Mild endotoxaemia and the inflammatory response induced by a marathon race

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1. To address the question of whether endotoxaemia could be involved in the inflammatory response induced by long-term strenuous exercise, 18 male marathon runners [mean age 41±2 (SEM) years] were studied. Their performance in the marathon ranged from 2 h 46 min to 4 h 42 min.

2. Four venous blood samples were drawn: at rest, just before the race (baseline); within 15 min following the completion of the marathon; after 1 h of recovery; and the morning after the race.

3. The following humoral markers of the inflammatory response to exercise were measured: polymorphonuclear myeloperoxidase (MPO), anaphylatoxin C5a (CSa), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Plasma endotoxin was measured by a sensitive and rapid chromogenic Limulus assay. All inflammatory markers were significantly increased (P<0.001) after the race, reaching in most cases peak values in the first blood sample drawn following the completion of the marathon [MPO, 298±19 ng/ml (SEM); CSa, 1.45±0.32 ng/ml; TNF-α, 20±3 pg/ml; IL-6, 88±13 pg/ml] when compared with baseline [MPO, 146±16 ng/ml (SEM); CSa, 0.27±0.2 ng/ml; TNF-α, 12±1.5 pg/ml; IL-6, 1.0±0.5 pg/ml]. Traces of plasma endotoxin (ranging from 5 to 13 pg/ml, with one exceptionally high value of 72 pg/ml measured in one runner) were detected in seven subjects within the first hour of recovery. An ELISA method was used to determine the endogenous IgG antibodies toward a range of Gram-negative bacterial lipopolysaccharides (LPSs) of different sizes and structures. A transient decrease in certain anti-LPS activities, mainly against rough LPS, occurred in most cases in the first blood sample drawn after the race. There was no correlation between the magnitude of the inflammatory response to exercise, as assessed by the increase in blood levels of humoral markers of inflammation, and the changes in circulating endotoxin levels of anti-LPS IgG activity following the race.

4. From these results, we conclude that the mild, transient endotoxaemia detected in some of our subjects does not play a major role in the observed inflammatory response to a marathon competition.

INTRODUCTION

It is at present firmly established that strenuous muscular exercise induces a sequence of cellular and humoral changes similar to those characterizing the inflammatory response to trauma or infection [1]. These changes include leukocytosis [2], the release of inflammatory mediators [1, 3, 4] and acute phase reactants [5], increase in various indices of tissue damage [6–10], the activation of the proteolytic cascades of complement [11, 12], coagulation and fibrinolysis [13], the production of free radicals [14, 15], and the priming of various leucocyte lines [16, 17]. Among these changes, the production of inflammatory mediators, especially cytokines, and leucocyte activation have been the most extensively studied over the last decade. From previous studies conducted in this field, there is considerable evidence that polymorphonuclear neutrophils (PMN) are activated during exercise [18]. Despite the volume of experimental work on the exercise-induced inflammatory response, its underlying mechanisms still remain poorly understood and open to conjecture.

Based on the increase in plasma endotoxin [lipopolysaccharide (LPS)] levels accompanied by a decrease in blood concentration of specific anti-LPS antibodies (IgG) observed in athletes who participated in endurance events [19, 20], it has been suggested that the biochemical indices of the inflam-
matory response to exercise (IRE) could be in part due to the release of endotoxin in blood [4]. To our knowledge this hypothesis has never been tested experimentally. In the present study we have therefore measured plasma levels of various humoral markers of IRE such as cytokines and C5a anaphylatoxin (C5a) in runners before and after a marathon race. PMN activation was assessed by measuring the plasma concentration of myeloperoxidase (MPO), while the occurrence of exercise-induced endotoxaemia was studied by measuring plasma endotoxin and anti-LPS IgG antibodies toward a range of complete and incomplete [core glycolipid (CGL component)] LPSs from various Gram-negative bacteria.

METHODS

Subjects

Eighteen male marathon runners aged 41 (range: 24–64 years) ±2 (SEM) years volunteered to participate in this study. Ethical permission for the study was obtained from the Committee for Medical Ethics (Faculté de Médecine, Université Libre de Bruxelles, Brussels, Belgium). Written informed consent was obtained from all volunteers.

Blood sampling

Blood samples of 20 ml (i.e. two 10-ml Vacutainer) were drawn by venipuncture: at rest just before the start of the marathon (baseline); at \( t = 0 \) h, as soon as possible after the end of the race (sampling time between 5 and 15 min after completion of the race); at \( t = 1 \) and 24 h, after 1 and 24 h recovery respectively.

Blood used for the determination of MPO, tumour necrosis factor-\( \alpha \) (TNF-\( \alpha \)), interleukin-6 (IL-6), and C5a, was collected in Vacutainers containing EDTA as anticoagulant and centrifuged at 2500 \( g \) for 10 min. Plasma was removed and frozen immediately at \(-70^\circ \)C until assay.

Blood for the measurement of endotoxin was collected in pyrogen-free tubes (Endo tubes ET, Chromogenix AB, Mölndal, Sweden) containing heparin at a final concentration of 30 units/ml. Platelet-rich plasma was prepared by centrifugation at 2200 \( g \) for 15 min at \( 4^\circ \)C, and samples were stored at \(-20^\circ \)C.

Blood used for the determination of specific anti-LPS and anti-lipid A antibodies was allowed to clot at room temperature and then centrifuged at 2 200 \( g \) for 15 min. Serum was immediately removed and refrigerated until assay.

All measurements were performed in duplicate, and all samples of each individual were analysed in the same run. Plasma levels of IL-6 and TNF-\( \alpha \) were obtained by using immunoenzymic assay kits (Medgenic Diagnostics, Fleurus, Belgium). An enzyme immunoassay test kit was employed to detect the C5 split product C5a (Behring, Bruxelles, Belgium). Plasma MPO concentration was assessed according to the radioimmunological method described by Pincemail et al. [21].

Plasma concentrations of MPO, C5a, IL-6, TNF-\( \alpha \) and endotoxin were adjusted for changes in plasma volume during and after exercise.

Endotoxin assay

All samples were tested with Coatest Plasma-endotoxin (Chromogenix AB), a chromogenic Limulus amoeocyte lysate assay according to the manufacturer’s instructions.

To minimize non-specific plasma inhibitors, samples were diluted with pyrogen-free water and heat-activated at \( 75^\circ \)C for 10 min. Escherichia coli 0111:B4 reference endotoxin was used for the standard curve (Chromogenix AB). The limit of detection was 5 pg/ml.

Anti-LPS antibody assay

LPS antigens. Smooth LPS was extracted by the method of Westphal and Jann [22] from 13 bacterial strains and species (E. coli 0111:B4, 0127:B8, 055:B5, 026:B6, K235 and 0128:B12, Salmonella typhimurium, S. enteritidis, S. minnesota, Shigella flexneri 1A, Serratia marcescens, Pseudomonas aeruginosa, serotype 10 and Klebsiella pneumoniae). Rough LPS (CGL-component) was extracted by the method described by Galanos et al. [23] from nine bacterial strains and species (S. typhimurium TV119, SL1181 and SL684, E. coli F583, J5 and EH100, S. minnesota R7, R5 and R595). Two monophosphoryl lipids A, prepared by acid hydrolysis [24] of phenol-extracted LPS from E. coli F583 and S. minnesota R595, were also used. The antigens were supplied by Sigma Chemical Co (St Louis, MO USA).

General procedure. The presence of endogenous anti-LPS antibodies (IgG) to the panel of smooth LPSs, rough LPSs, and lipid A antigens was measured by a direct ELISA, as previously described with minor modifications [25]. Briefly, the plates (Nunc) were coated either with a mixture of the 13 smooth (S)-form LPSs (10 \( \mu \)g/ml of each), with a mixture of the nine rough (R)-form LPSs (10 \( \mu \)g/ml of each), or with individual purified smooth or rough LPSs at a concentration of 10 \( \mu \)g/ml and lipid A at a concentration of 5 \( \mu \)g/ml. All samples of any given marathon runner’s serum were assayed simultaneously and screened at duplicate 1:100 and 1:1000 dilutions with negative controls (uncoated wells). Goat anti-human IgG, alkaline-phosphate-conjugated, was used as an indicator antibody, and the absorbance of wells was read at 405 nm. Throughout our experiments, a serum sample was included in each set of ELISAs and on most plates as a methodological control. The results were arbitrary expressed as mean absorbance minus any back-
ground absorbance (negative control). Any fall in anti-LPS antibodies occurring after the race was expressed as a percentage of the initial value.

Criteria for the detection of exercise-induced endotoxaemia

The occurrence of exercise-induced endotoxaemia was studied by the measurement of circulating endotoxin levels and serum activity of IgG antibodies toward lipid A and LPSs from various S- and R-form bacterial strains. Based on the criteria used to detect endotoxaemia (i.e. the presence of circulating endotoxin and changes in the level of endogenous anti-LPS and anti-lipid A IgG antibodies), the subjects were divided into four different groups. It was assumed that runners in whom measurable amounts of circulating endotoxin (≥5 pg/ml) were measured had endotoxaemia [26]; those subjects were classified as group LPS+ (n = 7). Those in whom LPS was not detected were assigned to group LPS− (n = 11). Based on the changes in the ELISA assay of IgG antibody activity to the various LPSs and the two lipids A, it was assumed that subjects with a reduction in their IgG activity (net absorbance values) against five or more antigens have been challenged (at least transiently) with endotoxins; these subjects were assigned to group IgG+ (n = 7). The other volunteers were placed in group IgG− (n = 11).

Statistical analysis

Values throughout this study are expressed as means±SEM. Repeated measure analysis of variance (two-way ANOVA) was used to detect significant change over time. Where appropriate, comparisons with baseline values were performed using the paired t-test with Bonferroni’s adjustment. The unpaired t-test was used to determine whether there were differences in the variables measured in subjects assigned to groups LPS+ and IgG+ compared with groups LPS− and IgG− respectively.

A P value <0.05 was considered to represent statistical significance.

RESULTS

The mean time to finish the marathon race was 3 h 38 min (±7 min), with a range from 2 h 46 min to 4 h 42 min. The extreme performances were obtained in two subjects aged 32 and 64 years respectively. As expected, running time was found to be significantly correlated with subject age (r = 0.65; P <0.05).

The time courses of mean plasma concentrations of the humoral markers of the inflammatory response to the marathon race are illustrated in Figs. 1 and 2. Resting MPO and C5a concentrations were 146±16 and 0.27±0.02 ng/ml respectively. Immediately after the marathon, these variables were significantly elevated (P <0.001), reaching peak values of 298±19 and 1.45±0.32 ng/ml for MPO and C5a respectively (Fig. 1). Concentrations of these markers decreased during recovery. The plasma MPO level 60 min after the race was 214±19 ng/ml. This value was statistically higher than the baseline value (P <0.001). Twenty-four hours later, plasma MPO levels did not differ significantly from resting values (178±18 ng/ml). The mean concentrations of C5a seen 1 and 24 h after the marathon (0.44±0.08 and 0.24±0.03 ng/ml respectively) were not statistically different from baseline values. There was a significant correlation between plasma concentrations of MPO and C5a (r = 0.45; P <0.01).

Resting plasma levels of TNF-α and IL-6 were 11.6±1.5 and 1.1±0.5 pg/ml respectively. Immedi-
After cessation of exercise, plasma IL-6 concentration was found to be markedly increased, at $88 \pm 13 \text{ pg/ml}$ ($P < 0.001$) (Fig. 2). One hour later, this variable had decreased ($51 \pm 8.5 \text{ pg/ml}$ at time $t = 1 \text{ h}$), but still remained significantly elevated when compared with the baseline value ($P < 0.001$).

Mean plasma concentrations of TNF-$\alpha$ increased significantly toward values which reached $19 \pm 2$ and $20 \pm 3 \text{ pg/ml}$ immediately after the marathon and 1 h later respectively ($P < 0.001$) (Fig. 2).

Of the blood samples drawn in our subjects, traces of endotoxin $\geq 5 \text{ pg/ml}$, ranging from 5 to 72 pg/ml, were detected in nine of them (Fig. 3). Endotoxin was detected most frequently (6 cases out of 9) in blood samples drawn 1 h after the end of the race. An exceptionally high value of $72 \text{ pg/ml}$ was detected in one subject (age, 31 years; running time, 2 h 58 min) within the first hour of recovery. No subject had circulating endotoxin detected in the blood samples taken at rest, just before the race.

The effects of participation in a marathon on endogenous IgG antibody activity toward E. coli 0127:B8 LPS are illustrated in Fig. 4. Anti-LPS IgG activity was detected in serum samples taken from four different subjects at rest, just before the race. A mean decrease in absorbance of approximately 50% was observed immediately after completion of the marathon. Levels of anti-LPS IgG activity became almost undetectable in one subject immediately after the race, and remained so 1 h later. It should be pointed out, however, that although the main picture of change in IgG activity over time exhibited a trend toward a reduction in absorbance following the marathon in most subjects, there was a marked inter-individual variability with regard to the pattern of reactivity to the LPS antigens used in our study, as well as to the magnitude of changes in absorbance after exercise. We found that circulating levels of such antibodies, particularly those specific for the incomplete CGL-component LPS, were more consistently reduced compared with baseline levels (results not shown). In this report, we show the responses to a series of LPSs of different sizes from smooth and rough Salmonella mutants, and a variety of LPSs from smooth bacterial strains as well as complete or incomplete rough core types of E. coli. These data illustrate the overall trend of changes in IgG antibodies to various LPSs that were detected in these runners.

The anti-LPS IgG antibodies that were detected in our subjects are listed in Fig. 5. For any given specific antibody, the abscissa represents the number of marathon runners in whom one or more decreases in IgG activity over time were observed. Depending on the antibody analysed, the number of subjects in whom the marathon induced a reduction in anti-LPS IgG activity ranged between 3 (against...
S. minnesota R595 LPS) and 15 (against a panel of smooth E. coli LPSs).

The mean plasma concentration of MPO, C5a, IL-6 and TNF-α measured at the different time points in groups LPS+ and LPS−, as well as in groups IgG+ and IgG−, are listed in Table 1. Statistical analysis (unpaired t-test) revealed that there were no significant differences in the plasma levels of these variables at any time point between groups LPS+ and LPS−. When applied to groups IgG+ and IgG−, this statistical analysis led to the same conclusion, with the exception of TNF-α. The mean values of this variable measured at rest and after 1 h of recovery in group IgG+ were about twice as high as those obtained at the same time points in group IgG−. These differences were statistically significant (P<0.03).

DISCUSSION

The marked, highly significant increases in levels of the humoral markers of inflammation seen in our subjects are in agreement with an increasing body of data showing that strenuous exercise is accompanied by an inflammatory response involving, among others, the production of cytokines and the activation of complement and circulating PMN [1, 3, 11−14, 16−18]. Of interest were the similar time profiles of MPO and C5a. As shown in Fig. 1, the plasma levels of these substances were markedly elevated after the race and then decreased in parallel during recovery. This finding confirms the results of a previous study in which these variables were measured in healthy male subjects submitted to 20 min of dynamic exercise at 80% maximum aerobic power on a cycle ergometer [12]. From these observations, it is tempting to speculate that C5a, and probably other anaphylatoxins derived from the activation of complement, could play a role in exercise-induced PMN degranulation. Because of the lack of additional data concerning changes in the plasma concentrations of other anaphylatoxins, the question of whether the alternative and/or classical pathways of complement were involved in the observed increase in C5a levels cannot be answered by the present study. Further studies conducted in a greater number of subjects and involving measurement of additional markers of complement activation are needed to verify the hypothesis that the activation of this proteolytic cascade is involved in the process of PMN degranulation during exercise.

Data from the literature dealing with the production of cytokines during exercise appear to be, to some extent, contradictory. One, perhaps superficial, explanation for the discrepancies in this field is differences in the intensity and duration of exercise. Nevertheless, it seems at present well accepted that strenuous exercise is accompanied by increased production of some of these compounds [1, 3, 4, 16, 17]. Consistent with this view are the pronounced elevations of plasma TNF-α and IL-6 levels following the marathon (Fig. 2). It is generally believed that production of these cytokines primarily reflects activation of mononuclear cells by exercise [3, 16, 17]. Whereas IL-6 concentrations decreased over the first hour of recovery, there was practically no change in plasma TNF-α level at this time point. Whether the difference in the post-exercise profiles of these substances is due to sustained production of TNF-α by the persistence of specific stimuli after exercise, or to alterations in the clearance of this cytokine from blood is unknown. Nevertheless, the present results clearly show that caution should be exerted when data from studies based on different sampling protocols are compared to draw conclusions on the time course of the inflammatory response to exercise.

The presence of traces of endotoxin in blood samples drawn in seven subjects clearly shows that a marathon race can be accompanied in certain cases by a moderate, transient endotoxaemia. In agreement with this finding was the decrease in levels of specific anti-LPS IgG antibodies noted immediately

<table>
<thead>
<tr>
<th>MPO (ng/ml)</th>
<th>C5a (ng/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (h)</strong></td>
<td><strong>LPS+ (n = 7)</strong></td>
<td><strong>LPS- (n = 11)</strong></td>
<td><strong>LPS+ (n = 7)</strong></td>
</tr>
<tr>
<td><strong>Rest</strong></td>
<td>125.4±23.1</td>
<td>131.4±6.5</td>
<td>0.36±0.10</td>
</tr>
<tr>
<td>0 h</td>
<td>232.4±28.0</td>
<td>282.4±23.8</td>
<td>1.77±0.48</td>
</tr>
<tr>
<td>1 h</td>
<td>211.2±27.8</td>
<td>215.2±19.6</td>
<td>0.36±0.06</td>
</tr>
<tr>
<td>24 h</td>
<td>152.9±34.6</td>
<td>179.1±20.1</td>
<td>0.25±0.04</td>
</tr>
</tbody>
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**Table 1. Comparison of the mean plasma concentration (+SEM) of MPO, C5a, IL-6 and TNF-α measured at the different time points in groups LPS+, IgG+ versus LPS− and IgG−.**: P<0.03.
after the race in several runners. While endotoxaemia has been reported in athletes who took part in a triathlon competition [19] and an 89.4 km road race [20], its occurrence during exercise of shorter duration has, to our knowledge, not yet been documented.

The precise mechanisms underlying these phenomena, and their possible physiopathological implications during endurance events, if any, are hypothetical. By analogy with some pathological states where intestinal ischaemia and/or hypoxia are central causes of gut barrier alteration leading to LPS and bacterial translocation, it has been inferred that exercise-induced endotoxaemia probably results from reduction of splanchnic blood flow due to the effects of adrenergic activation, resulting in deviation of a proportion of cardiac output to active muscles and skin vessels and away from the abdominal viscera [27–29]. It is also thought that splanchnic hypoperfusion could be compounded by dehydration, favoured by excessive hyperthermia and inadequate fluid intake during exercise [28, 29]. If this hypothesis is correct, more information on the role of these different factors in the genesis of exercise-induced endotoxaemia is required. It should be pointed out, however, that increased permeability of the gut barrier has been found to occur in human subjects submitted to long-term strenuous exercise [30]. Based on these findings, it thus seems reasonable to assume that a transient alteration of the gut barrier, even of moderate proportion, could pave the way for the entry of endotoxins into the blood.

It is at present firmly established that the LPS molecule from the outer membrane of Gram-negative bacteria is a potent activator of cellular and humoral host-defense systems [31, 32]. Upon activation by bacterial endotoxins, blood monocytes and tissue macrophages secrete several cytokines, including IL-6 and TNF-α [33, 34]. Bacterial LPSs also directly activate the complement system [35]. Cytokines and complement cleavage products are potent stimulators of PMN [36–39]. Therefore, the present study was designed to verify the hypothesis that strenuous exercise could trigger an inflammatory response via transient endotoxaemia. For that purpose, we measured circulating endotoxin levels and four humoral inflammatory markers. In addition, we measured IgG antibodies to a range of Gram-negative bacterial LPSs of different sizes and structures [40]. Statistical analysis revealed that there were no significant differences in the mean plasma concentrations of MPO, C5a, TNF-α and IL-6 measured at any time point between the subjects assigned to groups LPS+ and LPS−. When applied to groups IgG+ compared with IgG−, this analysis led to the same conclusion, with the exception of the plasma concentration of TNF-α measured at rest and after 1 h of recovery. It is thus tempting to conclude that although the data collected from our subjects provide experimental evidence that transient gut-barrier alteration and LPS translocation had occurred, the relationship of these events to the inflammatory response to exercise is, at least in quantitative terms, far from being established. Further support of this view comes from the lack of correlation between the maximal concentration of the humoral markers of the inflammatory response to exercise and the initial levels of anti-endotoxin antibodies (results not shown). Because these molecules serve to inhibit the biological effects of endotoxin, an inverse relationship between the release of inflammatory mediators and baseline anti-LPS IgG activities might reasonably be expected if endotoxaemia was significantly involved in the exercise-induced inflammatory response. The absence of correlation between these two variables further suggests that the observed humoral changes following exercise related to other factors. However, the extent to which this assumption could still hold true for exercise of longer duration, especially in subjects who exercise too vigorously under poorly controlled conditions, in such as those characterized by hyperthermia compounded by dehydration, remain to be tested experimentally. With this in mind, the possibility that endotoxaemia of greater magnitude could play a significant role in the inflammatory response to exercise and be considered to be a potential hazard associated with strenuous physical work performed in extreme conditions, cannot be ruled out by the present results. In our opinion, this hypothesis deserves further study.

Whereas the circulating anti-LPS IgM molecules are primarily synthesized in and confined in the blood, substantial amounts of anti-LPS IgG antibodies are found not only in the blood, but also in the interstitial compartment, forming a 'reservoir' from which the blood IgG pool can be replenished when decreased by endotoxaemia. Migration of IgG from the interstitial pool to the blood and/or increased synthesis by activated lymphocytes might contribute to the rise of IgG levels following their transient depletion (Fig. 4).

Several methodological issues in the performance of our study should be kept in mind. For ethical and technical reasons, it was not possible to draw a number of tightly spaced blood samples; this of course, would have characterized the time course of the measured variables during the race and the subsequent recovery period. Our protocol would not detect translocation of LPS occurring during the marathon or later during the recovery period, leading to an under-evaluation of this phenomenon. Another factor which could contribute to this under-evaluation is the binding of endotoxin to several ligands, such as membrane and soluble CD14 receptors and LPS-binding protein. The endotoxin bound to these proteins would not be detected by the chromogenic Limulus amoebocyte lysate assay. The extent to which exercise could alter the binding of endotoxin to these ligands and the clearance of endotoxin is unknown. On the other hand, this compound may disappear so rapidly from the circulation...
that it becomes undetectable by the time its effects on the complement system and target cells are manifest.

Caution should also be exercised when data on anti-LPS IgG activities are used to monitor exercise-induced endotoxaemia. It should also be kept in mind that our results represent the general trend of changes in levels of IgG antibodies to different commercially available LPS preparations. Antibodies to other relevant LPS serotypes, or to other antigenic determinants of the outer membrane, were not measured in this study. Those may, however, have also entered the circulation and contributed to the inflammatory response to exercise [41]. As described previously [42, 43], we observed a great inter-individual variability in the anti-LPS IgG response; each runner had his own particular specificity pattern of antibodies directed toward different components of endotoxin. Furthermore, the scatter of absorbance values measured in different subjects at the different time points was quite large. As a consequence, it was not possible to combine all aspects of experimental data on LPS and anti-LPS IgG into a general scheme. In contrast with this finding, the relatively low scatter of MPO, C5a, TNF-α and IL-6 values suggests that the time profile of these variables does not markedly differ from one subject to another and argues for the hypothesis that factors other than endotoxaemia, perhaps operating with a common mechanism, probably play a major role in the exercise-induced inflammatory response. To our knowledge, these factors, and the precise sequence of events leading to the observed humoral changes characterizing this phenomenon, remain to be elucidated.

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REFERENCES