (P = 0.1 and P = 0.07; Fig. 5, A and B, respectively).

Effect of previous bout. When comparing the changes in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> cells expressing the CD69 molecule in the One trial with those in the Two trial, we observed no effect of a previous bout of exercise (Ex-M) in either mitogen-stimulated cells (CD4<sup>+</sup>, P = 0.2; CD8<sup>+</sup>, P = 0.2; Fig. 4, A and B, respectively) or in unstimulated cells (data not shown). However, in the CD56<sup>+</sup> cells, there was a larger decrease in the percentage of stimulated cells expressing the CD69 molecule during Ex-A in the Two compared with the One trial (t = 2.67, P = 0.03; Fig. 4C). This was not observed in the unstimulated cells (P = 0.3, data not shown).

Regarding the CD69 fluorescence intensity ratio, we observed no significant difference in the fluorescence intensity changes between the Two and One trials during Ex-A for CD56<sup>+</sup> cells nor for CD4<sup>+</sup> and CD8<sup>+</sup> cells (P = 0.2, P = 0.07, and P = 0.09, respectively; Fig. 5, A and B, respectively).
5). However, the decrease in fluorescence intensity ratio for CD56$^+$ cells from pre- to postexercise was larger in the Two trial ($t = 2.76, P = 0.02$) but not in the One ($t = 1.40, P = 0.2$) compared with the Rest trial (Fig. 5C).

**DISCUSSION**

The main finding of this study was that the second bout of high-intensity endurance exercise on the same day was associated with higher concentrations of neutrophils, lymphocytes, and CD4$^+$, CD8$^+$, and CD56$^+$ cells compared with a single bout of endurance exercise performed at the same time of the day. Furthermore, at the end of the second bout of exercise, we observed both a decreased percentage of CD56$^+$ cells expressing the activation marker CD69 and a decreased density of these molecules on the CD56$^+$ cells after stimulation with mitogen.

In contrast to previous investigations on leukocyte responses to repeated exercise (11, 19, 31, 35, 46, 48), the present study used a design that controlled for potential confounding effects of diurnal variations in blood constituents (Fig. 1). Consequently, this allowed us to compare the cellular responses to a single (first) bout of exercise with the responses to a second bout of exercise on the same day.

In a study using three bouts of 6-min “all-out” rowing, separated by 4 h of rest, Nielsen et al. (35) reported increased levels of neutrophils, lymphocytes, and CD4$^+$, CD8$^+$, and CD16$^+$ cells, as well as increased NK-cell activity at the end of the second and third bout of exercise. Another study from the same laboratory investigated athletes performing three repeated bouts of cycle ergometer exercise at 75% of $V_{O_2\ max}$, lasting 60, 45, and 30 min and separated by 2 h of rest (46). Successively increasing concentrations of neutrophils...
During the three bouts were found, whereas the magnitude of the lymphocytosis, including both T cells and NK cells, did not differ among the three exercise bouts. The fact that the duration of exercise in the latter study was reduced from 60 min on the first bout to 45 and 30 min on the second and third bouts complicates the comparison of the results.

A more pronounced increase in neutrophils, but not in lymphocytes, was also found during and after the second bout of exercise in two other studies: one using two 30-min bouts of exercise at 70% of peak O₂ uptake separated by 3 h of rest (31), and the other using two 30-min bouts of exercise at 50% of peak O₂ uptake separated by only 45 min of rest (48). In the latter study, no difference between the first and second bout was observed with regard to recruitment of T cells and NK cells or NK-cell activity (7, 48). The observation of an increased neutrocytosis associated with the second bout of exercise in the present study supports these previous findings. However, the increased lymphocytosis also found in the present study has only been demonstrated by Nielsen et al. (35) with their 6-min maximal exercise protocol. Therefore, this finding may be more linked to the intensity rather than the duration of exercise.

The reduced percentage of CD56⁺ cells expressing the CD69 molecule and the decreased density of CD69 observed after the second bout of exercise in the present study could have at least two explanations, not mutually exclusive. A subpopulation of CD56⁺ cells with a lower capacity of expressing CD69 after stimulation with anti-CD2 MAbs may have been recruited to the circulation, for example, because of decreased expression of the CD2 molecule. Alternatively, the second bout of exercise suppressed the functional capacity of the existing CD56⁺ cells in peripheral blood, resulting in a reduced number of CD69 molecules on the CD56⁺ cells after mitogen stimulation.

On activation, the CD69 molecule is expressed on cells of most hematopoietic lineages, including lympho-
cytes, neutrophils, and eosinophils, whereas it is constitutively expressed on monocytes, platelets, and epidermal Langerhans cells (29). In lymphocytes, expression of CD69 has been linked to cell activation, proliferation, apoptosis, and cytotoxicity (5, 51, 54). Cloning of the molecule has shown that it belongs to the NK-cell gene complex of signal transduction receptors (9). The physiological function of CD69 is presently not established, although it has been suggested to be involved in the pathogenesis of certain diseases. Therefore, the clinical significance of our findings concerning changes in mitogen-induced expression of CD69 is uncertain. However, the wide cellular distribution of CD69 and its linkage to intracellular signal transduction suggest a central role for this molecule in the function of hematopoietic cells.

NK cells are defined by the expression of CD56 and/or CD16 and the absence of CD3. The CD2 molecule, which we used in stimulation of the cells, is expressed on 70–90% of NK cells and >95% of T cells. Thus the majority of the CD56^+ cells expressing CD69 after stimulation were most likely NK cells (45). Hence, it is likely that the reduced state of activation of the CD56^+ cells observed after the second bout of exercise was associated with a decreased cytotoxic activity in NK cells. This assumption is supported by the findings of Borrego et al. (4), who demonstrated a parallel impairment of CD69 expression and killing capacity of interleukin-2-stimulated NK cells in elderly people using a ^51Cr release assay. Furthermore, Rhode et al. (46) observed suppressed lymphokine-activated killer cell activity after the last of three bouts of exercise on the same day, thus substantiating our suggestion that repeated bouts of high-intensity endurance exercise are associated with suppressed NK-cell activity. However, because the CD69 expression of CD56^+ cells in the present study was normalized at 4 h post-exercise, this change is probably temporary.

Fig. 4. Mean ± SE percentage of CD4^+ (A), CD8^+ (B), and CD56^+ (C) cells that expressed the CD69 molecule at 5 time points during the 3 trials (Rest, One, and Two). Light and dark shaded bars show Ex-M and Ex-A, respectively.
Several exercise experiments suggest that epinephrine and, to a lesser degree, norepinephrine mediate the early exercise effects on lymphocyte subpopulations, whereas cortisol is more involved in the postexercise concentration changes (6, 15, 21, 27, 33). The immediate exercise effect on neutrophils is associated with changes in both catecholamines and growth hormone (42). In a recent publication (47), our laboratory described the endocrine changes in the subjects participating in the present study. We observed significantly larger increases in plasma levels of epinephrine, norepinephrine, growth hormone, ACTH, and cortisol associated with the second bout of exercise. This suggests that the increased mobilization of leukocytes into the circulation found in the present study is associated with an increased neuroendocrine response.

Moreover, a close relationship between changes in concentrations of catecholamines, β-adrenergic antagonists, as well as cortisol and altered expression of various adhesion molecules on vascular endothelial cells and circulatory leukocytes has been documented (2, 3, 8, 32, 41). Decreased synthesis, internalization, or shedding of these surface molecules can induce a subsequent detachment of specific leukocyte subpopulations from the vascular bed in peripheral tissues and organs. Thus the mobilization of different leukocyte subpopulations into the circulation during strenuous exercise may at least in part be determined by the level of stress hormones and their effect on adhesion molecules expressed on leukocytes and endothelial cells (13, 52).

Our findings of more pronounced changes in leukocyte counts and lymphocyte responsiveness associated with the second bout of exercise demonstrate that there is a carryover effect on these immune cells from the previous exercise session. Thus one could speculate whether successive training sessions without complete recovery eventually may lead to a suppression of an athlete’s immunocompetence and subsequently in-

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Fig. 5. Mean ± SE CD69 fluorescence intensity ratio (FIR) of CD4⁺ (A), CD8⁺ (B), and CD56⁺ (C) cells at 5 time points during the 3 trials (Rest, One, and Two). FIR is expressed as the geometric mean of CD69 fluorescence from stimulated cells (10 μg of anti-CD2/CD2R) divided by nonstimulated cells (0 μg of anti-CD2/CD2R). Light and dark shaded bars show Ex-M and Ex-A, respectively.
creased risk of infections. However, the link between exercise-induced immunosuppression and increased risk of infections has not yet been proven conclusively. To provide evidence for such a link, further investigations could apply an intensive repeated exercise protocol over a longer time period. In addition to the immune parameters studied here, examination of in vivo cellular responses to immunological challenges, such as skin tests, may be performed along with registration of infectious episodes verified by microbiological and/or serological tests.

Conclusion

This investigation has demonstrated that a second bout of high-intensity endurance exercise on the same day was associated with a more pronounced change in concentrations of neutrophils, lymphocytes, and CD4\(^+\), CD8\(^+\), and CD56\(^+\) cells compared with a single bout of exercise performed at the same time of the day. Moreover, at the end of the second bout of exercise, there was a decreased mitogen-induced expression of the CD69 activation marker on CD56\(^+\) cells, both in terms of percentage of cells expressing CD69 and density of this molecule on each CD56\(^+\) cell. The mechanisms behind these alterations could be neuroendocrine-induced changes in expression of adhesion molecules on both leukocytes and vascular endothelial cells, mobilization of different leukocyte subpopulations into the circulation, suppression of intracellular functions in the circulating lymphocytes, or, most likely, a combination of these factors.

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