Leukocytosis, muscle damage and increased lymphocyte proliferative response after an adventure sprint race

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Abstract

The effect of an adventure sprint race (ASR) on T-cell proliferation, leukocyte count and muscle damage was evaluated. Seven young male runners completed an ASR in the region of Serra do Espinhaço, Brazil. The race induced a strong leukocytosis ($6.22 \pm 2.04 \times 10^3$ cells/mm^3 before vs $14.81 \pm 3.53 \times 10^3$ cells/mm^3 after the race), marked by a significant increase of neutrophils and monocytes (P<0.05), but not total lymphocytes, CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells. However, the T-cell proliferative response to mitogenic stimulation was increased (P=0.025) after the race, which contradicted our hypothesis that ASR, as a high-demand competition, would inhibit T-cell proliferation. A positive correlation (P=0.03, r=0.79) was observed between the proliferative response of lymphocytes after the race and the time to complete the race, suggesting that the proliferative response was dependent on exercise intensity. Muscle damage was evident after the race by increased serum levels of aspartate amino transferase (24.99 ± 8.30 vs 50.61 ± 15.76 U/L, P=0.003). The results suggest that humoral factors and substances released by damaged muscle may be responsible for lymphocyte activation, which may be involved in muscle recovery and repair.

Key words: Strenuous exercise; Lymphocyte; Proliferation; Aspartate amino transferase; Immunosuppression

Introduction

Strenuous endurance exercise can induce a variety of modifications in several physiological systems. It is well recognized that strenuous exercise is marked by an acute inflammatory response, characterized by leukocytosis and leukocyte activation, release of inflammatory mediators and tissue damage (1-3). Muscle damage and/or altered muscle cell permeability lead to cellular protein leakage, and increased serum activity of many enzymes. These include creatine kinase, lactate dehydrogenase and aspartate amino transferase (AST), which have been used as biochemical indicators of exercise-induced tissue damage (4).

Strenuous exercise is commonly associated with increased symptoms of upper respiratory tract infection in athletes (5-7), suggesting that high-volume exercise may have an immunosuppressive effect, as indicated by the suppression of lymphocyte proliferation, neutrophil phagocytosis and microbicidal activity (7), as well as increased lymphocyte and neutrophil apoptosis (8). The immunological mechanisms that underlie this apparent increase in infection susceptibility in athletes are unknown, but are likely to be multi-factorial and include perturbations of the T-cell compartment. The proliferative capacity of lymphocytes is a key event in the adaptive immune response, and the host capacity to respond to an immunological challenge can be significantly compromised if lymphocyte proliferation is not adequate.

Adventure racing is a fast growing ultra-endurance sport, during which participants are required to perform multiple disciplines that require considerable skill and physical capacity (9,10). The competition is physically demanding, and because the aim is to reach as many checkpoints as possible within a given time interval, competitors must cover large distances as quickly as possible. Adventure racing includes sprints (<6 h), intermediate (6-12 h), long (12-36 h), and expedition races (>36 h).

Despite its growing popularity, data on the effects of adventure sprint races (ASRs) on immune responsiveness and exercise-induced inflammatory responses are scarce. Additional data would be useful for understanding and management of the physiological response to competition. Recent reports on the effects of adventure racing on lymphocyte and neutrophil viability have been reported (11), and showed no changes in neutrophil phagocytic capacity after the race despite increased neutrophil death by necrosis and lymphocyte apoptosis. Although increased lymphocyte apoptosis can potentially be involved in exercise immunosuppression, lymphocyte function, i.e., proliferative response, was not evaluated by Levada-Pires et al. (11). Thus, the impact of this kind of competition on immune responsiveness has not been fully investigated. In this context, the present research evaluated the effect of ASR on mitogen-induced proliferative response of lymphocytes, leukocytosis and muscle damage. We hypothesized that, since ASR is a strenuous competition, it could result in leukocytosis, muscle damage and reduced lymphocyte proliferative response.

Material and Methods

Subjects and race description

The present study enrolled 8 male nonsmoking athletes (27.7 ± 5.9 years). These volunteers did not report any infectious or autoimmune diseases and were not taking any anti-inflammatory drugs. All were amateur cyclists who regularly participated in state and national competitions, but had little or no prior experience in adventure races. They participated as individual competitors in an adventure sprint race in the Serra do Espinhaço region, Diamantina, MG, Brazil (-18° 13′ 58″ S, -43° 39′ 02″ W) in October 2010. The competition included 12 km of trekking, 100 m of vertical techniques, and 30 km of mountain biking, covering a total distance of 42.1 km. Serra do Espinhaço is a rugged region of cerrado vegetation, with elevations of 665-1143 m. During the competition the recorded temperature was $20.8 \pm 4.3°C$ with a relative humidity of $49.9 \pm 18.9\%$. Nutritional and hydration strategies were the responsibility of each competitor; race organizers provided nutrition and hydration stops for the competitors.

The Internal Review Board of the Universidade Federal dos Vales do Jequitinhonha e Mucuri approved the study. All volunteers signed an informed consent form, thereby agreeing to participate in the study.

Precompetition evaluation

Five days before the race, resting and maximum heart rate, body mass, percentage of body fat, and peak oxygen consumption ($VO_{2\ peak}$) were measured. The Balke cycle ergometer protocol was employed to estimate aerobic capacity ($VO_{2\ peak}$). Briefly, the protocol consisted of progressive exercise performed at 50-60 rpm, with an initial load of 1.5 kg. The load was increased by 0.5 kg every 2 min until fatigue. Resting and maximum heart rate were measured using a heart rate monitor (Polar® RS800, USA). Body fat percentage was estimated according to Jackson and Pollock (12), using the tricep, suprailiac and calf skinfolds.

Competition outcomes

Peripheral venous blood was collected from the antecubital vein 24 h before and immediately after the race, using heparinized vacuum tubes. The pre-race blood collection was performed 1 h after breakfast, and volunteers were instructed to have the same breakfast as planned for the day of competition. On race day, volunteers were asked about the breakfast before the beginning of the competition, and all confirmed that they ate the same breakfast as before the pre-race blood collection.

Total and differential leukocyte counts were performed using an automated cell counter (CC550, Celm, Brazil) and blood smears were prepared with Giemsa and May-Grünwald stains.

A blood sample (approximately 1 mL) was centrifuged (400 g, 15 min, 25°C) and the plasma was collected for the measurement of AST and alanine amino transferase (ALT) activities. The measurements of ALT and AST activities were carried out by monitoring the rate of NADH oxidation in a coupled reaction system employing lactate and malate dehydrogenase, respectively, using commercially available kits (K049 and K048, Bioclin, Brazil) and a semi-automatic analyzer (PW-3000M, Pioway, China). The oxidation of NADH to NAD$^+$ was accompanied by a decrease in absorbance at 340 nm. Under circumstances in which the ALT and AST activities are rate limiting, the rate decrease is directly proportional to the ALT or AST activity in the sample.

The peripheral blood mononuclear cells (PBMC) were isolated from the remaining blood sample by centrifugation (400 g, 30 min, 25°C), using Histopaque® 1077 (Sigma, USA), as described by Bicalho et al. (13). A sample of the PBMC (5×10^5 cells) was suspended in phosphate-buffered saline (PBS) and stained with anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies (BD Biosciences, USA), conjugated with specific fluorochromes, to determine the *ex vivo* percent of TCD4$^+$
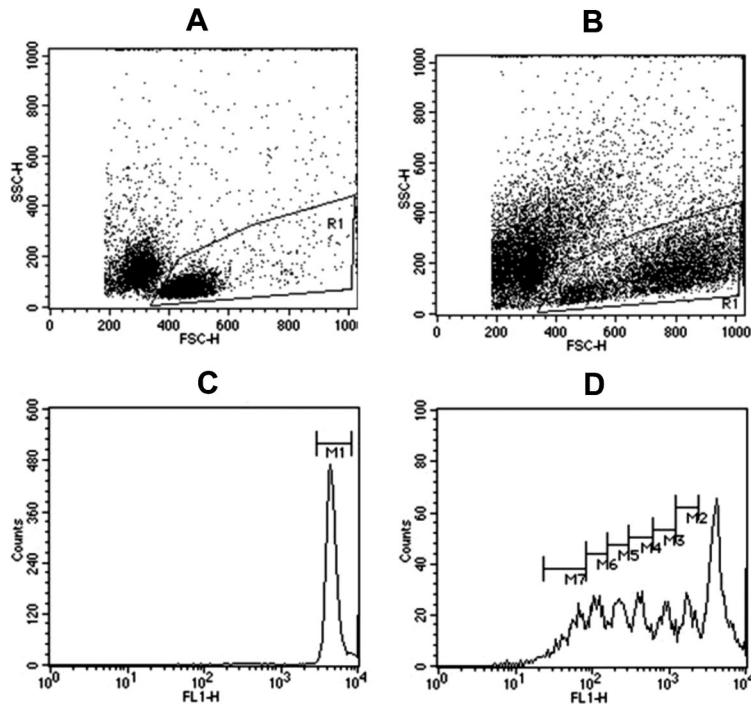
Figure 1. Analysis of cell proliferation using carboxyfluorescein succinimidyl ester (CFSE) by flow cytometry. *A*, Control for unstimulated cultures; *B*, phytohemagglutinin-stimulated cultures; *C*, CFSE-stained cells derived from unstimulated cultures; *D*, CFSE-stained cells in cultures stimulated with PHA. SSC and FSC = side and forward scatter, respectively.

and TCD8$^+$ cells, as described by Openshaw et al. (14).

The proliferative response of lymphocytes was evaluated by the decay of carboxyfluorescein succinimidyl ester (CFSE) fluorescence as previously described (15). Briefly, PBMC (1×10^7 cells/mL) were suspended in PBS with 0.1% bovine serum albumin and labeled with 10 μM CFSE (Sigma) (16). The cells were washed and suspended in RPMI (Sigma) supplemented with 2 mM L-glutamine (Sigma), antibiotic/antimycotic solution (100 IU/mL penicillin G, 100 μg/mL streptomycin and 2.5 μg/mL fungizone; Sigma), and 10% fetal calf serum (Gibco, USA). Then, PBMC were stimulated with 1 μg/mL mitogen phytohemagglutinin (PHA, Sigma) for 5 days at 37°C and 5% CO_2. After the culture period, cells were washed twice in PBS (200 *g*, 10 min, 4°C), suspended in PBS/2% paraformaldehyde, for analysis by flow cytometry (FACScan, Becton Dickinson, USA).

Data acquisition was performed using the region of small lymphocytes and blast cells according to the size and granularity profiles [forward (FSC) *vs* side scatter (SSC)]. CFSE fluorescence was measured in the FL1 channel; 50,000 events in the R1 region were acquired for later analysis. The acquired data were analyzed using the Cell Quest program (Becton Dickinson).

Regions of analysis were defined as follows. The R1 region was defined in dot plots of FSC $\times$ SSC and comprised lymphocytes and blast cells for unstimulated (Figure 1A) and PHA-stimulated cultures (Figure 1B). The events in R1 were then analyzed for CFSE fluorescence in histograms of FL1 (Figure 1C and D). The M1 region

was defined as CFSE-stained cells derived from unstimulated cultures, which represented the peak of quiescent cells (Figure 1C). The M2 to M7 regions were defined according to the peaks of different CFSE intensities in cultures stimulated with PHA (Figure 1D).

The proliferative index was calculated from the data in the histograms considering that two cells with a given CFSE intensity were produced by mitosis of a single cell, possessing a CFSE intensity immediately above, using the following formula (17): proliferative index = (100-Y)/Y, where Y (%) = $X0 + X1/2 + X2/4 + X3/8 + X4/16 + X5/32 + X6/64 + X7/128$, X0 represents the percentage of T cells that did not divide (located at M1), and X1-7 represents the peak of gradual division (located from M2 to M7).

Statistical analysis

GraphPad Prism version 5.00 for Mac OSX (GraphPad Software, USA, http://www.graphpad.com) was used for the statistical analysis. $P \leq 0.05$ was considered to be statistically significant. Data are reported as means $\pm$ SD. The Shapiro-Wilk test was used to evaluate the normalcy of the data. Since the dependent variables were normally distributed/transformed, parametric tests were used for statistical analysis. The Student paired *t*-test was used to compare the total and differential leukocyte count, percentage of TCD4$^+$ and TCD8$^+$ cells, activity of the enzymes ALT and AST and the proliferative response of T cells before and after the race. Pearson's correlation was used to evaluate the relationship between the biological aspects studied and race duration.

Table 1. Subject characteristics.

Characteristics	Means ± SD
Age (years)	27.7 ± 5.9
Body mass (kg)	63.2 ± 3.6
Height (cm)	170.2 ± 3.4
Body fat (%)	6.9 ± 1.7
$VO_{2\ peak}$ (mL $O_2 \cdot kg^{-1} \cdot min^{-1}$)	60.8 ± 1.8

Results

One of the 8 athletes initially enrolled in the study did not complete the race because he got lost during the trekking stage and did not finish the race within the time frame. Table 1 describes the mean age, body mass, body fat percentage, height, and $VO_{2\ peak}$ of the subjects. The mean race duration was 4 h 38 min 32 s ± 48 min 12 s. The first athlete finished the race in 3 h 54 min 57 s and the last in 6 h 7 min 52 s.

The race caused significant alterations in leukocyte count profiles (Table 2). A 2.3-fold increase (P = 0.0006) in total leukocyte count was observed after the competition, which was due mainly to the increased number of neutrophils (P < 0.0001) and monocytes (P = 0.02). No effect of the race on the number of circulating lymphocytes was observed (P = 0.7). The race did not have an effect on the percentages of $CD3^+CD4^+$ or $CD3^+CD8^+$ cells (Table 3).

However, the lymphocyte proliferative response to stimulation by PHA significantly increased from 1.73 ± 0.96 before the race to 2.66 ± 1.61 after the race (P = 0.025). We also evaluated the plasma levels of the enzymes AST and ALT (Figure 2). The plasma levels of AST increased (P = 0.003) from 24.99 ± 8.30 before to 50.61 ± 15.76 U/L after the race. The race had no effect on the ALT levels (P = 0.06; Figure 2B).

Considering that leukocytosis, proliferative response and muscle enzyme leakage can be related to exercise intensity we evaluated whether the alterations observed could be related to the time to complete the race. No correlation was observed between AST activity and leukocyte number and the time to finish the race (data not shown). However, a positive correlation (P = 0.03, r = 0.79, r^2 = 0.63, Pearson's correlation) was observed

Table 3. Adventure sprint race had no effect on the percentage of T helper ($CD3^+CD4^+$) and T cytolytic ($CD3^+CD8^+$) lymphocytes and CD4/CD8 ratio.

	Before race	After race
% $CD3^+CD4^+$ cells	40.6 ± 3.6	42.9 ± 5.5
% $CD3^+CD8^+$ cells	10.6 ± 5.8	11.8 ± 3.6
CD4/CD8 ratio	4.7 ± 1.8	3.9 ± 1.2

Data are reported as means ± SD. CD4/CD8 ratio represents the quotient between the percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. The Student paired t-test was used for analyses.

between the proliferative response of lymphocytes after the race and the time to complete the race (Figure 3).

Discussion

We report the effect of an adventure sprint race on leukocytosis, lymphocyte proliferation and skeletal muscle damage of amateur runners. As expected, we observed a 2.3-fold increase in leukocyte number after the race, and also increased serum levels of AST, but not of ALT. Also, lymphocyte proliferative response to mitogenic stimulation was increased after the race.

Leukocytosis is the most consistent hematological alteration observed in response to prolonged exhaustive exercise, and the magnitude of change of the number of circulating leukocytes is proportional to the intensity and duration of the exercise. During long-duration exercise, leukocytosis primarily results from elevated granulocytes (18,19), which are composed mainly of neutrophils. Consistently, in the present study racers experienced a 3.5-fold increase in neutrophils, and a 2-fold increase in monocytes following the race. Lymphocyte count, however, was not affected by the competition, nor was the frequency of T helper ($CD3^+CD4^+$) or T cytotoxic ($CD3^+CD8^+$) cells. To our knowledge, there have been no reports about exercise-induced leukocytosis following a sprint adventure race, but Davidson et al. (20) and Kratz et al. (21) also reported increased absolute numbers of neutrophils and monocytes, shortly after a marathon race (mean duration of 4 h and 30 min), but not of circulating lymphocytes. However, Lucas et al. (22) observed increased numbers of circulating neutrophils (166%),

Table 2. Adventure sprint race increased total leukocyte, neutrophil and monocyte but not lymphocyte counts.

	Before race	After race
Total leukocyte ($\times 10^3$ cells/mm^3)	6.2 ± 2.0	14.8 ± 3.5*
Neutrophils ($\times 10^3$ cells/mm^3)	3.5 ± 1.6	12.1 ± 3.3*
Monocytes (cells/mm^3)	200.0 ± 100.0	430.0 ± 190.0*
Lymphocytes ($\times 10^3$ cells/mm^3)	2.2 ± 0.5	2.3 ± 0.7

Data are reported as means ± SD. *P < 0.05, compared to before race (Student paired t-test).
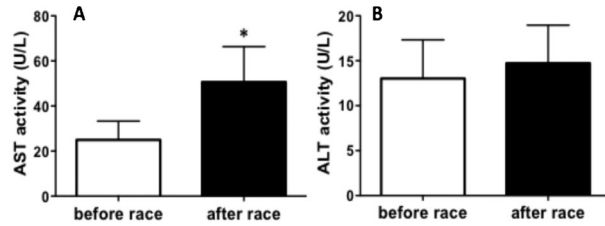
Figure 2. Plasma levels of AST (*A*) but not of ALT (*B*) were elevated after an adventure sprint race. Data are reported as means ± SD. AST: aspartate amino transferase; ALT: alanine amino transferase. *P<0.05, compared to before race (Student paired *t*-test).

Figure 3. Correlation between lymphocyte proliferative response (proliferation index) and time to complete race. Data are reported as means ± SD (dotted lines).

lymphocytes (42%) and monocytes (131%) in the first part (15 to 30 h) of a 411-km adventure race, a difference that can be attributed to the race duration.

It is generally assumed that leukocytosis after a race is mainly caused by the demargination of leukocytes induced by increased blood flow, although catecholamines and cortisol secreted during exercise may also contribute to leukocyte demargination (23-25). However, the observation of this and other studies of an increase in the absolute number of neutrophils and monocytes, but not of lymphocytes, argues that some of the leukocytosis observed in runners might be due to an inflammatory response caused by tissue injury (26). In an acute inflammatory response, neutrophils and monocytes are the first cells to reach the injured site, followed 24 to 48 h later by lymphocytes if tissue is not properly repaired. Consistent with this, we observed increased plasma levels of AST after the race that was positively correlated with leukocyte count. Others have also observed elevated AST levels in the blood of runners after a 21-km race (27) and after a marathon (21). Plasma elevation of AST and other intracellular proteins such as creatine kinase, lactate dehydrogenase and myoglobin, may indicate exertional rhabdomyolysis and leakage of proteins from skeletal muscle cells (4,28), whereas ALT, a more specific marker for hepatic injury, showed little change in response to exercise. Since ALT, but not AST, was increased in the present study, plasma levels of ALT might be attributed mainly to leakage from muscle and not liver cells.

It has been suggested, mainly from epidemiological studies, that exhaustive exercise leads to a temporary immunosuppression, since athletes report more episodes of respiratory infections during periods of intense training and following competition (7,29,30). In order to establish an effective adaptive immune response, lymphocytes must proliferate when stimulated by pathogens. Reduced lymphocyte proliferation following prolonged/exhaustive exercise, like marathons and triathlons, has been reported by many authors (31-33). Since an adventure sprint race is a high-demand competition (the aim is to reach as many checkpoints as possible within the allowed stage times, and in which competitors have to cover large
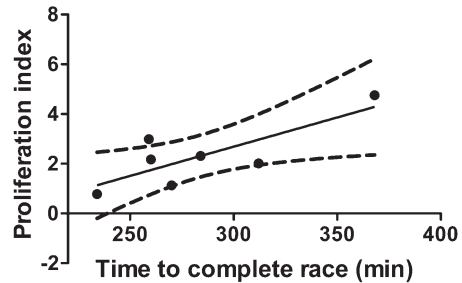
distances as quickly as possible), the proliferative response of lymphocytes to PHA stimulation after the race was evaluated to address the potential immunosuppressive effect of the competition. To our surprise, PHA stimulation of the lymphocyte proliferative response was not reduced, but instead it was increased after the race. This suggests that this kind of competition may lead to temporary immunosuppression, but that it cannot be attributed to a reduced T-cell proliferation response. It has been suggested that exhaustive competitions, like adventure races (11) and triathlons (34), increase lymphocyte death, thus compromising lymphocyte function, but T-cell function was not evaluated in those reports.

The observed increased lymphocyte proliferative response indicates that factors produced and/or secreted during the race might be the mitogenic stimulus priming these cells. It is well known that prolonged exercise elicits the secretion of a series of soluble factors, like cytokines and hormones, many of which have potential lymphocyte priming activity. As reported by Radom-Aizik et al. (35), Jurkat cells, an immortalized T-cell line, have increased proliferative response to the stimulation by proliferating nuclear cell antigen when treated with serum obtained from healthy, young donors after 30 min of exercise. This indicates that serum factors present after exercise are able to modulate the proliferative response of lymphocytes *in vitro*. It can also be suggested that other factors released from damaged skeletal muscle cells, for example, can activate lymphocytes to engage in the inflammatory response to exercise. Heat shock proteins (Hsp) are a candidate factor, released from cells during exercise (36), capable of stimulating lymphocytes (37,38). As demonstrated by Haug et al. (39), human Hsp70 complexed to antigenic peptides enhances activation and proliferation of human memory TCD4+ cells. Although we did not find a correlation between AST levels and the proliferative response of lymphocytes, increased AST levels reflect leakage of proteins from muscle, which may include Hsp, amongst others. A positive correlation between lymphocyte proliferative response and time to complete the race was observed, suggesting that the proliferative response of lymphocytes following the race

was dependent on exercise intensity. Many potential lymphocyte-activating factors released during exercise are also induced in an intensity-dependent way, reinforcing the hypothesis that factors released during the race may be involved in lymphocyte activation, resulting in increased lymphocyte proliferative response. However, the identification of such factors, as well as their potential immunomodulatory action in response to exercise, has not yet been conducted.

In summary, this study showed that an adventure sprint race induced a strong leukocytosis, marked by increased numbers of circulating neutrophils and monocytes, which were correlated with plasma AST levels in response to long duration/exhaustive exercise, consistent with observations of other investigators. Contrary to the exercise-induced immunosuppression hypothesis, increased lymphocyte proliferative response was also induced by the competition. Compared with traditional or controlled long-duration exercise, adventure races impose a series of additional stresses to the athlete during competition, which include environmental and soil conditions, nutritional and hydration strategies, amongst others. This race was conducted in a region of rugged terrain with great variation in altitude, characterized by high solar incidence and large variations in temperature and humidity. This imposed high physical, thermoregulatory and technical demands on the competitors, which undoubtedly contributed to the observed effects. Leukocytosis and increase of lymphocyte proliferative response might be due to soluble and/or cellular factors released in response to exercise, and may be involved in the regenerative inflammatory response to exercise.

Acknowledgments

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