Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-α levels after prolonged running

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Starkie, R. L., J. Rolland, D. J. Angus, M. J. Anderson, and M. A. Febbraio. Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-α levels after prolonged running. Am J Physiol Cell Physiol 280: C769–C774, 2001.—The present study was undertaken to examine the effect of prolonged running on monocyte intracellular cytokine production and plasma cytokine concentration. Blood samples were collected 1 h before, immediately after, 2 h after, and 24 h after a competitive marathon run. There was no change in the number of cells spontaneously producing tumor necrosis factor (TNF)-α; however, there was a decrease in the number of cells producing interleukin (IL)-1α and IL-6 (P < 0.01) postexercise. In contrast, there was an increase in the number of cells producing monocyte IL-1β and IL-6 (P < 0.01) postexercise. Plasma IL-6, TNF-α, epinephrine, norepinephrine, and cortisol concentrations were markedly increased (P < 0.01) postexercise. These data demonstrate that circulating monocytes are not the source of elevated levels of plasma IL-6 and TNF-α after prolonged running. In addition, it is likely that stress hormones result in a decrease in the amount of cytokine produced by LPS-stimulated cells postexercise.

Flow cytometry; epinephrine; cortisol

PLASMA LEVELS OF SEVERAL CYTOKINES, namely, interleukin (IL)-1β (15), tumor necrosis factor (TNF)-α (15, 27), and IL-6 (11, 14–16, 22, 24, 28), have been shown to increase during and after intense prolonged exercise. It has been hypothesized that stress associated with strenuous exercise alters leukocyte cytokine production (2). IL-1, TNF, and IL-6 are recognized as key components of the immune response against infections (2), and alterations in their production could leave individuals vulnerable to invading pathogens after exercise. Numerous cells in a variety of tissues produce proinflammatory cytokines (25); therefore, alterations in plasma concentrations are not necessarily indicative of changes in production by circulating leukocytes. In support of this, Ostrowski et al. (16) observed IL-6 mRNA expression in skeletal muscle subjected to prolonged running. These previous data provide indirect evidence that increases in plasma cytokines may be due not only to altered circulating leukocyte production but also to changes in other tissues, such as contracting muscle. In the present study, intracellular cytokines in blood leukocytes were analyzed by flow cytometry to determine the association between exercise and the number of circulating monocytes producing IL-1α, TNF-α, and IL-6, as well as the amount of cytokine they produce. This method has the advantage of rapid analysis of cytokine production by a large number of individual cells, permitting reliable detection of even small proportions of cytokine-positive cells (18). Recent work from our laboratory (23) employed this method to study the effect of prolonged, submaximal cycling exercise on monocyte cytokine production. We observed that circulating monocytes are not likely to be the source of the small increase in plasma IL-6, because no change was observed in spontaneous monocyte IL-6 production postexercise. Furthermore, exercise increased the number of monocytes producing cytokines upon stimulation; however, these cells produced less cytokine postexercise compared with preexercise. Notably, plasma cortisol levels were not elevated postexercise. Cortisol infusion has been demonstrated to decrease IL-1α, TNF-α, and IL-6 production upon stimulation (7); therefore, elevations in cortisol may affect cytokine production. As yet, it is not known whether exercise that causes substantial elevations in plasma cortisol levels has a similar effect on spontaneous and stimulated monocyte intracellular cytokine production.

Damaged muscle releases cellular fragments into the circulation that, in turn, may activate immune cells (6). It has been reported that muscle and joint trauma results in activation of circulating monocytes, which, in turn, produce large quantities of proinflammatory IL-1, IL-6, and TNF (20). Because cycling was the exercise mode employed in our previous study, it is unlikely that a large amount of muscle damage would have occurred. In addition, the rise in plasma IL-6 was small (<2 pg/ml), whereas studies that have used running (16) report much higher levels (>90 pg/ml). As...
yet, it is not known whether exercise, which causes substantial elevations in plasma IL-6, has a similar effect on spontaneous and stimulated monocyte intracellular cytokine production.

Hence, the aim of the present study was to investigate whether prolonged, strenuous running affects the ability of circulating monocytes to produce cytokines upon stimulation and whether spontaneous cytokine production is responsible, in part, for the increased plasma cytokine concentration. It was hypothesized that exercise would decrease cytokine production by stimulated circulating monocytes and that these cells would not be the source of elevations in plasma cytokines.

METHODS

Subjects. Five male entrants (76.9 ± 3.5 kg; 182 ± 4.7 cm) in the Melbourne Marathon volunteered for this study. Each subject was informed of the experimental protocol and possible risks and signed a letter of informed consent before participating. Subjects had been free from infection for 6 wk before the study, were exempt from symptoms of respiratory illnesses, and were not taking any medication. For 2 mo leading up to the marathon, subjects had been running 84 ± 22 km/wk. On the day of the marathon, the temperature was 21.2°C and the humidity was 71%. The marathon commenced at 8:00 AM, and subjects completed the race in 169 ± 20 min (range 151–205 min). Experiments were approved by the Human Research Ethics Committee of The University of Melbourne.

Experimental procedures. A resting blood sample was obtained from a vein in the antecubital fossa 1 h before commencement of the race (preexercise). Blood was also collected immediately upon completion of the marathon (post-exercise) and after 2 h (2 h postexercise) and 24 h of recovery (24 h postexercise). During the race, intake of drinks was ad libitum, and during the 2-h rest period, subjects were given water and Gatorade ad libitum. Preexercise, postexercise, and 2-h postexercise blood samples were analyzed for alterations in leukocyte counts and spontaneous and lipopolysaccharide (LPS)-stimulated monocyte cytokine production. In addition, plasma IL-6, TNF-α, glucose, lactate, cortisol, catecholamines, and creatine kinase were analyzed in all blood samples.

Leukocyte counts. Blood (3 ml) was placed in EDTA tubes and analyzed for differential white cell counts as routinely performed by the hematology laboratory at Alfred Hospital (Melbourne, Victoria, Australia). This analysis included the determination of total white blood cell numbers and neutrophil, monocyte, and lymphocyte numbers to detect changes in circulating white blood cell populations with exercise.

Intracellular cytokines. Blood (2 ml) was placed in heparin sodium tubes and kept at room temperature until the end of the experiment for measurement of intracellular cytokine production. The tubes were gently inverted and rolled periodically. Whole blood was incubated for 4 h with (stimulated) or without (spontaneous) 1 μg of LPS at 37°C in a humidified incubator. Brefeldin-A (10 μg/ml) was added to all samples at the commencement of incubation to inhibit intracellular transport of proteins, thus retaining cytokines produced within the cell. Aliquots (100 μl) of stimulated and nonstimulated blood were then incubated for 30 min with CD33 (PECy5)-conjugated monoclonal antibody (Immunotech, Marseille, France) for staining of monocytes. Red blood cells were lysed (0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 1 mM EDTA) for 10 min, and the samples were spun in a centrifuge (350 g) for 5 min. The supernatant was decanted, and the pellet was resuspended in 500 μl of 4% paraformaldehyde for 20 min. Samples were again spun (350 g) for 5 min, and the supernatant was decanted. The fixed cells were permeabilized with 500 μl of permeabilizing solution (Becton Dickinson, San Jose, CA) for 20 min, washed (1% fetal calf serum, phosphate-buffered saline, and 0.02 M sodium azide), and spun (350 g) for 5 min, and the supernatant was decanted. The cells were then incubated with monoclonal antibodies against IL-6 [fluorescein isothiocyanate (FITC); Pharmingen, San Diego, CA], IL-1α [r-phycoerythrin (PE)], TNF-α (FITC), and/or control (PE/Cy5/FITC/PE; Becton Dickinson) for 30 min. After the samples had been washed and then spun (350 g) for 5 min, the pellet was resuspended in 500 μl of wash buffer. All incubations took place at room temperature in the dark. The percentage of cytokine-positive monocytes was determined by flow cytometry (FACScan; Becton Dickinson). Monocytes were separately gated on viable cells on a side scatter vs. CD33 (FL3) cytogram. Data for 2 × 10⁶ events within this gate were acquired. Analysis of data was then performed by using the Cell Quest program (Becton Dickinson) with gates for positive set on isotype controls (Fig. 1). Results are expressed as the percentage and number of cytokine-producing cells in CD33 + populations. The absolute count was determined by multiplying the percentage of cytokine-positive monocytes by the concentration of monocytes in peripheral blood. For quantification of the amount of cytokine within positive cells, the mean fluorescence intensity of positive events was obtained.

Plasma IL-6 and TNF-α. Blood (3 ml) was collected into EDTA tubes and spun for 4 min at 6,000 g. The supernatant was removed and stored at −80°C until analysis. The concentrations of IL-6 and TNF-α were measured by using commercially available chemiluminescent ELISA kits (R&D Systems, Minneapolis, MN), which detect both soluble and receptor-bound IL-6 and TNF-α. All measurements were performed in duplicate.

Hormones and metabolites. Blood (5 ml) was placed in heparin lithium tubes for analysis of cortisol, glucose, and lactate, and blood (2 ml) for catecholamine analysis was placed into tubes containing 20 μl/ml EGTA and reduced glutathione. Blood was then spun for 4 min at 6,000 g. The supernatant was removed and stored at −80°C until analysis. Cortisol concentration was determined by RIA (Diagnostic Products, Los Angeles, CA). Samples were analyzed for plasma catecholamines by using a modification of the single isotope ³H radioenzymatic assay (TRK 995; Amersham, Amersham, UK). Plasma glucose and lactate were analyzed by using enzymatic automated analysis (EML-105; Electrolyte Metabolite Laboratory, Radiometer, Copenhagen, Denmark).
Creatine kinase. Blood (2 ml) was collected into heparin lithium tubes, spun for 4 min at 8,000 rpm, and stored at −80°C until analysis. Samples were analyzed for creatine kinase by using enzymatic automated analysis (Hitachi System 747; Boehringer Mannheim Diagnostica, Mannheim, Germany).

Statistical analysis. Analysis of the measured variables revealed that the data were not normally distributed. To ensure homogeneity of the data, data were log transformed before statistical analysis. A one-way analysis of variance (ANOVA) with repeated measures on the time factor was used to compare blood metabolites, hormones, white blood cell counts, and cytokine-positive cells. Newman-Keuls post hoc tests were used to locate differences when the ANOVA revealed a significant interaction. Descriptive data are presented as means ± SD, and comparative data are presented as means ± SE. The level of significance to reject the null hypothesis was set at \( P < 0.05 \).

RESULTS

Leukocyte numbers. There was an increase \( (P < 0.01) \) in circulating monocyte and neutrophil numbers postexercise and 2 h postexercise compared with preexercise, resulting in an increase \( (P < 0.01) \) in total circulating leukocyte numbers (Table 1). Lymphocyte numbers remained at preexercise levels after the marathon and were suppressed \( (P < 0.01) \) 2 h postexercise (Table 1). Cell counts had returned to resting values at 24 h postexercise.

Spontaneous cytokine production. There was a decrease \( (P < 0.01) \) in the percentage of monocytes spontaneously producing TNF-\( \alpha \) postexercise; however, there was no change in the number of cells producing TNF-\( \alpha \) as a result of exercise (Table 2). In addition, the amount of TNF-\( \alpha \) per cell, as indicated by fluorescence intensity, was decreased \( (P < 0.01) \) postexercise and 2 h postexercise (Table 2). This decrease indicates that cells entering circulation during exercise are not spontaneously producing TNF-\( \alpha \) and that previously productive cells continue to produce TNF-\( \alpha \), but in lesser amounts. The percentages of cells spontaneously producing IL-1\( \alpha \) and IL-6 were decreased \( (P < 0.01) \) postexercise and 2 h postexercise, and the numbers of cells spontaneously producing IL-1\( \alpha \) and IL-6 were decreased \( (P < 0.01) \) postexercise (Table 2). This decrease indicates that monocytes entering the circulation during exercise are not producing IL-6 and IL-1\( \alpha \) spontaneously and that cells previously producing IL-1\( \alpha \) and IL-6 cease production after exercise. Exercise had no effect on the amount of IL-1\( \alpha \) \( (P = 0.35) \) and IL-6 \( (P = 0.52) \) in positive cells (Table 2).

Stimulated cytokine production. Upon stimulation with LPS, the percentage of IL-1\( \alpha \)-positive monocytes was depressed postexercise \( (P < 0.05) \), and this remained low \( (P < 0.01) \) at 2 h postexercise (Table 3). There was no change in the percentages of TNF-\( \alpha \)- or IL-6-positive monocytes postexercise. However, because of the increase in circulating monocyte numbers postexercise and 2 h postexercise \( (P < 0.01) \), the absolute number of cytokine-positive monocytes was increased \( (P < 0.01) \) at these times (Table 3). The amounts of IL-1\( \alpha \), TNF-\( \alpha \), and IL-6 in positive cells...
were decreased ($P < 0.05$) in stimulated samples post-exercise and 2 h post-exercise (Table 3).

**Plasma measurements.** Plasma IL-6 (Fig. 2) and TNF-α (Fig. 3) concentrations were elevated ($P < 0.01$) post-exercise and 2 h post-exercise, and the increase in plasma IL-6 was maintained 24 h post-exercise. Exercise had no effect on plasma glucose concentration; however, plasma lactate was increased ($P < 0.01$) in post-exercise samples (Table 4). Plasma epinephrine, norepinephrine, and cortisol concentrations were elevated ($P < 0.01$) post-exercise (Table 4). Cortisol and norepinephrine levels remained elevated 2 h post-exercise ($P < 0.01$) but had returned to pre-exercise levels by 24 h post-exercise. Creatine kinase activity (Table 4) was elevated post-exercise, 2 h post-exercise, and 24 h post-exercise ($P < 0.01$).

**DISCUSSION**

Numerous studies have examined the effect of exercise on the plasma concentration of proinflammatory cytokines; however, it is acknowledged that few studies have examined the cellular origin of these cytokines (2). Previous studies have demonstrated that exercise does not increase IL-1α, IL-1β, TNF-α, and IL-6 mRNA in blood mononuclear cells, even though plasma concentrations of IL-1β, TNF-α, and IL-6 increase with exercise (10, 26). Moldoveanu et al. (10) and Ullum et al. (26) suggested that monocytes were unlikely to be the source of the rise in plasma IL-6 with exercise. In the present study, the decrease in percentage and number of cells producing cytokines, as well as the fluorescence intensity of cytokine-positive cells, post-exercise provides evidence that circulating monocytes are not likely to be the source of elevated plasma concentrations of TNF-α or IL-6.

It has been suggested that the source of this increase in plasma cytokines is the contracting skeletal muscle. Bruunsgaard et al. (4) observed higher levels of plasma IL-6 when comparing eccentric with concentric exercise. They also observed an increase in plasma creatine kinase activity, leading them to conclude that the cytokine response to exercise is related to muscle damage. Further research reported plasma IL-6 concentration to be higher after running (which has a large eccentric component) than after cycling (little eccentric component) exercise (13). In our previous study, in which subjects performed 2 h of cycling at 70% peak $O_2$ consumption, plasma IL-6 was <2 pg/ml post-exercise (23), compared with 120 pg/ml in the present study. It is well recognized that repetitive eccentric contractions cause more damage to skeletal muscle than concentric contractions (1, 9, 19). Muscle damage invokes an immune response, and it is possible that this process stimulates local production of inflammatory cytokines (17). Monocytes are involved in the muscle tissue inflammatory response to muscle injury (29), and thus infiltrating monocytes may be the source of elevated plasma IL-6. Indeed, IL-6 mRNA has been reported to be expressed in skeletal muscle after prolonged running (16). Both the identification of the cytokine-producing cell within the muscle and the question as to whether the increase in cytokine mRNA results in protein translation during acute exercise remain to be elucidated. The present study demonstrates increased levels of plasma IL-6 and TNF-α post-exercise, yet the

**Table 4. Plasma hormones and metabolites**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Pre</th>
<th>Post</th>
<th>2 h Post</th>
<th>24 h Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>5.5 ± 0.1</td>
<td>6.1 ± 0.7</td>
<td>5.3 ± 0.5</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.5 ± 0.1</td>
<td>4.3 ± 0.7a</td>
<td>1.9 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Epinephrine, nmol/l</td>
<td>0.3 ± 0.1</td>
<td>1.4 ± 0.4ab</td>
<td>0.3 ± 0.1</td>
<td>nm</td>
</tr>
<tr>
<td>Norepinephrine, nmol/l</td>
<td>1.0 ± 0.3</td>
<td>6.6 ± 0.5a</td>
<td>2.1 ± 0.5a</td>
<td>nm</td>
</tr>
<tr>
<td>Cortisol, nmol/l</td>
<td>654 ± 511</td>
<td>192 ± 83a</td>
<td>863 ± 83a</td>
<td>408 ± 58</td>
</tr>
<tr>
<td>Creatine kinase, U/l</td>
<td>188 ± 41</td>
<td>427 ± 53a</td>
<td>697 ± 149a</td>
<td>2,810 ± 1,150a</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 5$ subjects) and represent concentrations of plasma hormones and metabolites before, immediately after, 2 h into recovery, and 24 h into recovery after a marathon race. nm, Not measured. *$P < 0.01$ vs. Pre.
data show a decrease and no change in the number of circulating monocytes spontaneously producing IL-6 and TNF-α, respectively. In addition, mean IL-6 fluorescence intensity was not altered in response to exercise, demonstrating that any cells that were spontaneously producing IL-6 postexercise did not increase their production. A decrease in mean TNF-α fluorescence intensity postexercise and 2 h postexercise suggests that cells spontaneously producing TNF-α decreased their production at these time points; therefore, it is not likely that these cells are the source of the increase in plasma levels. It is possible, however, that these cells were productive in the early stages of exercise and were in a refractory period at the time of collection. Despite this possibility, it is not likely that these previously produced cytokines were still present and significantly contributing to the elevated cytokine concentration at the time of collection because cytokines are unstable and are removed from the circulation. This suggests that IL-6 and TNF-α are coming from a source other than circulating monocytes. Because prolonged running was the mode of exercise employed, and high levels of plasma creatine kinase were observed, local production in damaged skeletal muscle was a likely source.

Decreased blood flow to the splanchnic bed during exercise may be sufficient to induce an ischemic state resulting in gut wall bacterial translocation (2). Endotoxemia has been observed after marathon running (5); therefore, it is possible that subjects in the present study had elevated levels of endotoxin. Endotoxins are lipopolysaccharides of gram-negative bacteria, and it is possible that this powerful monocyte stimulant contributes to elevations in plasma proinflammatory cytokines. The results from the present study demonstrate a decrease in spontaneous cytokine production by circulating monocytes postexercise; therefore, if endotoxemia does contribute to increased levels of plasma cytokines, it is not due to its stimulatory effect on circulating monocytes.

There are several reports that the risk of upper respiratory tract infections is higher in athletes undertaking heavy training or competing in endurance events compared with that in nonathletes (8, 12). It is possible that exercise modifies leukocyte cytokine production, thereby affecting immune function. In the present study, exercise resulted in an increase in circulating leukocytes; therefore, there were more monocytes in circulation to respond to stimulation. However, it is important to note that monocytes responding to stimulation were producing less cytokine than they were preexercise. Hence, exercise may have resulted in an increase in the number of cytokine-positive cells in response to stimulation, but these cells were producing less cytokine postexercise than preexercise. The overall impact of these observations on immune function and the question as to whether cytokine production is a limiting factor in immune protection postexercise remain unclear.

Neuroendocrine hormones have been shown to regulate the immune response, and direct neuroimmune communication occurs (21). Proinflammatory cytokines activate both the hypothalamic-pituitary-adrenal axis and the sympathoadrenergic system (24), both of which exert potent anti-inflammatory actions that limit production of proinflammatory cytokines (2). Incubation of whole blood with epinephrine (3) or norepinephrine (26) decreases IL-6 and TNF-α production. Furthermore, administration of cortisol at levels comparable to those in the present study decreased LPS-stimulated IL-1, TNF-α, and IL-6 production (7). In the present study, cells spontaneously producing IL-1α and IL-6 ceased production after exercise, and the amount of TNF-α produced by each cell was reduced postexercise. In addition, cells produced less cytokine upon stimulation postexercise. Because there was an increase in the concentration of plasma epinephrine, norepinephrine, and cortisol postexercise, it is possible that these hormones had a role to play in decreasing cytokine production.

In conclusion, it is likely that elevations in stress hormones during exercise cause a decrease in monocyte cytokine production postexercise. The results of this study indicate that circulating monocytes are not likely to be the source of elevations in plasma TNF-α and IL-6 after prolonged, strenuous running.

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REFERENCES


