

Effects of submaximal cycling and long term endurance training on neutrophil phagocytic activity in middle aged men

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Abstract

Objective—To investigate the effects of long term (> 10 years) endurance training and submaximal exercise on the phagocytic activity of circulating neutrophil granulocytes.

Methods—The ability of stimulated blood neutrophils isolated from well trained cyclists [$n = 8$; $\dot{V}O_{2,max}$ 61.0(SD 8.8) ml·kg⁻¹·min⁻¹; age 38(4) years] and age matched sedentary controls [$n = 8$; $\dot{V}O_{2,max}$ 37.4 (6.6) ml·kg⁻¹·min⁻¹] to ingest nitroblue tetrazolium was assessed at rest and following a standardised submaximal bout of exercise on a cycle ergometer.

Results—Trained subjects had a lower resting blood neutrophil count ($P < 0.01$). Acute exercise caused a rise ($P < 0.01$) in the blood neutrophil count irrespective of training status, but the magnitude of the rise was smaller in the trained subjects ($P < 0.05$). The circulating neutrophil phagocytic capacity was approximately 70% lower in trained individuals at rest compared with the control subjects ($P < 0.01$). Acute submaximal exercise increased this variable in both groups, but phagocytic capacity remained substantially lower in the trained subjects compared with the controls ($P < 0.05$) despite the observation that a higher proportion of the circulating neutrophils were stimulated to undergo phagocytosis in the trained subjects [57(14)% *v* 32(7)%; $P < 0.01$].

Conclusions—Although neutrophil phagocytic activity is only one variable that contributes to immunological status, prolonged periods of endurance training may lead to increased susceptibility to opportunistic infections by diminishing this activity at rest.

(Br J Sports Med 1996;30:125-129)

Key terms: exercise; training; humans; neutrophils

Neutrophils constitute 50–60% of the circulating blood leucocyte pool and have an important role in non-specific host defence against a variety of microbial pathogens including bacteria, viruses, and protozoa. Neutrophils kill microbes by ingestion (phagocytosis) followed by enzymatic attack and digestion within intracellular vacuoles, utilising granular hydrolytic enzymes and reactive oxygen species.¹ Disorders of neutrophil function and neutropenia are associated with recurrent infections.¹ There is now a convincing body of

evidence to show that highly trained athletes are more susceptible to infection than their relatively sedentary counterparts.²⁻⁶ Frequent intense training has been shown to impair various aspects of specific immunity.⁵⁻⁸ Low blood leucocyte counts are also commonly reported in athletes.²⁻⁶ An impaired or depleted neutrophil function could possibly be an important contributing factor to the increased susceptibility to infection of athletes.⁹

Smith *et al*¹⁰ showed that neutrophil phagocytic activity remains increased for six hours after a bout of submaximal exercise. However, activated neutrophils become depleted of their granular enzymes and have a decreased capacity to produce and/or release oxygen derived free radicals upon subsequent stimulation with zymosan.¹¹ It is possible, therefore, that repeated bouts of exercise may result in a circulating population of neutrophils that are functionally depleted, and less capable of responding to a pathogenic challenge. The large number of intense training sessions that elite athletes undertake each week could leave a significant proportion of their circulating neutrophil population in a chronically sub-optimal state.⁹

Very few studies have compared the effects of training on the neutrophil response to exercise. Both Lewicki *et al*¹² and Hack *et al*¹³ reported no differences in circulating neutrophil numbers and in vitro phagocytic capacity in neutrophils isolated from athletes and untrained controls, either at rest or after graded exercise to exhaustion on a treadmill. However, in a more recent study, Hack *et al*¹⁴ reported a training period dependent impairment in neutrophil function: trained subjects performing longer and more frequent training (running) bouts had lower neutrophil counts at rest and reduced phagocytic activity at rest and after exercise compared than when they were performing lower training loads and compared with sedentary controls. Smith *et al*¹⁰ have also showed that the capacity of isolated neutrophils to produce H₂O₂ and HOCl upon stimulation with opsonised zymosan was about 50% lower in trained cyclists compared with untrained subjects. However, an acute bout of submaximal exercise increased the ability to produce microbicidal reactive oxygen species, irrespective of training status. In contrast, Ortega *et al*¹⁵ reported that neutrophils isolated from elite female basketball players had a greater phagocytic capacity than those from sedentary women. The controversy regarding the effect of training status on neutrophil

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Accepted for publication 13 September 1995

counts and function may be because investigators have employed different training regimens or different durations of training periods.

In order to determine if neutrophil function is modified by very long term endurance training we compared neutrophil phagocytic activity in trained male cyclists who had been exercising on a regular basis for over 10 years and age matched untrained controls. Both qualitative and quantitative indices of phagocytic activity were measured under resting conditions and following an acute standardised bout of submaximal exercise.

Methods

SUBJECTS

Sixteen middle aged men (35–45 years) volunteered to take part in the study which was approved by the Coventry research ethics committee. Eight were highly trained cyclists and at the time of the study were cycling distances of 120(SD 30) km per week. They had all been regularly exercising for at least the past 10 years. The other eight subjects were age matched controls who did not participate in any form of strenuous physical activity in their leisure time. The physical characteristics of the subjects are shown in table 1. The untrained subjects were of similar age, height, and body mass but had a lower maximum oxygen uptake compared with the cyclists, at 37(7) *v* 61(9) ml·kg⁻¹·min⁻¹; *P* < 0.01). All the subjects were in good health and they had been without medication for at least four weeks before the study. They refrained from eating food and drinking alcohol, coffee, or tea for 12 h before the exercise tests. For the cyclists, their last training bout occurred 36–48 h before the exercise tests.

BLOOD SAMPLING AND EXERCISE PROTOCOL

Subjects reported to the laboratory at 09.00–11.00 h. They were rested (seated) for 30 min before a pre-exercise blood sample was taken by venepuncture from an antecubital vein. Shortly after, the subjects exercised on an electrically braked cycle ergometer for 15 min at each of three work rates (50, 100, and 150 W). Subjects were required to maintain a pedal cadence of 60–65 rev·min⁻¹. Room temperature was 20(2)°C and relative humidity was 45(10)%. Heart rates were monitored continuously and recorded at 5 s intervals using a telemetric heart rate system (Sports Tester PE3000, Polar Electro, Finland). A further venous blood sample was taken immediately after exercise.

Table 1 Physical characteristics of the subjects. Values are means (SD)

	Control	Trained
Age (years)	37.8 (3.5)	38.0 (4.0)
Height (m)	1.77 (0.05)	1.75 (0.04)
Body mass (kg)	71.4 (8.3)	72.5 (7.0)
VO ₂ max (ml·kg ⁻¹ ·min ⁻¹)	37.4 (6.6)	61.0 (8.8)†
HR (beats·min ⁻¹) pre-exercise	69 (14)	52 (8)*
HR (beats·min ⁻¹) post-exercise	161 (19)	127 (13)†

Significant differences between trained and control subjects: **P* < 0.05; †*P* < 0.01.

VO₂max, maximum rate of oxygen uptake; HR, heart rate.

BLOOD ANALYSIS

Ten millilitres of blood were taken from each subject before and after exercise. One aliquot of blood was placed into a 4.5 ml K₃EDTA tube for analysis of haematological variables, including a differential leucocyte count using a Technicon H-2 system. Changes in plasma volume after exercise were calculated from the changes in blood haemoglobin concentration and packed cell volume relative to the pre-exercise sample, as described by Dill and Costill.¹⁶

One millilitre of blood was pipetted into a siliconised collection vial containing 20 units of heparin. In triplicate, 50 μl of heparinised blood were added, together with 100 μl of 0.1% (wt/vol) nitroblue tetrazolium (NBT) solution and 5 μl of freeze dried bacterial extract stimulant solution to siliconised vials, as described by the Sigma Diagnostics NBT reduction test kit bulletin (Sigma Chemical Company Ltd, Poole, Dorset, UK). The mixture was incubated at 37°C for 10 min and then left to stand at room temperature for a further 10 min. Approximately 50 μl of the mixture was then transferred onto a glass slide, smeared, stained using Wright stain (Sigma), and allowed to dry before being microscopically inspected using an oil immersion objective. For each slide, a total of 100 neutrophils was manually counted. Those neutrophils showing blue/black formazan deposits were recorded as positive phagocytic cells. The circulating number of active phagocytic neutrophils was calculated by multiplying the proportion of positive cells by the number of circulating neutrophils per litre of blood.

Four millilitres of blood was placed into a beaker containing K₃EDTA, to which was added 16.0 ml of ice cold isotonic ammonium chloride solution in order to lyse the red blood cells. The mixture was mixed well, incubated at 4°C for 15 min and then centrifuged at 160 *g* for 10 min. The supernatant was removed and the cell pellet gently resuspended in 10 ml of 0.15 M phosphate buffered saline (PBS; pH 7.2). Neutrophils were washed twice in PBS and the cells resuspended in 0.5 ml of RPMI 1640 cell culture medium (Sigma). A quantitative NBT test as described by Chapel and Gooi¹⁷ was carried out in triplicate on 100 μl samples of the neutrophil suspension, using latex beads (0.8 μm diameter) to stimulate phagocytosis. Following incubation at 37°C for 15 min, 5.0 ml of HCl (1.0 M) was added. After centrifugation at 1000 *g* for 5 min, the supernatant was discarded. The formazan was extracted from the neutrophils using pyridine and the absorbance of the extract was measured at 515 nm against a reagent blank. The quantitative circulating phagocytic capacity (CPC) was then calculated according to the following formula:

$$\text{CPC} = (\text{absorbance at 515 nm}) \cdot (\text{litre of blood})^{-1}$$

STATISTICAL ANALYSIS

The experimental data established in triplicate are given as means (SD) unless otherwise stated. Comparisons between trained and

untrained subjects were performed using Student's unpaired *t* test. For comparisons within groups, the paired *t* test was used. The accepted level of significance employed was $P < 0.05$.

Results

HEART RATE AT REST AND DURING EXERCISE

Resting heart rate was lower ($P < 0.05$) in the trained subjects than in the untrained controls (table 1). During the final minute of the exercise bout at a work rate of 150 W, heart rate was also significantly lower in the trained subjects, at 127(13) *v* 161(19) beats·min⁻¹ ($P < 0.01$). The relative exercise intensity during the final exercise period was 69(7)% and 87(10)% of age predicted maximum heart rate for the trained and control group, respectively.

RESTING HAEMATOLOGICAL VARIABLES AND LEUCOCYTE RESPONSES TO EXERCISE

At rest, cell counts of circulating leucocytes, lymphocytes, and neutrophils were all significantly lower in the trained than in the control subjects (table 2). Packed cell volume was not significantly different [trained 42.4(2.3)%; controls 44.0(1.5)%; $P > 0.05$], but the blood haemoglobin concentration was slightly lower in the trained subjects [trained 14.1(0.8) g·dl⁻¹; controls 15.1(0.4) g·dl⁻¹; $P < 0.05$].

Plasma volume fell by 7.3(4.4)% ($P < 0.05$) in the control subjects after exercise, and by a similar amount [6.3(3.0)%; $P < 0.05$] in the trained subjects. Immediately after exercise the total number of leucocytes, lymphocytes, and neutrophils increased significantly compared with resting values in both groups of subjects (table 2). However, the magnitude of the increases in leucocytes, lymphocytes, and neutrophils were all significantly smaller in the trained subjects compared with the controls.

NEUTROPHIL PHAGOCYTIC ACTIVITY

The percentage of neutrophils that ingested formazan deposits after stimulation with bacterial extract was not different in the cyclists compared with the controls at rest (table 3). Exercise increased the percentage of actively phagocytic cells in both groups ($P < 0.01$), but to a greater extent in the trained subjects.

The circulating number of stimulated (positive) phagocytic neutrophils was higher in the controls compared with the trained subjects at rest ($P < 0.05$) and increased in both groups following exercise ($P < 0.01$). However, after exercise there was no significant differ-

Table 3 Neutrophil phagocytic activity and number of circulating active phagocytic neutrophils. Values are means (SD)

	Phagocytic activity (% positive cells)	
	Pre-exercise	Post-exercise
Control	15 (3)	32 (7)†
Trained	19 (3)	57 (14) ^b
Number of circulating active phagocytic neutrophils ($\times 10^9 \cdot \text{litre}^{-1}$)		
	Pre-exercise	Post-exercise
Control	0.61 (0.08) ^a	1.53 (0.33)†
Trained	0.47 (0.10)	1.70 (0.41)†

Significant differences compared with resting values: * $P < 0.05$; † $P < 0.01$. Significant differences between control and trained subjects: a $P < 0.05$; b $P < 0.01$.

ence in the circulating number of stimulated phagocytic neutrophils in the controls and trained subjects (table 3).

The CPC, a quantitative measure of formazan ingestion per unit volume of blood, was greater ($P < 0.01$) in the sedentary controls than in the trained subjects at rest (table 4). Exercise increased the CPC in both trained and untrained subjects, but the CPC was still higher in the controls than in the trained subjects immediately after exercise ($P < 0.05$).

Discussion

Our study provides further evidence that both acute exercise and endurance training affect neutrophil phagocytic activity. We recognise that there are definite weaknesses in cross sectional studies and it is obviously important to be sure that any observed differences are not simply due to incomplete recovery from the last training session. A cross sectional comparison is necessary in the present situation because of the very prolonged (> 10 years) training period. For our trained cyclists, the last training bout was performed 36–48 hours before the exercise tests. Most studies (for example, Smith *et al*¹⁰) indicate that cell counts and neutrophil phagocytic function return to pre-exercise levels within 24 hours of a strenuous exercise bout. Acute exercise, in general appears to elicit an initial activation of neutrophils.^{8 18 19} Previous studies have indicated that both submaximal cycling exercise in young untrained subjects²⁰ and progressive incremental cycling¹² or running¹³ to fatigue in young highly trained subjects increase neutrophil phagocytic function. Our findings show that neutrophil phagocytic activity is increased in both trained and untrained middle aged subjects in response to a standardised submaximal exercise bout.

Acutely, exercise caused an increase in the blood leucocyte count (table 2). During short term exercise (less than one hour) this is largely

Table 2 Blood cell counts of circulating leucocytes, lymphocytes, and neutrophils in control (C) and trained (T) subjects. Values are means (SD)

Cells ($\times 10^9 \cdot \text{litre}^{-1}$)		Leucocytes	Lymphocytes	Neutrophils
Pre-exercise	C	6.62 (0.87)	2.02 (0.27)	3.83 (0.86)
	T	4.36 (1.15) ^b	1.36 (0.20) ^b	2.46 (0.87) ^b
Post-exercise	C	8.76 (1.43)†	2.89 (0.53)†	4.78 (1.30)†
	T	5.51 (1.07) ^b	1.87 (0.34)*	2.99 (0.79)*
Increase due to exercise	C	2.14 (0.62)†	0.87 (0.34)†	0.95 (0.31)†
	T	1.02 (0.45)†	0.46 (0.26)*	0.53 (0.28)*

Significant differences compared with resting values: * $P < 0.05$; † $P < 0.01$. Significant differences between control (C) and trained (T) subjects: a $P < 0.05$; b $P < 0.01$.

Table 4 Circulating phagocytic capacity (CPC) in control and trained subjects ($A_{515nm} [\text{litre blood}]^{-1}$). Values are means (SD)

CPC	Pre-exercise	Post-exercise
Control	141 (41)	189 (64)*
Trained	36 (9) ^b	106 (48)†

Significant differences compared with resting values: * $P < 0.05$; † $P < 0.01$. Significant differences between control and trained subjects: a $P < 0.05$; b $P < 0.01$.

due to demargination of leucocytes that are normally adhered to the vascular endothelium at rest. Increased cardiac output and raised plasma concentrations of catecholamines are thought to be mostly responsible for demargination.²¹ Very few studies are available on the influence of training status on the leucocyte response to exercise. One study reported no differences between trained and untrained subjects during submaximal and maximal exercise,²² while another reported larger increases in trained subjects after exhaustive exercise.²³ However, neither of these studies compared trained and untrained subjects at the same absolute or relative exercise intensities. Indeed, in both studies the trained subjects exercised at a higher work rate or for a longer duration. Our data establish that the magnitude of the increase in circulating leucocytes in response to the same absolute submaximal work rate is significantly smaller in trained subjects. A likely explanation for this is the reduced catecholamine response to exercise after training²⁴ and a lesser degree of demargination. Our findings and those of Hack *et al*¹³ contrast with a recent study by Benoni *et al*,²⁵ who noted a significant increase in circulating neutrophils after exercise in trained subjects but not in untrained subjects who performed 10 minutes of cycling at the same relative exercise intensity (heart rate of 150 beats·min⁻¹). However, it may be that the intensity or duration of the exercise was not sufficient to elicit a significant response in their control group.

In the present study an acute bout of exercise increased the percentage of activated phagocytic neutrophils irrespective of training status. However, a higher proportion of the neutrophils from the trained subjects became activated after exercise compared with the sedentary controls (table 3). This could be due to an altered receptor density after training, increasing the sensitivity of the neutrophils to factors that stimulate phagocytosis. Acute exercise is known to increase the expression of complement receptors on the neutrophil plasma membrane.¹⁹ Further studies are required to establish the time course of this effect and to determine if a similar effect can be induced by training.

Alternatively, exercise may exert an intensity-dependent effect on neutrophil function. Dziedziak²⁶ showed that the neutrophil oxidative burst was enhanced after light exercise (50% $\dot{V}O_{2max}$), but decreased after more intense exercise (80% $\dot{V}O_{2max}$). Our finding that the proportion of actively phagocytic cells was greater in the trained subjects after exercise, may be a reflection of the lower relative exercise intensity for these subjects compared with the controls.

Neutrophil phagocytosis, oxidative burst and degranulation can be induced by a number of factors including endotoxin, complement cleavage products, platelet activating factor, interleukin-1, leukotriene B₄, and fibronectin.¹⁻⁹ Once activated, neutrophil microbicidal activity can be further enhanced by other soluble factors including growth hormone.²⁷ Exercise is associated with complement activation,¹⁸ and

increased plasma concentrations of growth hormone^{24, 28} and interleukin-1.²⁹ These factors may be responsible for the exercise induced activation of neutrophils, as evidenced by the appearance of granular enzymes in plasma¹⁹ and increased *in vitro* phagocytic activity³⁰ following an acute bout of exercise. Alternatively, the increased activity of neutrophils observed after exercise could be due to a redistribution of neutrophils, with demargination of a sub-population of cells having a greater intrinsic activity entering the circulating pool. However, this possibility was discounted by Smith *et al*¹⁰, since brief maximal cycling depressed the capacity of neutrophils to produce reactive oxygen species in spite of significant demargination.

The markedly lower neutrophil cell counts and circulating phagocytic capacity (CPC) of the blood of the trained subjects at rest compared with the sedentary controls suggests a depletion of the neutrophil system as a result of prolonged training. Even after exercise, the CPC remained higher in the controls than in the trained subjects (table 4), despite a similar circulating number of active phagocytic neutrophils (table 3), indicating that the capacity of individual stimulated neutrophils to ingest foreign particles is decreased by endurance training. This conclusion is supported by the finding of Smith *et al*¹⁰ that postexercise neutrophil oxidative activity at low particle concentration was lower in athletes than non-athletes, implying a lower affinity of neutrophils in trained athletes. The mechanism causing this diminution of phagocytic function at rest in well trained subjects is not clear, but could be related to the chronically raised plasma concentrations of stress hormones, including adrenaline¹⁴ and cortisol,³¹ which have been reported in highly trained individuals at rest. Both these hormones are known to decrease adherence and chemotaxis of neutrophils.³² Another possible explanation is that repeated bouts of exercise in which neutrophils are activated^{18, 19} (as indicated by the release of granular enzymes into the plasma) cause neutrophils to become functionally depleted. It has been shown that neutrophils have a reduced capacity to produce and release oxygen free radicals following repeated stimulation.¹¹ Hence, a high training load or very prolonged periods of training may leave a significant proportion of the athlete's neutrophils in a chronically depleted state, making the individual more prone to opportunistic infections.

Innate non-specific immunity is heavily dependent on the capacity of the circulating blood pool of neutrophils to react to a potentially pathogenic microbial challenge.¹⁻⁹ This capacity is determined to a significant degree by the phagocytic capacity, oxidative capacity, and circulating numbers of neutrophils, all of which appear to be diminished in highly trained individuals. This may be one explanation for the observation that athletes engaged in heavy or very prolonged training programmes show an increased susceptibility to infection compared with their more sedentary counterparts.²⁻⁶

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