Impact of a Competitive Marathon Race on Systemic Cytokine and Neutrophil Responses

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ABSTRACT

SUZUKI, K., S. NAKAJI, M. YAMADA, Q. LIU, S. KURAKAKE, N. OKAMURA, T. KUMAE, T. UMEDA, and K. SUGAWARA. Impact of a Competitive Marathon Race on Systemic Cytokine and Neutrophil Responses. *Med. Sci. Sports Exerc.*, Vol. 35, No. 2, pp. 348–355, 2003. **Purpose:** To investigate whether cytokines and neutrophils mediate exercise-related pathogenesis, we examined their responses and possible association after exhaustive exercise. **Methods:** Plasma and urine samples were obtained from 10 male runners before and after a 42.195-km marathon race. Major cytokines and neutrophil activation markers [myeloperoxidase (MPO) and lactoferrin (LTF)] were measured by enzyme-linked immunosorbent assays. Functional modulation of standard neutrophils and monocytes by plasma was determined *in vitro* on their luminol-dependent chemiluminescence responses. **Results:** The race induced peripheral neutrophilia accompanied by an increase in band neutrophils and monocytosis. Plasma MPO and LTF concentrations increased significantly by 1.8 and 1.4 times after the race. There was a greater increase in urine concentrations of MPO and LTF, 12.3 and 3.5 times after exercise, respectively, suggesting that neutrophil activation occurred and that renal clearance exceeded the increase in plasma concentrations. Plasma interleukin (IL)-6, IL-8, IL-10, granulocyte colony-stimulating factor (G-CSF), macrophage CSF (M-CSF), and monocyte chemotactic protein 1 (MCP-1) increased significantly after the race, and urine IL-1β, IL-6, G-CSF, M-CSF, and MCP-1 increased significantly. The plasma IL-6 responses correlated with the increases of band neutrophil count ($r = 0.860$, $P < 0.01$), suggesting IL-6-mediated bone marrow release of neutrophils. Furthermore, the increases in urine MPO concentration were correlated with increases in urine IL-6 ($r = 0.868$, $P < 0.01$) and G-CSF ($r = 0.875$, $P < 0.01$), suggesting that these cytokines promoted neutrophil activation *in vivo*. However, *in vitro* preincubation of neutrophils and monocytes with postexercise plasma could not cause priming responses, possibly because of the exercise-induced enhancement of plasma antioxidant activity. **Conclusion:** Although many cytokines recruiting and priming neutrophils and monocytes were secreted and functional after exhaustive exercise, overwhelming antioxidant and antiinflammatory defenses were induced, preventing exercise-induced oxidative stress. **Key Words:** INFLAMMATION, LEUKOCYTES, CHEMOKINES, ADHESION MOLECULES, FREE RADICALS

Cytokines are potent intercellular signaling molecules that regulate inflammation and immune responses. Cytokines act locally at extremely low concentrations in a paracrine or autocrine manner. However, systemic spillover of proinflammatory cytokines from a damaged site can be elicited by a variety of serious insults (i.e., severe trauma, burns, hemorrhagic shock, sepsis, and ischemia-reperfusion injuries), which are integrated as a systemic inflammatory response syndrome (SIRS) (1,11,12,31,32). In SIRS, cytokines can induce peripheral neutrophilia that is associated with an increase in the percentage of immature band (nonsegmented) neutrophils (a shift to the left). This provides definitive evidence for the release of neutrophils from the bone marrow (32) and is one diagnostic criterion for SIRS (1). It has been suggested that these newly released neutrophils are less deformable and thus become trapped in the microvasculature, mediating endothelial injury (32). Although tumor necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β), and IL-6 are known to be main inducers of SIRS, these cytokines can also cause a priming of circulating neutrophils and monocytes. Priming of these cells is incited by a substance such that an augmented response of these cells produce reactive oxygen species (ROS) without direct stimulatory actions upon the cells themselves (11,22). In response to cell stimulation, these primed cells can become activated to release ROS and lysosomal enzymes, which in turn destroy cells and tissues (1,2,11,22). Chemokines and colony-stimulating factors are also considered important in the pathogenesis of SIRS because of their priming and stimulatory actions (11,32). For example, IL-8, also called neutrophil activating peptide 1 (NAP-1), is a potent neutrophil chemotactic protein, whereas monocyte chemotactic protein 1 (MCP-1) promotes monocyte activation and extravasation into inflammatory tissues (31). Granulocyte colony-stimulating factor (G-CSF), granulocyte

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macrophage CSF (GM-CSF), and macrophage CSF (M-CSF) induce proliferation, maturation, and functional priming of neutrophils and monocytes (11,31). In contrast to these proinflammatory cytokines, antiinflammatory cytokines prevent inflammatory tissue damage (12,31,33). In particular, IL-10 is a strong antiinflammatory and immunosuppressive cytokine that inhibits both proinflammatory cytokine production and ROS production by activated neutrophils (12). In addition to antiinflammatory cytokines, free radical scavengers work to counteract oxidative tissue damage by ROS (5,9,25).

Exhaustive exercise induces subclinical SIRS, characterized by hypercytokinemia (see reviews in refs. 19,22,23,31). Proinflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-8, and G-CSF, and antiinflammatory cytokines such as IL-6, IL-10, and IL-1 receptor antagonist (IL-1ra) are secreted systemically after exhaustive exercise or eccentric exercise (3,4,8,19–24,29–31,35). Exercise also induces a delayed-onset neutrophilia, as cells are released from the bone marrow reserve (28,29,35), and enhances the capacity of neutrophils and monocytes to produce ROS (15,18, 22,27–29). Furthermore, exercise induces neutrophil and cytokine accumulation in damaged muscle (6,8,19,31), which then releases myocellular proteins such as creatine kinase (CK) and myoglobin into the circulation in a delayed-onset manner (3,29,30). The involvement of neutrophils in muscle damage has been demonstrated in animal experimentation in which administration of antineutrophil antibody to deplete blood neutrophils prevented postexercise muscle proteolysis due to neutrophil infiltration (16). Studies on humans have also shown that exercise-induced neutrophilia, neutrophil priming, and/or IL-6 responses were positively correlated with, but appeared earlier than, myocellular protein efflux (3,29,35). Taken together, these findings suggest that circulating neutrophils mobilized and primed by proinflammatory cytokines, and possibly endotoxins (4,10), might be involved in the exercise-induced pathogenesis. Although inflammatory damage occurs in muscle tissues, exercise rarely results in SIRS-related multiple organ dysfunction syndrome (1), such as rhabdomyolysis accompanied by acute renal failure and circulatory shock. It can be hypothesized that adaptive mechanisms that protect the body from disease progression, such as antiinflammatory and antioxidative defenses, might be induced by exercise or training.

Marathon running enhances plasma IL-6 levels as much as 100 times and other cytokines to a lesser extent (4,20,23,30,31). Among the possible biological effects of IL-6 (2,33) are induction of acute phase protein synthesis in the liver (10), altered energy substrate turnover (23), stimulation of xanthine oxidase on vascular endothelia (8), and recruitment and priming of neutrophils (28,31,35). Exhaustive exercise also induces systemic secretion of IL-8 (20–21,23,29–31) and G-CSF (30,31,35). There is, at present, inconclusive evidence for the precise regulation of these leukocyte responses by cytokines and possible interactions among leukocytes and vascular endothelia. The present investigation used marathon running, which induces such a massive proinflammatory state, to study the various interactions in the inflammatory process. Our aim was to assess cytokine concentrations in plasma and urine samples to examine possible associations among cytokines and neutrophil mobilization and functional status *in vivo*.

Plasma concentrations of myeloperoxidase (MPO) and lactoferrin (LTF) were measured as markers of neutrophil activation, and intercellular cell adhesion molecules were assessed as indicators of neutrophil-interaction with the endothelial lining (8,14,25,34). Phagocyte priming can be induced *in vitro* by preincubating cells with proinflammatory mediators such as cytokines and endotoxins (2,11,13,22), and our method can differentiate stimulation, priming, down-modulation, or antioxidant activities of the tested samples (7,14). To determine whether *in vivo* phagocyte priming reported previously (15,18,22,27–29) can be reproduced *in vitro*, phagocytes were preincubated with plasma samples obtained before and after exercise, and stimulated responses of them were compared. In addition, indices of muscle damage, renal impairment, and the turnover of energy substrates were assessed to determine any association with elevated cytokine and neutrophil responses. Our hypothesis is that although exhaustive exercise causes mobilization and activation of neutrophils that might be involved in tissue damage, these responses might be counteracted by antioxidative and antiinflammatory defense mechanisms, so that the balance between pro- versus anti-inflammatory agents were addressed in *in vivo* and *in vitro* investigations.

METHODS

Subjects. Ten male entrants (age 31.7 ± 5.0 (mean ± SD) yr, height 171.0 ± 5.4 cm, and weight 63.4 ± 3.4 kg) participating in the 48th Beppu-Oita Mainichi Marathon, held on February 7, 1999, volunteered for this study. They have experienced a mean of eight standard marathon races (42.195 km) and had been regularly running a distance of 70–140 $km \cdot wk^{-1}$. All were nonsmokers in good health with no history of febrile disease in the month before the race. None were taking any medication or vitamin supplements within 2 wk before the race, and alcohol and caffeine were prohibited 1 d before and on the race day. Subjects did not exercise before the prerace blood sampling. The protocol was approved in advance by the Ethics Committee of the Hirosaki University School of Medicine. Subjects were informed of the experimental procedures and possible risks, and signed a letter of informed consent before participating.

Marathon race. The race began at 12:00 p.m. The weather was clear, with the atmospheric pressure between 1025 and 1027 hPa. The ambient temperature during the race ranged from 12.2 to 14.0°C. Relative humidity was 33–39%. Wind velocity was between 0.6 and 3.6 $m \cdot s^{-1}$. In a written questionnaire, no subjects reported that the weather adversely affected their perception of their performance. During the race, fluid ingestion was allowed *ad libitum*, and drinks were provided at 10 places of 4-km intervals on the running course. The total running distance

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was 42.195 km, and the mean finishing time was 2.62 h (range, 2.55–2.68 h).

Blood and urine sampling. Peripheral blood samples were drawn by antecubital venipuncture with the subjects in the sitting position. With the subjects resting quietly, the prerace blood and urine samples (Pre) were collected on the day before the race (1:30–3:00 p.m.), compatible to the time of day when subjects would be completing the race the next day. The postrace blood and urine samples (Post) were collected within 10 min after completion of the race. Whole blood was collected in Vacutainers containing no additive or disodium EDTA and/or heparin as anticoagulants to obtain serum and plasma samples, respectively. Whole blood samples containing no additives were allowed to clot at room temperature for 30 min before processing, whereas blood samples containing EDTA or heparin were processed immediately. Whole blood and urine samples, to remove sediments, were centrifuged at 1000 *g* for 10 min, and the supernatants were stored in multiple aliquots at −80°C until assayed. All samples were thawed only once.

Hematological tests. Complete blood cell counts, hemoglobin, and hematocrit were determined on EDTA-treated venous blood using an automatic blood cell counter (Sysmex K-2000, Kobe, Japan). Cover-slipped whole blood smears were prepared using freshly drawn whole blood and May-Giemsa-stained as previously described (27,28). Neutrophil morphology, the band (nonsegmented) or segmented nucleus, cell disruption, and pseudopod extension as a reflection of cytoskeletal activation were examined (27). Large granular lymphocytes (LGL) as a marker of natural killer (NK) cells were also determined. The percentages of these morphologic features were recorded for each cell type. The absolute number of each cell type was calculated from the total leukocyte count and the percentage of each differential count.

Assays for cytokines, neutrophil activation markers, and adhesion molecules. Cytokine concentrations were measured in EDTA-plasma and urine samples with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions. Concentrations of IL-1β, IL-6, G-CSF, GM-CSF, and M-CSF were measured with Quantikine kits (R&D Systems, Inc., Minneapolis, MN); all kits except for M-GSF were high sensitivity. The IL-1β kit does not detect the inactive precursor form of IL-1β, and the IL-6 kit directly measures the total amount of IL-6 without the interference of soluble IL-6 receptor. IL-10 and TNF-α concentrations were measured with a series of Ultra Sensitive ELISA kits (BioSource Europe S.A., Fleurus, Belgium). MCP-1 was measured with ELISA kits (BioSource Europe S.A.). For detecting IL-8, an Enzyme Amplified Sensitivity Immunoassay (EASIA) kit (BioSource Europe S.A.) was used. This kit is similar to an ELISA except that microwells are coated with several monoclonal antibodies directed against distinct epitopes of the target substance; this avoids hyperspecificity and allows high-sensitive assays. As markers of the release of neutrophil granule contents in plasma and urine, MPO and LTF concentrations were measured with ELISA kits (OXIS International, Inc., Portland, OR). Plasma concentrations of intercellular adhesion molecule-1 (ICAM-1), E-selectin, L-selectin, and P-selectin were determined with Parameter ELISA kits (R&D Systems, Inc.). For all assays, absorbance was measured spectrophotometrically on a microplate reader (Model 550, Bio-Rad, Hercules, CA), and the concentration of each substance was calculated by comparison with a calculation curve established in the same measurement.

Functional modulation of phagocytes by plasma. A luminol-dependent chemiluminescence technique was used in *in vitro* experiments of functional modulation of standard neutrophils and monocytes using plasma samples. Neutrophils and mononuclear cells were obtained from a healthy 30-yr-old man (blood type O) at the afternoon sampling using a double-density gradient separation method as described before (7,27). Briefly, heparinized venous blood was diluted twofold with Hanks' balanced salt solution (HBSS) and was layered onto equal volumes of Histopaque-1077 and Histopaque-1119 (Sigma Diagnostics, St. Louis, MO). After centrifugation at 500 *g* for 30 min at 4°C, the mononuclear cell fraction located at the plasma/1077 interface and the neutrophil fraction at the 1077/1119 interface were separately harvested and washed twice with HBSS. Cells were then resuspended in HBSS at a concentration of 1×10^6 cells·mL^{-1} using an automatic blood cell counter (Micro Diff II, Beckman Coulter, Tokyo, Japan), which can differentiate neutrophils, monocytes, and lymphocytes.

Cell suspensions of 1×10^5 cells were preincubated at 37°C in the presence of 1 mM luminol (5-amino-2,3-dihydro-1,4-phatalazinedione: Sigma) with or without 10% heparin-plasma samples at final concentrations in the wells of a 96-well black flat-bottom plastic microplate (Greiner Japan, Tokyo, Japan) for 0, 15, 30, and 60 min, and then neutrophils and monocytes were stimulated with opsonized zymosan (1 mg·mL^{-1}). Throughout the preincubation period and after cell stimulation up to 60 min, the chemiluminescence responses were recorded using a 1-min interval in Lumi Box H-1000 (Microtec, Funabashi, Japan) (7,14). Blanks (HBSS) in the absence of plasma were also used for comparison. Indeed, good response curves for ROS production were confirmed, and obtained data could be compared depending on the experimental conditions (i.e., pre- versus postrace plasma). The areas under the curve of the chemiluminescence responses for 60 min were calculated by integration.

Biochemical measurement. Total proteins, albumin, glucose, free fatty acids (FFA), creatinine (Cre), blood urea nitrogen (BUN), uric acid (UA), and creatine kinase (CK) activity were measured in serum samples with biochemical assay kits prescribed for a kinetic enzyme analyzer Paramax (Baxter Diagnostics Inc., Deerfield, IL). Urine osmolarity was determined using an auto-osmometer (OSMOSTAT, Kyoto Daiichi Kagaku, Kyoto, Japan).

Statistical analyses. Data are presented as mean ± SD. Statistical validation was made by Student's paired

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t-test. Associations among measured variables were determined using Pearson's linear regression (coefficient, r). Significance was evaluated at $P < 0.05$ or $P < 0.01$.

RESULTS

Hematological data. Hemoglobin and hematocrit values had not changed significantly after the race (Table 1), indicating that hemoconcentration was not prominent; consequently, the postexercise raw data were not adjusted. Total leukocyte count increased significantly (+3.2× over resting values) after the race. This leukocytosis could be attributed to the mobilization of neutrophils (+4.6×) and monocytes (+2.9×). In particular, band neutrophil mobilization was remarkable (+11.8×), and the band/total neutrophil ratio as an index of a shift to the left increased significantly (+2.5×). The band neutrophil/total leukocyte ratio, which is one of the diagnostic criteria for SIRS (≥10%) (1), was also elevated (+3.7×) after the race. The absolute numbers of lymphocytes and basophils did not change significantly, but the numbers and percentages of LGL (NK cells) and eosinophils decreased significantly after the race.

Neutrophil activation markers and adhesion molecules. Plasma concentrations of MPO (+1.8×) and LTF (+1.4×) increased significantly after the race (Table 2). Urine concentrations of MPO (+12.3×) and LTF (+3.5×) rose more markedly. When corrected for the urine concentrations based on either urine osmolarity or Cre that were measured in parallel (Table 3), the increase in MPO was still prominent (data not shown), indicating that MPO was excreted from systemic circulation into urine after exercise. Plasma P-selectin concentration rose significantly (+1.2×) after the race, whereas L-selectin was not changed significantly and E-selectin and ICAM-1 could not be measured below the detectable assay limits.

Cytokines. Plasma IL-6 (+80×), IL-8 (+10×), IL-10 (+4.1×), G-CSF (+2.9×), M-CSF (+1.6×), and MCP-1 (+2.8×) concentrations increased significantly after the race. Urinary IL-6 (+8.2×), IL-1β (+4.2×), G-CSF (+4.6×), M-CSF (+4.0×), and MCP-1 (+6.0×) concentrations were significantly elevated after exercise. There was a trend for urinary IL-10 to increase after exercise. Plasma TNF-α and IL-1β, and urinary GM-CSF concentrations did not change significantly after the race. Urinary TNF-α, IL-8,

TABLE 1. Changes of hematological parameters and leukocyte morphological features following a 41.195-km marathon race.

	Pre	Post	Significance
Hemoglobin (g·dL^{-1})	13.7 ± 0.9	13.7 ± 0.7	NS
Hematocrit (%)	42.0 ± 2.2	41.7 ± 1.9	NS
Total leukocytes (cells·μL^{-1})	5380 ± 1620	17330 ± 4460	$P < 0.01$
Total neutrophils (cells·μL^{-1})	3170 ± 1340	14570 ± 3910	$P < 0.01$
Band neutrophils (cells·μL^{-1})	310 ± 220	3650 ± 1700	$P < 0.01$
Segmented neutrophils (cells·μL^{-1})	2880 ± 1410	10760 ± 3960	$P < 0.01$
Band/total neutrophil ratio (%)	10.1 ± 6.1	25.3 ± 8.7	$P < 0.01$
Band/total leukocyte ratio (%)	5.7 ± 3.5	21.2 ± 7.1	$P < 0.01$
Neutrophil pseudopod (%)	36.3 ± 20.6	39.9 ± 15.7	NS
Neutrophil disruption (%)	3.0 ± 2.0	4.6 ± 3.5	NS
Total lymphocytes (cells·μL^{-1})	1740 ± 590	1750 ± 980	NS
Total LGL (cells·μL^{-1})	465 ± 173	299 ± 313	$P < 0.09$
LGL/total lymphocyte ratio (%)	27.7 ± 9.4	15.0 ± 8.7	$P < 0.05$
Total monocytes (cells·μL^{-1})	314 ± 162	925 ± 409	$P < 0.01$
Eosinophils (cells·μL^{-1})	124 ± 98	43 ± 100	$P < 0.01$
Basophils (cells·μL^{-1})	35 ± 41	43 ± 57	NS

Data are mean ± SD (N = 10). Pre, before the race; Post, after the race; LGL, large granular lymphocytes as a morphological feature of natural killer (NK) cells.

TABLE 2. Changes of neutrophil activation markers and cytokines in plasma and urine following a 42.195-km marathon race.

Variable	Detection Limits	Pre	Post	Significance
Neutrophil activation markers				
MPO				
Plasma (ng·mL^{-1})	0.4	19.2 ± 7.6	33.6 ± 12.3	$P < 0.01$
Urine (ng·mL^{-1})	0.4	1.85 ± 1.35	22.8 ± 16.8	$P < 0.01$
LTF				
Plasma (ng·mL^{-1})	1.0	224 ± 60	315 ± 53	$P < 0.01$
Urine (ng·mL^{-1})	1.0	71.9 ± 66.1	247.0 ± 128.6	$P < 0.01$
L-selectin				
Plasma (ng·mL^{-1})	30	1525 ± 1394	1628 ± 838	NS
P-selectin				
Plasma (ng·mL^{-1})	10	89.1 ± 22.4	106.0 ± 30.9	$P < 0.05$
Cytokines				
TNF-α				
Plasma (pg·mL^{-1})	0.05	0.31 ± 0.44	0.29 ± 0.38	NS
Urine (pg·mL^{-1})	0.05	ND	ND	
IL-1β				
Plasma (pg·mL^{-1})	0.1	0.43 ± 0.27	0.52 ± 0.23	NS
Urine (pg·mL^{-1})	0.1	1.7 ± 3.7	7.1 ± 5.1	$P < 0.05$
IL-6				
Plasma (pg·mL^{-1})	0.5	1.27 ± 1.19	101.40 ± 50.34	$P < 0.01$
Urine (pg·mL^{-1})	0.5	2.86 ± 6.91	23.60 ± 19.94	$P < 0.05$
IL-8				
Plasma (pg·mL^{-1})	0.5	1.16 ± 0.70	11.06 ± 6.95	$P < 0.01$
Urine (pg·mL^{-1})	0.5	ND	ND	
IL-10				
Plasma (pg·mL^{-1})	0.21	8.0 ± 2.1	32.8 ± 14.5	$P < 0.01$
Urine (pg·mL^{-1})	0.21	19.3 ± 6.3	22.8 ± 3.8	$P < 0.083$
G-CSF				
Plasma (pg·mL^{-1})	0.2	18.0 ± 7.8	52.9 ± 17.0	$P < 0.01$
Urine (pg·mL^{-1})	0.2	0.40 ± 0.49	1.83 ± 1.44	$P < 0.05$
M-CSF				
Plasma (pg·mL^{-1})	40	1340 ± 334	2100 ± 372	$P < 0.01$
Urine (pg·mL^{-1})	40	3270 ± 1850	12940 ± 3830	$P < 0.01$
GM-CSF				
Plasma (pg·mL^{-1})	0.1	ND	ND	
Urine (pg·mL^{-1})	0.1	6.05 ± 3.76	5.58 ± 6.81	NS
MCP-1				
Plasma (pg·mL^{-1})	20	32.7 ± 5.4	90.1 ± 15.9	$P < 0.01$
Urine (pg·mL^{-1})	20	72.3 ± 28.1	436.4 ± 243.2	$P < 0.01$

Data are mean ± SD (N = 10). Pre, before the race; Post, after the race; NS, not significant; ND, not detected below the detection limits of the assays; MPO, myeloperoxidase; LTF, lactoferrin; TNF, tumor necrosis factor; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemotactic protein 1.

TABLE 3. Changes of biochemical parameters following a 42.195-km marathon race.

Variable	Pre	Post	Significance
Total protein (g·dL^{-1})	7.08 ± 0.42	7.52 ± 0.50	$P < 0.01$
Albumin (g·dL^{-1})	4.66 ± 0.22	4.99 ± 0.30	$P < 0.01$
Glucose (mg·dL^{-1})	100 ± 17	104 ± 13	$P < 0.01$
FFA (μEg·L^{-1})	143 ± 88	910 ± 506	$P < 0.01$
Cre (mg·dL^{-1})	0.79 ± 0.09	1.17 ± 0.19	$P < 0.01$
BUN (mg·dL^{-1})	16.6 ± 3.7	24.1 ± 5.5	$P < 0.01$
UA (mg·dL^{-1})	4.52 ± 1.17	6.08 ± 1.22	$P < 0.01$
CK (IU·L^{-1})	215 ± 103	410 ± 164	$P < 0.01$
Cre (g·L^{-1})	1.17 ± 0.45	2.40 ± 1.03	$P < 0.01$
Osmolarity (mOsm·kg^{-1}H_2O)	782 ± 162	676 ± 190	NS

Pre, before the race; Post, after the race; NS, not significant; FFA, free fatty acid; Cre, creatinine; BUN, blood urea nitrogen; UA, uric acid; CK, creatine kinase.
Data are mean ± SD (N = 10). Variables analyzed in serum are total protein, albumin, glucose, free fatty acid, creatinine, blood urea nitrogen, uric acid, and creatine kinase. Variable analyzed in urine are creatinine and osmolarity.

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and plasma GM-CSF concentrations were below detection limits of the assays.

Functional modulation of phagocytes by plasma. Using *in vitro* experiments, we examined whether the increases in circulating cytokines induce functional modulation of neutrophils and monocytes. When added to isolated standard neutrophils and monocytes during the preincubation period, postrace plasma had no stimulatory effect on luminol-dependent chemiluminescence, and postrace plasma did not enhance stimulation-induced responses of these cells. In the presence of plasma, only suppressed responses were observed independently on the preincubation time, indicating that plasma alone exerted a potent antioxidant activity. Figure 1 shows the chemiluminescence responses of neutrophils and monocytes in the absence (blank) and presence of plasma samples at a final concentration of 10% without preincubation (0 min). The prerace plasma, compared with the blank, inhibited the chemiluminescence responses of neutrophils (−41.2%) and monocytes (−73.4%) and indicated that plasma has a scavenger activity. In contrast, the mean inhibition rates by postrace plasma compared with the prerace values more markedly increased in neutrophils (−34.3%) than in monocytes (−24.6%; Fig. 1).

Biochemical data. Serum levels of total proteins (+6%), albumin (+7%), FFA (+7.6×), Cre (+48%), BUN (+45%), UA (+35%), and CK (+91%) rose significantly after the race (Table 3). There was a trend for an increase in serum glucose concentration after the race. Urine Cre concentration increased significantly (+2.1×), whereas urine osmolarity tended to decrease after the race.

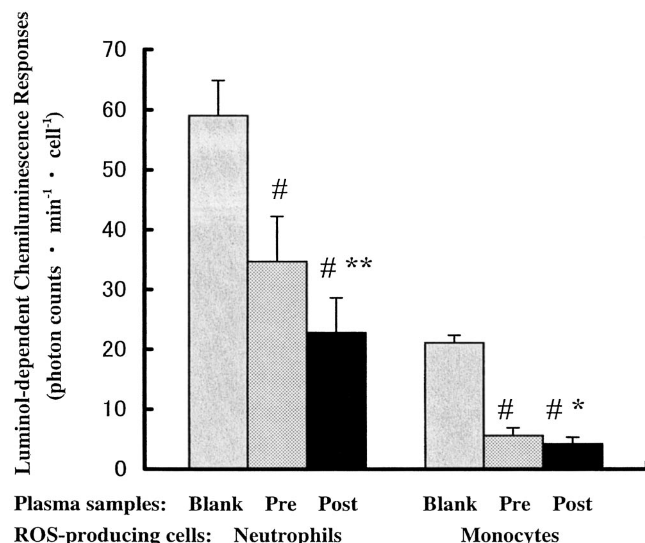

FIGURE 1—Effects of plasma, obtained before and after a 42.195-km marathon race, on luminol-dependent chemiluminescence responses of isolated standard neutrophils and monocytes stimulated with opsonized zymosan. Heparinized plasma samples accounted for 10% concentration in the reaction mixture. The blank, i.e., absence of plasma, was set using medium (Hanks' balanced salt solution). Values are mean ± SD; # $P < 0.01$ from corresponding blank; * $P < 0.05$ and ** $P < 0.01$ from corresponding prerace values.

Associations between measured parameters. There was a positive correlation between the percentage increases in plasma IL-6 concentration and band neutrophils (r = 0.860, $P < 0.01$) and band/total neutrophil ratio (r = 0.780, $P < 0.01$). In addition, the percentage increases in plasma M-CSF concentration were positively correlated with increases in neutrophil count (r = 0.713, $P < 0.05$). The percentage increases of urine MPO concentration were positively correlated with increases in urine concentrations of IL-6 (r = 0.868, $P < 0.01$) and G-CSF (r = 0.875, $P < 0.01$). There were no significant correlations between the other measured parameters.

DISCUSSION

Endurance exercise increases the circulating number of leukocytes, of which neutrophils exhibit the greatest changes in cell count and function (4,15,18,21,27–30,35). As observed previously (28,29,35), the current study confirmed neutrophilia after marathon running, with a shift to the left, suggesting that neutrophils were mobilized from the bone marrow reserve as well as from the marginated peripheral blood neutrophil pool (27). Indeed, the greatest change in existing hematological parameter was the postrace increase in band neutrophils, by more than 10 times. Previous studies have noted that delayed-onset neutrophil mobilization from the bone marrow is mediated by cortisol (22,28,29). However, recent basic studies show that corticosteroid-induced neutrophilia occurs primarily by demargination of cells from the blood vessel walls, with a minor contribution from the bone marrow (17). On the other hand, exercise-induced neutrophilia occurs in the absence of a cortisol response (29), suggesting the existence of other mobilizing factors. We have recently suggested that IL-6, IL-8, and G-CSF might be associated with the delayed-onset neutrophil mobilization from the bone marrow reserve (29,35). In the present study, IL-6 and M-CSF responses were positively correlated with neutrophil mobilization after exercise. Consistent with this view is a finding that a bronchoalveolar lavage (i.e., saline solution instillation into the normal lung) does not induce TNF-α, IL-1β, and GM-CSF secretion but does induce increases in IL-6 and G-CSF plasma levels, which correlate with neutrophil mobilization from the bone marrow (32). Other basic investigations have provided evidence that IL-6, IL-8, and colony-stimulating factors exhibit potent bioactivity to mobilize neutrophils from the bone marrow reserve (22,26). It can be postulated that cytokines play an important role in mediating exercise-induced neutrophil mobilization from the bone marrow, although cortisol is also involved to a smaller degree (29).

When activated, neutrophils release intracellular granule contents extracellularly, and plasma concentrations of these substances have been used as markers of neutrophil activation (11). Elastase and MPO are located in the primary (azurophilic) granules, whereas LTF is found in the secondary (specific) granules (2,22). Changes in plasma levels of these substances are used as markers for neutrophil activa-

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tion after exercise (4,21,22,29). MPO and LTF might be prone to excretion into urine, because they do not form complexes as in the case with elastase that combined with α_1-proteinase inhibitor. Thus, we investigated both plasma and urinary concentrations of MPO and LTF. The current study found that these substances were cleared from the systemic circulation into urine, suggesting that such deducted plasma values may not be sensitive markers for neutrophil activation. On the other hand, we detected exercise-induced neutrophil activation as observed by increased urine excretion of MPO, which has not been reported previously. Interestingly, the increased excretion of MPO was correlated with responses of IL-6 and G-CSF; these cytokines by themselves can facilitate neutrophil activity, including degranulation (2,11). Because of the rapid renal clearance of MPO, we suggest that urinary MPO concentration might be a better measure of systemic neutrophil activation than that measured in the plasma. In addition, in the present study, there were significant increases in other priming cytokines for phagocytes, such as IL-8, M-CSF, and MCP-1, after the race. To our knowledge, the effect of exercise on MCP-1 was demonstrated for the first time in the present study. Taken together, these findings suggest that these cytokines, in combination with mobilization from the bone marrow reserve, promote neutrophil activation, and that degranulated substances are excreted promptly into the urine. We have also reported previously that maximal exercise rapidly increases plasma levels of IL-6, IL-8, MCP-1, G-CSF, and M-CSF, although to a lesser extent (31,35). These findings suggest that early and continuous cytokine release may depend on both the exercise stimulus and exercise duration, respectively.

In contrast, independent research groups have repeatedly attempted to detect changes in plasma levels of TNF-α, IL-1β, and interferon gamma (IFN-γ) after various types of exercise using immunoassay kits. In many studies, these cytokines could not be detected, or only very small changes were measurable, in plasma after prolonged exercise (10,20,21,23,29,30, see a review in ref. 31). Sprenger et al. (24) showed two different patterns of urine excretion—rapid increases in IFN-γ and TNF-α, and slower and more persistent rises in IL-1β and IL-6—after 20-km running within 2 h. We could only duplicate the latter observation, possibly because the marathon race was longer than the 2-h run in the study by Sprenger et al. (24). However, we showed that the urine osmolarity exhibited a trend toward a decrease after the race than before. In the study by Sprenger et al. (24), cytokine concentrations were adjusted for changes in urinary osmolarity, but raw data were not shown. Presented urine adjusted data might be overcorrected by the postrace lower osmolarity and exaggerated, whereas delayed-onset excretion of IL-1β and IL-6 were in accordance with our results of the marathon race. In the present study, plasma levels of IL-1β and TNF-α were stable. In contrast to these proinflammatory cytokines, we demonstrated that plasma IL-6 concentration increased by 80 times, and IL-8, IL-10, G-CSF, M-CSF, and MCP-1 were secreted systemically. IL-6 and IL-10 inhibit the release of IL-1β and TNF-α (12,23,33), whereas IL-6, IL-8, G-CSF, M-CSF, and MCP-1 are capable of recruiting and priming neutrophils and monocytes (2,11,22,31). Because of the large changes and biological characteristics of these latter cytokines, it is possible that they play a role in immunomodulation of endurance exercise.

Cytokines are produced by a wide variety of cell types. However, evidence concerning the source of systemic cytokine release after exercise is at present inconclusive, with the possible exception of IL-6 production by exercised muscle (23,31). One of the major secretory pathways of IL-8 is exocytosis from not only platelets but also Weibel-Palade bodies in microvascular endothelial cells, which also contain P-selectin and platelet-activating factor involved in the initial step of neutrophil adhesion to endothelial cells (34). In the present study, we found a significant increase in plasma P-selectin concentration. Because neutrophils accumulate in the exercised muscle (6,8,29), it is possible that the microvascular endothelia or exercised muscular cells are the source of at least some IL-8 released during exercise. Endothelial cells can also produce other cytokines including IL-6, G-CSF, M-CSF, and MCP-1 (31,34), the concentrations of which also increased during the race. Furthermore, recent reports show that ROS and MPO are strong inducers of leukocyte adhesion and cytokine secretion in the endothelium (13,25). Therefore, the role of vascular endothelia in exercise-induced cytokine secretion and neutrophil emigration warrants future investigation.

We have previously reported that endurance exercise enhances the capacity of neutrophils isolated from peripheral blood to produce ROS, as assessed *ex vivo* by luminol-dependent chemiluminescence responses (27–29); this response also reflects disease severity of SIRS (11). In the present study, we used an *in vitro* system to examine whether high concentrations of proinflammatory cytokines in plasma alter neutrophil and monocyte activity; this was intended to mimic an *in vivo* environment after exercise. Surprisingly, *in vitro* incubation of standard cells with postrace plasma did not induce priming or activation of neutrophils and monocytes. Suppressed responses were only observed independent of the preincubation time; this might indicate plasma antioxidant activity. Furthermore, antiinflammatory mediators such as IL-10 and cortisol, secreted in response to exercise, might be involved in the suppression of ROS production by neutrophils (12,20,30). Considering the capacity of phagocytes to produce ROS in response to humoral modulators, it appears that oxidative damage is not easily induced, at least in the systemic circulation.

NK cells mediate cytotoxic reactions against a variety of neoplastic and virally infected cells. The present study showed that LGL (NK cell) counts in peripheral blood decreased after the race. It has been demonstrated that circulating NK cells increase in response to short-term exercise depending on the intensity of exercise (27), whereas exhaustive endurance exercise decrease NK cell count and cytotoxicity 40–60% for at least 6 h (19). Such impaired immunity and an increased susceptibility to infections after exhaustive exercise are referred to as “open window” (19).

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Our results may explain part of the immunosuppression due to stress and overloading.

Although exercise-induced generation of ROS has attracted much attention, it has also been shown that the antioxidant defense system is induced by acute and chronic endurance exercise (5,9,13,25). In the present study, the luminol-dependent chemiluminescence responses of standard neutrophils and monocytes were markedly inhibited by the presence of only 10% plasma. Monocytes, which mainly produce superoxide (O_2^-) and hydrogen peroxide (H_2O_2) among many ROS, were inhibited by prerace plasma more than neutrophils, suggesting that the plasma of our well-trained subjects contain scavengers for O_2^- and H_2O_2. Miyazaki et al. (15) reported enhancement of resting enzymatic activities of superoxide dismutase (SOD, a scavenger for O_2^-) and glutathione peroxidase (a scavenger for H_2O_2 and lipid peroxide) by endurance training. Although acute exhaustive exercise causes an increase in the capacity of neutrophils to produce ROS (15,18,22,27–29), the magnitude of the increase is also attenuated with training (15,22,28,29). As suggested by the present study, this might reflect an increased turnover of the cells or induction of antioxidant enzymes. On the other hand, inhibition by postrace plasma was greater in neutrophils, which produce more toxic ROS such as hypochlorous acid (HOCl), hydroxyl radicals (·OH), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$) derived from O_2^- and nitric oxide (NO) (14), the latter of which increases after marathon running (21,30). Based on the findings that luminol is more sensitive to these toxic ROS (7,14), it is suggested that scavengers for toxic ROS might be induced in response to exhaustive exercise, whereas endurance training might enhance resting SOD and glutathione peroxidase activities. In the present study, we observed an increase in albumin (a scavenger for HOCl and ·OH) and UA (a scavenger for HOCl, ·OH and $^1O_2$); these might result from both increased UA production through induction of xanthine oxidase (8) and inhibition of renal clearance as observed in the present study. Child et al. (5) also demonstrated that a half-marathon run enhanced serum total antioxidant capacity, as determined by chemically induced oxidation reactions of luminol, which was attributed to the increase in serum UA concentration after exercise. Others have reported no plasma lipid peroxidation after a full marathon race; this is thought to be due partly to the action of sulfhydryl groups including glutathione (9). In our subsequent study, we demonstrated enhanced enzymatic activities of plasma SOD and catalase (a scavenger for H_2O_2) and plasma concentration of vitamin C (ascorbate: a scavenger for O_2^-, ·OH, $^1O_2$, and other oxidants) after the marathon race despite no changes in lipid peroxide levels (K. Suzuki et al., unpublished observation). These findings suggest that induced free radical scavengers act during exercise to prevent oxidative stress, which seems to be in agreement with our present results of *in vitro* experiment using plasma. Furthermore, it is demonstrated that free radical scavengers prevent not only oxidation of molecules in the body but also adhesion of neutrophils to the endothelial lining, inhibiting neutrophil infiltration (8,25). Therefore, our results indicate that trained athletes seem to be well protected from inflammatory tissue damage due to ROS produced after exercise. We must admit that the methodology to duplicate *ex vivo* neutrophil was biased by mobilized antioxidants in the present study and the *in vitro* experiment might fail from this viewpoint. However, this study provides an important discovery from a viewpoint of antioxidant defense. For further precise differentiation, we are going to test the *in vitro* study by removing plasma after preincubation or modulation of neutrophil MPO-releasing activity by pre- and postrace plasma.

There is some debate whether the systemic responses of proinflammatory cytokines and neutrophils are associated with the exercise-induced pathogenesis represented by muscle damage (3,6,8,27–30). In the present study, serum CK activity rose about twofold after the race, but clear association was not detected by correlational analysis with measured variables. This lack of correlation may arise partly because blood was sampled only at one time after the race. Moreover, the marathon is a complex exercise mode, including both concentric and eccentric muscle actions. Our previous study using a simpler exercise protocol (cycling) and sampling at multiple times showed positive correlations between myocellular protein release and neutrophil/cytokine responses (29). On the other hand, in the present study, exercise-induced impairment of renal clearance was observed, as indicated by a rise in Cre and BUN. This may be due to the decreased renal blood flow during exercise, because prolonged exercise leads to a redistribution of blood flow to supply oxygen and substrates to the exercising muscle. We, however, did not examine renal tubular damage after exercise. Future studies might assess this variable to help explain the observed cytokines excretion, neutrophil-derived toxic substances, and their mutual interactions in urine after the race. Similarly, in SIRS, decreased blood flow to the gastrointestinal tracts causes gut ischemia-related leakage of endotoxins into the blood stream (the proposed "gut hypothesis") (1), which may be associated with cytokine induction during exercise (4,8,10).

It has been suggested recently that IL-6 might work in a hormone-like fashion mediating glucose and fat mobilization during exercise (23). In the present study, there was no correlation between IL-6 and changes in glucose and FFA concentrations, although such a simple statistical analysis may not necessarily exclude the possibility of a biological relationship. Further precise and specific interventional studies are needed to elucidate the associations between cytokine induction and exercise-induced pathogenesis.

In conclusion, we demonstrated that exhaustive exercise induced systemic release of IL-6, IL-8, G-CSF, M-CSF, and MCP-1, which might mediate recruitment and activation of neutrophils and monocytes. On the other hand, antiinflammatory cytokines, IL-6 and IL-10, and plasma free radical scavenging activity were induced to a much greater extent, as to suppress *in vitro* neutrophil and monocyte responses. Although free radical generation and cytokine production are overestimated by their potent bioactivities, these results suggest that the oxidative stress and exercise-induced patho-

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genesis might be prevented via antiinflammatory and antioxidant defense mechanisms induced during exercise.

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