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The immune system and serum glutamine during a triathlon

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Abstract This study examined the influence of a triathlon on the immune system and on serum amino acid concentrations. Eight male triathletes swam 2500 m, bicycled 81 km, and ran 19 km. The concentration of total serum amino acids decreased during the race, with the lowest values occurring 2 h postexercise. Similarly, serum glutamine concentration declined from 468 (SEM 24) (prerace) to 318 (SEM 20) $\mu mol l^{-1}$ (2 h postrace) and the natural killer (NK) and lymphokine activated killer (LAK) cell activities were suppressed 2 h postexercise ($P < 0.05$). Blood mononuclear cell proliferation decreased during exercise with the lowest value observed after running. The leucocyte concentration increased during and after exercise due to an increase in the concentration of neutrophils and monocytes. There was no significant change in lymphocyte concentration during or after the exercise. The plasma concentration of interleukin-6 did not change and the plasma concentration of interleukin-1β and tumor necrosis factor-α were below detection limits. The LAK cell cytotoxicity, but not NK cell activity or proliferative response, was significantly correlated with serum glutamine concentrations ($r = 0.39$, $P < 0.01$). This study confirms that prolonged endurance exercise results in changes in the cytotoxic function of the NK and LAK cells as well as the proliferative response. The time-course of changes in serum glutamine concentrations were best parallelled by changes in LAK cell activities.

Key words Lymphocyte proliferation · Lymphocyte subpopulations · Lymphokine activated killer cells · Natural killer cells · Glutamine

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Introduction

Intense long-duration exercise has been associated with an increased risk of upper respiratory tract infections (URTI) (Heath et al. 1991; Nieman et al. 1990; Peters and Bateman 1983). This has been thought to be a consequence of postexercise immunosuppression, which is characterized by a suppression of natural killer (NK) cell activity, lymphokine activated killer (LAK) cell activity (Tvede et al. 1993) and lymphocyte proliferation (Tvede et al. 1989). Glutamine has been shown to be an important substrate for the cells of the immune system including the blood mononuclear cells (BMNC) (Ardawi and Newsholme 1984; Parry Billings et al. 1990) which utilize glutamine for both energy supply and nucleic acid synthesis (Ardawi and Newsholme 1982). Specifically, glutamine influences BMNC function, including both the proliferative response and LAK cell activity (Rohde et al. 1995). Furthermore, it has been shown that optimal lymphocyte function *in vitro* is obtained when the plasma glutamine concentration is at a physiological level of 500–600 $\mu mol \cdot l^{-1}$ (Parry Billings et al. 1990; Rohde et al. 1995).

Forearm venous plasma glutamine concentrations have been reported to decrease following long-term, strenuous exercise (Keast et al. 1995; Parry Billings et al. 1992a) and it has been suggested may contribute to the postexercise immunosuppression (Parry Billings et al. 1992a). However, it is not clear whether the low glutamine concentration contributes to, or just parallels the changes in immune function. The purpose of the present study was to examine the effects of long-term strenuous exercise (a triathlon) on immunological function and serum amino acid concentrations.

Methods

Subjects and protocol

The experimental protocol was approved by the local Ethics Committee and eight elite male triathletes of mean age 29.1 (SEM 2.6) years (range 20–45 years) swam 2500 m, bicycled 81 km and ran 19 km. The swimming was performed in an indoor 50-m pool and bicycling and running took place on country roads. The subjects were allowed to drink and eat normally before and during the event. The triathlon began at 8 a.m. and was only interrupted for blood sampling from an antecubital vein. Blood samples were obtained at rest (40 ml), after swimming, bicycling and running (20 ml each), 2 h postexercise and 2, 3 and 4 days postrace between 8 a.m. and 9 a.m. (40 ml). Samples were taken immediately after the end of each discipline except after bicycling where the samples were taken after a 10-min delay. The blood was used for the *in vitro* determination of immunological and metabolic parameters.

To evaluate the intensity of the work performed by the athletes during bicycling and running they were equipped with a portable heart rate (HR) monitor with a microcomputer, Polar Sporttester that has been described by Treiber et al. (1989) and Tsanakas et al. (1986) and which was programmed to record and store data every 15 s. After the race these values were down-loaded to a computer and the mean values for bicycling and running were calculated. Resting HR was measured during sleep and maximal oxygen uptake ($\dot{V}O_{2\,max}$) and maximal heart rate (HR_{max}) were determined during a treadmill test. The relative exercise intensity was calculated against HR_{max}.

Isolation of blood mononuclear cells

The BMNC were isolated by density gradient centrifuging (Lymphoprep Nyegaard, Oslo, Norway) on LeucoSep tubes (Greiner, Frickenhausen, Germany) and washed three times in Roswell Park Memorial Institute (medium) (RPMI) (1640 Gibco, Grand Island, NY., USA). Cells were frozen in freezing medium (50% RPMI, 30% foetal calf serum (FCS) (Gibco), 20% dimethylsulphoxide (Bie and Berntsen, Rødovre, Denmark)) and kept in liquid nitrogen until thawed for analysis.

Determination of amino acids

At each sampling time blood (3 ml) was taken in sterile glassware, kept on ice until coagulated (20 min) and thereafter centrifuged at 3000 rpm for 5 min. Serum was stored at $-20^{\circ}C$ and analysed in duplicate for amino acids by prior derivation with phenylisothiocyanate and high performance liquid chromatography (Heinrikson and Meredith 1984).

Examination of blood constituents

A 6-ml sample of blood was taken, at each sampling time, for estimation of the concentration of leucocytes, neutrophil granulocytes, lymphocytes, monocytes and creatine kinase. These analyses were carried out using standard laboratory procedures at the Department of Clinical Chemistry at Rigshospitalet.

Proliferation assay

The BMNC proliferation assay included cell cultures performed in triplicate in microtitre plates (NUNC, Roskilde, Denmark) (Tvede et al. 1989). The BMNC, 3.3×10^5 cells $\cdot$ ml^{-1}, were cultured for 72 h and were stimulated with phytohaemagglutinin (PHA), 3 µg $\cdot$ ml^{-1} (Difco laboratories, Detroit, Michigan, USA). During the last 24 h of the culture period of the cells were exposed to ^{3}H-thymidine. The cultures were collected on glass fibre filters with a harvesting machine (Mikromate 196, Packard) and ^{3}H-thymidine incorporation was measured in a β counter (Harvester, Matrix 96, Packard). For each triplicated sample the mean was recorded and the mean counts per minute (cpm) value of unstimulated controls was subtracted.

Cell surface marker analysis by flow cytometry

The BMNC subsets were estimated at each sampling point. The BMNC were washed once in phosphate buffer (PBS) with 3% FCS, resuspended in PBS containing FCS and one of the monoclonal antibodies anti-leu4 (CD3, pan T-cells), anti-leu2 (CD8, T-subpopulation), anti-leu3 (CD4, T-subpopulation), anti-leu11 (CD16, NK-cells), anti-leuM3 (CD14, monocytes), anti-leu19 (CD56, NK-cells) (Becton-Dickinson, Calif, USA) or anti-CD19 (B-cells) (Dako, Denmark). After storing the cells on ice for 30 min the cells were washed twice in PBS containing 3% FCS. Labelled cells were analysed by flow cytometry using a fluorescence-activated cell sorter (FACStar, Becton-Dickinson). Lymphocytes were distinguished from monocytes by their forward versus right angle scatter.

NK cell activity

The NK cell activity was measured using K562 target cells in a ^{51}Cr release assay (Pedersen and Kharazmi 1987). Effector cells were BMNC incubated in medium (1640 RPMI with 10% FCS) for 1 h at 37°C. In triplicate, 100 µl (5×10^6 cells $\cdot$ ml^{-1}) of effector cells and 100 µl (10^5 cells $\cdot$ ml^{-1}) of target cells were incubated in microtitre plates for 3 h at 37°C. The plates were centrifuged for 5 min at 2000 rpm and 100 µl of supernatant was transferred to a new tube and the radio-activity determined. The spontaneous release was determined by the incubation of 100 µl of target cells with 100 µl of medium. The maximal release was determined by the incubation of 100 µl of target cells with 100 µl of medium containing 10% Triton X-100. Percentage ^{51}Cr release (NK-cell activity) was determined as:

$$\%\,\text{lysis} = 100\% \times (\text{test-spontaneous})\text{cpm}/(\text{maximum-spontaneous})\text{cpm}$$

The NK cell activity was also adjusted per-NK cell (Nieman et al. 1993).

LAK cell activity

The LAK cell activity was measured using Daudi target cells in a ^{51}Cr release assay. The effector cells were established by incubation of 100 µl of BMNC (10^7 cells $\cdot$ ml^{-1}) with 100 µl of interleukin-2 (IL-2) (3×10^6 IU $\cdot$ ml^{-1}) and 800 µl of 10% FCS (1640 RPMI with 10% FCS) for 48 h at 37°C. In triplicate, 100 µl of effector cells (10^6 cells $\cdot$ ml^{-1}) and 100 µl of target cells (2×10^4 cells $\cdot$ ml^{-1} were incubated in microtitre plates for 4 h at 37°C. Otherwise the ^{51}Cr release assay was performed as described for the NK cell assay.

Cytokines

A 3-ml sample of blood was taken in glass tubes containing 11.7 µmol dipotassium-ethylenediaminetetra-acetic acid (EDTA) and 500 kallikrein inactivator units (KIU) of Trasylol (Bayer, Leverkusen, Germany). The tubes were kept on ice until centrifuging at

3000 rpm for 5 min. The plasma was stored at − 80°C. The concentration of cytokines in plasma was measured using commercially available ELISA-kits: Interleukin-1β (IL-1β) (Immunotech S.A., Marseille, France), tumor necrosis factor-α (TNF-α) (T-cell Diagnostics, Inc. Cambridge, Mass., USA) and interleukin-6 (IL-6) high sensitivity (R&D Systems, Minneapolis, USA).

Statistical analyses

To assess the effects of exercise on the BMNC proliferation and NK and LAK activities analyses of variance with two within-subjects factors (time of testing, and serum glutamine concentration) was used. If significance was indicated a Tukey's post hoc test was performed to determine where the significance had occurred. To assess the effects of exercise on the change from rest in amino acid and cytokine concentration an analysis of variance with Duncan's post hoc test (serum amino acids) or Tukey's post hoc test (cytokines) was used. To analyse the variation in the BMNC subpopulations and the clinical chemistry values a simple univariate Student's *t*-test was used. To examine the relationship between serum glutamine concentrations and the lymphocyte proliferative response, NK activity or LAK activity, Pearson's correlation coefficients were determined. Significance was accepted at $P < 0.05$.

Results

Performance duration and intensity

The subject's $\dot{V}O_{2max}$ was 5.3 (SEM 0.1) $l \cdot min^{-1}$ and HR_{max} was 186 (SEM 5) beats $\cdot min^{-1}$, while resting HR was 39 (SEM 1) beats $\cdot min^{-1}$. The mean swimming time was 43.0 (SEM 0.5) min, mean cycling time was 142 (SEM 2) min and mean running time was 77 (SEM 1) min. The total exercising time (excluding clothing changes and blood sampling) was 261 (SEM 4) min. The mean HR during cycling was 148 (SEM 2) beats $\cdot min^{-1}$ and during running was 159 (SEM 4) beats $\cdot min^{-1}$. The mean exercise intensities were calculated (% of HR_{max}) as 74 (SEM 2) % for cycling and 81 (SEM 3) % for running.

Serum amino acids

All concentrations of amino acids except for glutamate, taurine, arginine, tyrosine, methionine and phenylalanine were reduced from rest values by the end of the triathlon (Fig. 1). Similarly, 2-h postexercise all amino acid concentrations except glutamate, hydroxyproline, taurine and phenylalanine were lower than the rest values. However, 1 day after the completion of the race, all concentrations of amino acids except for ornithine had recovered to their prerace values.

Leucocyte subpopulations and creatine kinase

The leucocyte concentration had increased at the end of cycling, running and 2 h after the race due to an increased concentration of neutrophils, lymphocytes and monocytes, (Fig. 2). However, the change in lymphocyte concentration was not significant. The percentage distribution and concentration of CD3 +, CD4 +, CD8 +, CD16 +, CD56 +, CD19 +, and CD14 + cells did not change, except for a slight increase in the percentage of CD19 + cells 2 h postexercise (data not shown). There were no changes in the concentration of creatine kinase but the rest concentration was high [mean 1043 (SEM 403) U $\cdot l^{-1}$].

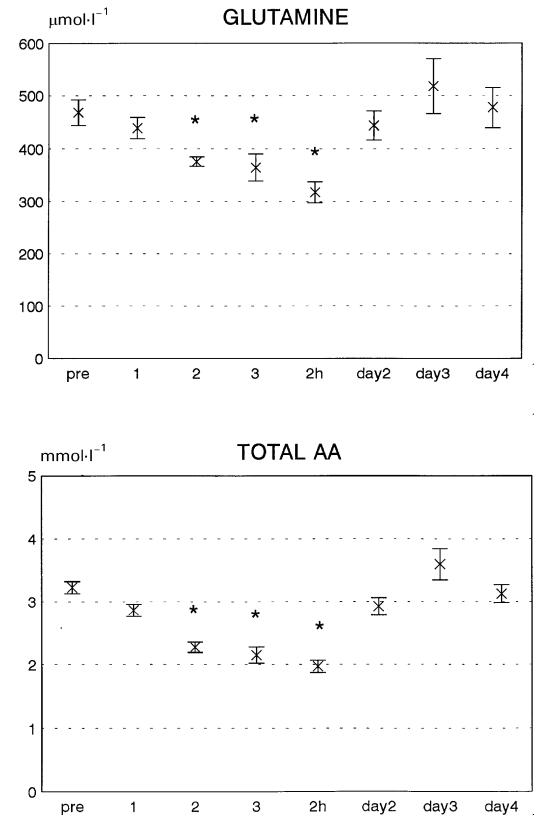

Fig. 1 The mean and SE of serum glutamine and total serum amino acid (total AA) concentrations before (pre), after swimming (1), after cycling (2), after running (3), 2 h postexercise (2h), day 2, day 3 and day 4. * Significant difference from pre-exercise value $P < 0.05$

Proliferation of BMNC

The proliferative response decreased with time reaching the lowest value after running (Fig. 3). There was no significant correlation between the serum glutamine concentrations and the proliferative response of BMNC.

NK activity

When BMNC were tested for cytotoxic activity against K562, the NK cell activity had declined 2 h postexercise (Fig. 3). During exercise there was no enhancement

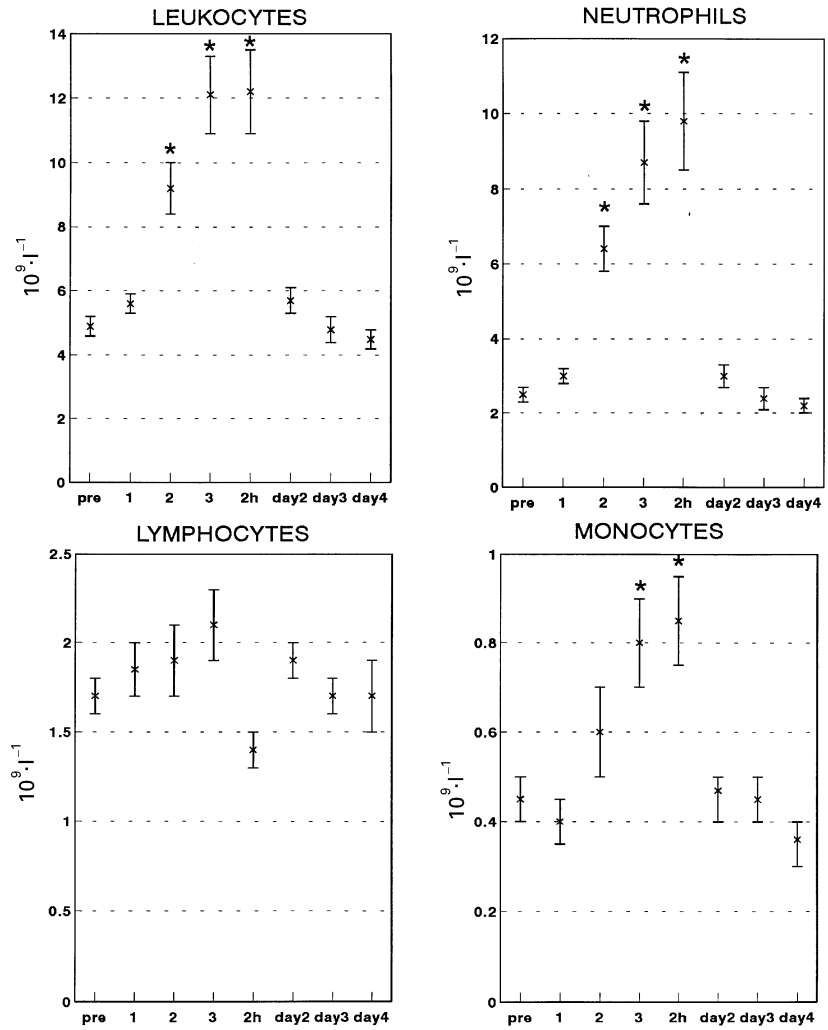

Fig. 2 Number of leucocytes, neutrophils, lymphocytes and monocytes in peripheral blood measured before (pre), after swimming (1), after cycling (2), after running (3), 2 h postexercise (2h), day 2, day 3 and day 4. *Significant difference from pre-exercise value $P < 0.05$

of NK cell activity. The NK activity returned to pre-exercise levels 24 h postexercise. When the NK cytotoxic activity was adjusted per CD16 + or CD56 + cell there were no significant changes (data not shown). The NK activity did not correlate significantly with serum glutamine concentrations.

LAK activity

The LAK cell activity declined after running and dropped further 2 h post-exercise ($P < 0.0001$; Fig. 3). At 24 h postexercise the LAK cell activity had returned to pre-exercise values. The LAK cell activity adjusted per individual CD16 + or CD56 + cell did not change significantly. A positive relationship between LAK activity and serum glutamine concentration was observed ($r = 0.39$, $P < 0.01$). Analysis of variance (model: LAK activity = constant + subject + time + serum glutamine) indicated an interaction of subject (individual variation), time ($P < 0.001$) and serum glutamine on the LAK cell activity.

Cytokines

The IL-6 concentrations were in the range 5.4 (SEM 1.3) (pre-exercise) to 2.4 (SEM 1.2) $pg \cdot ml^{-1}$ (day 5). There were no significant changes with time. Plasma concentrations of IL-1β and TNF-α were below detection limits.

Discussion

A major finding of this study was that a decrease in unadjusted NK- and LAK cell cytotoxic activities and a decrease in serum glutamine concentrations was observed 2 h after the end of a triathlon. The finding that glutamine concentration has a possible influence on the LAK cell activity, but not on the NK cell activity is in accordance with *in vitro* studies (Rohde et al. 1995). Although the glutamine concentration has been shown to influence the proliferative response (Parry Billings et al. 1990; Rohde et al. 1995), we found no correlation

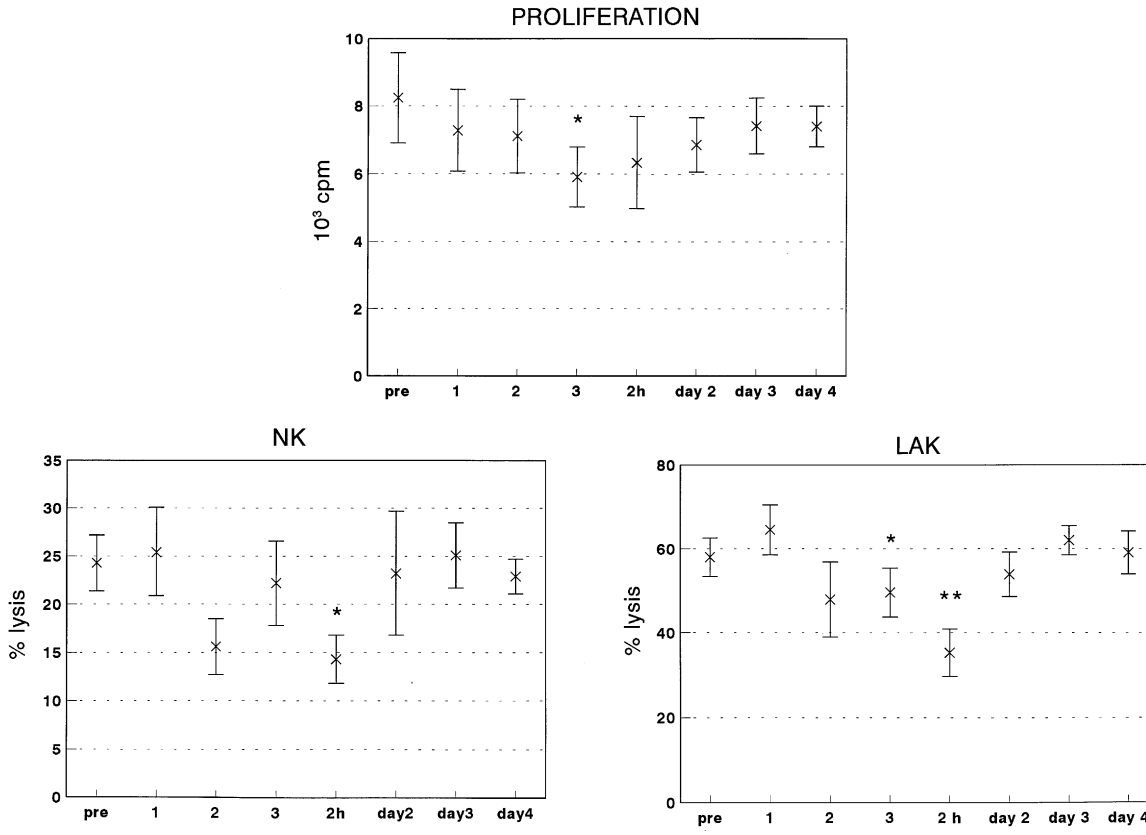

Fig. 3 The phytohaemagglutinin stimulated proliferative response, the natural killer (*NK*) cell activity (% lysis) and the lymphokine activated killer (*LAK*) cell activity (% lysis) of blood mononuclear cells isolated before (pre), after swimming (1), after cycling (2), after running (3), 2 h postexercise (2 h), day 2, day 3 and day 4. Significant difference from pre-exercise value $*P < 0.05$, $**P < 0.0001$

between the PHA proliferative response and serum glutamine concentrations. Both the proliferative response and the serum glutamine concentrations declined in relation to the exercise, but with different kinetics. For example, the PHA response was lowest during running, while serum glutamine was lowest 2 h post-exercise. The LAK activity and glutamine concentration were correlated, but it is unknown whether the low serum glutamine concentration was responsible for the decrease in LAK activity or whether the relationship was coincidental. It has been found that *in vitro* the LAK cell cytotoxicity is optimal when glutamine is around 600 $\mu mol \cdot l^{-1}$, but not significantly lower at 300 $\mu mol \cdot l^{-1}$ (Rohde et al. 1995), and in this study the glutamine concentration declined to 318 $\mu mol \cdot l^{-1}$.

The unadjusted NK and LAK cytotoxicities were suppressed with the lowest values 2 h postexercise. Due to the limited amount of blood available we were not able to test more than one effector to target cell ratio, and were therefore unable to calculate lytic units, which is the optimal way of presenting data from NK and LAK assays. However, when the NK or LAK activities were adjusted per CD16 + cell we found no significant changes. In the literature there is a fundamental disagreement on this topic. Several studies have suggested that per NK cell, NK cytotoxic activity is elevated following high intensity exercise (summarized in Nieman et al. 1993). Other studies (Pedersen et al. 1990) have indicated that the decrease in NK cytotoxic activity is caused by a prostaglandin-induced down-regulation of NK cytotoxic activity.

It has been reported the concentration of glutamine in plasma decreases in relation to severe exercise, overtraining, trauma, burns and surgery (Parry Billings et al. 1990, 1992a, 1992b; Vinnars et al. 1975). For example, following a marathon the venous plasma glutamine concentration has been observed to decline from approximately 600 $\mu mol \cdot l^{-1}$ to 500 $\mu mol \cdot l^{-1}$ (Parry Billings et al. 1992a). Other studies have shown a decrease in plasma glutamine concentration in relation to different exercise intensities (Keast et al. 1995), with substantial decreases in venous plasma glutamine concentrations after short-term exercise above 90% of $\dot{V}O_{2\,max}$. In contrast, Lehmann et al. (1995) have reported no changes in venous plasma glutamine concentrations following an ultra triathlon (7.5-km swimming, 360-km cycling and 85-km running). However, in that study the blood samples were taken within 30 min of

completing the race and it is possible that the glutamine concentrations may have decreased after this time. In this study we found a decrease in glutamine concentration from 468 to 318 $\mu mol \cdot l^{-1}$. It is apparent that glutamine concentrations decline in relation to both exercise and other stress situations. However, what is not known is the impact that this decline has on the immune system *in vivo*, or whether maintenance of the plasma glutamine concentration *in vivo* can prevent the postexercise immunosuppression.

The increase in leucocyte concentration following exercise has been shown to be due to an increase in both neutrophils and lymphocytes Hoffman-Goetz and Pedersen 1994; McCarthy and Dale 1988). In this study there was no significant increase in the concentrations of either total or individual lymphocyte subpopulations. Furthermore, the shift in BMNC subpopulations resulting in an increase in the percentage of CD16 + cells and thereby in NK and LAK cell activity that has been found at the end of exercise by Hoffman-Goetz and Pedersen (1994) and Pedersen and Ullum (1994) was not found in this study. There may be several explanations for the discrepancy between the present and former studies. It has been shown that the mechanism behind the recruitment of NK cells to the blood includes an increase in catecholamine concentration (Kappel et al. 1991a). Furthermore, heating of the body during exercise may also contribute to the modulation of the numbers of NK cells. For example, Kappel et al. have demonstrated that hyperthermia (39.5°C) enhanced the percentage of NK cells and thereby NK cell activity (Kappel et al. 1991b). However, during swimming in 26–28°C water the body temperature does not increase. This may explain why the lymphocyte count and the NK and LAK cell activities did not increase during swimming. Another explanation may be that these parameters have been shown to increase within a few minutes of the onset of exercise (Nielsen et al. in press). The lack of an increase immediately after cycling and running may have been due to the fact that the lymphocyte count and the NK and LAK cell activities had already peaked. Finally, the constancy in the subpopulations may be explained by adaptation of the immune system as a result of chronic intense exercise.

The concentrations of IL-6, IL-1β and TNF-α in plasma have been shown to be elevated in relation to strenuous exercise (Cannon et al. 1989; Northoff et al. 1990). However, in the present study we were unable to detect IL-1β or TNF-α in plasma. Using a high sensitivity IL-6 kit it was possible to detect IL-6, but there were no significant changes with time in this parameter. Previous studies have shown conflicting results regarding changes in the plasma/serum concentrations of cytokines in relation to exercise. The most consistent results have been obtained from IL-6 where elevated concentrations have been detected shortly after the end of severe exercise (Northoff et al. 1990). High intensity eccentric exercise in untrained subjects is most often associated with muscle damage. This damage is reflected by:

1. An increased level of myocellular enzymes in the circulation
2. Ultrastructural damage of the muscle cell
3. An acute inflammatory response occurring post-exercise (Evans et al. 1986).

Pedersen and Bruunsgaard (1995) have suggested that high intensity eccentric exercise, compared to concentric exercise, results in a more pronounced increase in plasma cytokine (Il-1β, TNF-α and Il-6) concentrations as a result of greater muscle damage. A triathlon consists mainly of concentric exercise and this may explain in part the lack of an increase in plasma cytokine concentrations observed in the present study. However, the running portion of a triathlon does contain an eccentric component. The subjects in this experiment were well-trained runners and were therefore most likely adapted to the eccentric component of running. This may be reflected by the finding that resting creatine kinase concentrations have been higher for well-trained athletes compared to untrained subjects and the increase in response to exercise is also less pronounced (Evans et al. 1986). In the present study there were no significant increases in the postexercise creatine kinase concentrations and this finding supports the suggestion that the subjects used were well trained and muscle damage was minimal.

In summary, this study shows that long-term intense exercise results in a reduction in NK and LAK cell activities and also a decrease in the serum glutamine concentration, but no changes in the plasma cytokine concentrations.

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