Phagocytic function in cyclists: correlation with catecholamines and cortisol

E. ORTEGA RINCÓN,1 J. M. MARCHENA,1 J. J. GARCÍA,1 A. SCHMIDT,2 T. SCHULZ,2 I. MALPICA,1 A. B. RODRÍGUEZ,1 C. BARRIGA,1 H. MICHLN,2 AND H. LÖTZERICH2

1Departamento de Fisiología, Facultad de Ciencias, Universidad de Extremadura, 06071 Badajoz, Spain; and 2Institute of Morphology and Tumor Research, Deutsch Sporthochschule Köln, 50933 Köln, Germany

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EXERCISE HAS BEEN EQUIVATED with health, and people who perform some type of sport regularly have been associated with having less susceptibility to infection compared with sedentary people, especially if the sport performed is of low intensity. However, this picture may not be true for competition athletes, who are in many cases more susceptible to infections than sedentary people (5, 20).

With respect to the effect of exercise on the function of lymphocytes, whereas the number of these cells in the blood is increased, it has been noted that their function can be impaired after intense and acute exercise but not after moderate exercise or in trained subjects (5). The reduction of the functional capacity of lymphocytes in situations of intense exercise could reflect a temporary immunosuppression that would allow microorganisms and viruses time to evade immunological recognition and to become established, giving rise to infections in athletes. This is probably why phagocytes, and the nonspecific defenses in general, including natural killer activity, play an important part in the defense against infection of athletes, probably preventing the entry and maintenance of the antigen in situations where the specific immune response seems to be depressed (12). In fact, phagocytosis is increased, in general, after both intense and moderate exercise, with or without training, and in both monocytes-macrophages and neutrophils (12, 25).

Some authors have indicated that the influence of exercise on the immune system is not due to the exercise itself but to the stress induced by the exercise, and that it also depends on the degree of stress experienced, with elite sportsmen and sportswomen being subjected to greater physical and psychological stress (8, 12). In vitro studies have shown that the phagocytic response of murine macrophages during exercise is mediated by stress hormones (6, 7, 14, 16). Hence, in evaluating the immunological status of athletes, it is important to take these mediators into account, above all those released from the hypothalamus-pituitary-adrenal axis (such as glucocorticoids) and sympathetic nervous system (such as catecholamines).

Therefore, given that it has been suggested that the nonspecific immune response may be a limiting factor when it comes to evaluating a sportsperson’s immune status, the aim of the present work was to determine the variations in phagocytic activity over the course of a training-competition season in a group of cyclists, and also to study any changes that might occur during this period in plasma catecholamine and cortisol levels, in a search for neuroendocrine markers that would allow an athlete’s immunological status to be readily identified.

METHODS

Subjects

The study was performed on a group of 14 amateur cyclists (men) of a high competitive level, with a mean age of 25 yr...
(range 23–28 yr). Their training-competition season was between January and November, with the main period of competition being between March and September. The mean distance cycled during the training-competition season was 25,000 km (range 15,000–36,000 km). The studies were performed in accordance with the ethical standards of the Committee on Human Experimentation.

**Extraction of Blood Samples**

A sufficient amount of blood was extracted from the ante-cubital vein of each participant in the study under basal conditions (no type of exercise was performed in the day before extraction) at 9:00 AM around the middle of each month in which determinations were made (January, March, May, July, September, and November). All individual sampling was done at the equal times relative to the training seasons. Only one sample of blood per month was taken from each participant. Immediately after the extraction, 2 ml of the blood were separated for the catecholamine assay. The rest of the blood was deposited in tubes with heparin.

For each of the indicated months, and for each cyclist, the percentage of monocytes and granulocytes with phagocytic capacity was determined. Also, plasma was obtained from each sample for the catecholamines (epinephrine and norepinephrine) and cortisol concentration evaluations.

**Plasma Separation**

For the cortisol assay, plasma was obtained from 1 ml of anticoagulated blood from each participant by centrifugation (300 g for 20 min).

For the catecholamine assay, before separation of the plasma, to 2 ml of blood of each sample, we added 40 μl of a stabilizing solution: 900 mg of EGTA and 700 mg of glutathione in 10 ml of NaOH (0.1 mol/l). The plasma was then isolated by centrifugation as in the above conditions. In both cases, plasma samples were conserved at −30°C until the time of measurement.

**Catecholamine (Epinephrine and Norepinephrine) Assays**

The determination of the plasma concentrations of catecholamines was performed by HPLC with electrochemical detection (Coultech II model). During the extraction of the samples (Chromsystems Instruments and Chemical, Munich, Germany), an internal standard (dihydroxybenzylamine) was added to them to allow the subsequent calculation of the exact original concentration avoiding losses during the process.

The column used was a C18 (Waters), the working potential was between 450 and 660 mV, the flow was 1 ml/min, and the pressure did not exceed 200 bar. After injection of 20 μl of the final processed sample, a chromatogram was obtained in which one peak was observed at a lag of ~4 min, corresponding to norepinephrine, and another at ~5 min, corresponding to epinephrine. The catecholamine concentrations, expressed as nanograms per millilitre, were obtained by processing the data with the program package MILLENIUM (Waters).

**Cortisol Assays**

The cortisol plasma concentrations were determined by radioimmunoasay (DRG Instruments).

**Study of the Monocyte and Neutrophil Phagocytic Function**

We used the test Phagotest (Orpegen). This test kit allows one to make quantitative determination of leukocyte phagocytosis in heparinized whole blood. It contains (FITC)-labeled opsonized bacteria (Escherichia coli-FITC). It measures the overall percentage of monocytes and granulocytes showing phagocytosis in general (ingestion of 1 or more bacteria per cell). The study of phagocytosis was performed by flow cytometry.

**Assay procedure.** The following steps were performed.

1) **Dispensing.** Heparinized whole blood was mixed (Vortex mixer), and 100-μl aliquots were put onto the bottom of 5-ml tubes. Before the bacteria were added, the blood samples were incubated in an ice bath for 10 min to cool them down to 0°C.

2) **Activation.** The precooled E. coli bacteria were mixed well (Vortex mixer), and 20 μl per test were added to the whole blood.

3) **Incubation.** All tubes were mixed once more. The control samples remained on ice. The test samples were incubated for 10 min at 37°C in a water bath. Incubation time and temperatures were monitored closely, and the water bath was closed and preheated.

4) **Quenching.** Precisely at the end of the incubation time, all samples together on one rack were simultaneously taken out of the water bath and placed on ice to stop phagocytosis. One hundred microliters of ice-cold quenching solution were added to each of the samples. The samples were mixed (vortex mixer).

5) **Washing.** Three milliliters of washing solution were added per tube. The samples were mixed, and the cells were spun down (5 min, 250 g, 4°C). The supernatant was discarded.

6) **Washing.** The samples were washed with 3 ml of washing solution once again (5 min, 250 g, 4°C), and the supernatant was discarded.

7) **Lysis and fixation.** The whole blood was lysed and fixed with 2 ml of prewarmed (room temperature) lysing solution. The samples were mixed and incubated for 20 min at room temperature. The cells were spun down (5 min, 250 g, 4°C). The supernatant was discarded.

8) **Washing.** The samples were washed once more with 3 ml of washing solution (5 min, 250 g, 4°C).

9) **DNA staining.** Two hundred microliters of DNA staining solution were added, followed by mixing and incubation for 10 min on ice (light protected in the ice bath). The cell suspension was measured within 60 min.

**Flow Cytometric Analysis**

Cells were analyzed by flow cytometry using a blue-green excitation light (488-nm argon-ion laser, Coulter EPICS XL3-Color).

**Measurement.** During data acquisition a “live” gate was set in the red fluorescence histogram on those events that have at least the same DNA content as a human diploid cell (i.e., exclusion of bacteria aggregates having the same light-scattering properties as leukocytes). Alternatively, bacteria can be excluded by using fluorescence triggering in the fluorescence channel. We collected 15,000 leukocytes per sample.

**Data evaluation.** The percentage of cells having performed phagocytosis (granulocytes and monocytes) was determined. For that purpose, the relevant leukocyte cluster was gated in the software program in the scatter diagram (linear forward vs. linear side scatter) and its green fluorescence histogram (fluorescence-1 (FL1)) analyzed. The control sample was used to set a marker in the FL1 histogram so that <1% of the events were positive. The percentage of phagocytizing cells in the test sample was then determined by counting the number of events above this marker position.
Statistical Analysis

All the graphs represent means ± SD of the determinations performed for the 14 participants of the study. Normal distribution of the data was verified (normality test). Comparison between months was made using the ANOVA Schef- fée’s F-test statistic, with P < 0.05 taken as the minimum significance level required. Correlations between phagocytic functional cells and catecholamines or cortisol were determined by multiple regression.

RESULTS

In the present work, we have investigated the variations of phagocytosis of granulocytes and monocytes during the training-competition season in a group of cyclists and their correlation with the concentration in blood of catecholamines and cortisol.

Fig. 1. Phagocytosis of granulocytes (A) and monocytes (B) during a training-competition season in high-level amateur cyclists. Values are means ± SD of 14 individuals. *P < 0.05 with respect to the values obtained in January. +P < 0.05 with respect to March. aP < 0.05 with respect to May. bP < 0.05 with respect to July.

Fig. 2. Norepinephrine (A) and epinephrine (B) plasma concentrations during a training-competition season in high-level amateur cyclists. Values are means ± SD of 14 individuals. *P < 0.05 with respect to the values obtained in January. bP < 0.05 with respect to May. cP < 0.05 with respect to September.

Results corresponding to the variations in phagocytosis of granulocytes and monocytes are shown in Fig. 1. As can be seen, a peak was found in the function of granulocytes during March, May, and July (P < 0.05 with respect to other months), with the highest values observed in May (Fig. 1A). Similarly, phagocytosis of monocytes also showed a peak in the same months, with the highest values in May (P < 0.05 with respect to January, September, and November) (Fig. 1B).

Plasma concentrations of catecholamines are represented in Fig. 2. Norepinephrine showed the lowest values in May (P < 0.05 with respect to January and March) (Fig. 2A), coinciding with the highest values found in phagocytosis. In contrast, epinephrine showed a maximum in the same month (P < 0.05 with respect to the values found in March, July, September and
November) (Fig. 2B). However, no statistical differences were found between the values of the central month of the season in plasma cortisol values, although a significant increase ($P < 0.05$ with respect to January) was observed in November (Fig. 3).

Taking together the results found for phagocytosis (in both monocytes and neutrophils) and for the concentrations of catecholamines and cortisol, we studied their possible correlations in the central months (March to September) of the training-competition season. The results only showed good correlations between epinephrine and phagocytosis, above all with monocytes ($r^2 = 0.998$) (Fig. 4).

**DISCUSSION**

Many studies have focused on the effects of exercise on immune cells. The exercise has been either acute or a randomized training program, and the results were compared with those before exercise or with sedentary people. However, the purpose of the present investigation was to evaluate the possible variations during a complete training-competition season in a group of cyclists. Several investigations have concluded that competition athletes are more susceptible to infection than sedentary people (5, 17). One of the causes of increased incidence of infection in athletes could be related to a temporary suppression of immune function after intense exercise (17, 23), and this effect could be mediated by stress hormones (8). It has been hypothesized that phagocytosis plays an important role in preventing the entry and maintenance of the antigen in situations where the specific immune response is depressed (4, 10, 12, 22). However, recent investigations support the idea that endurance training could also decrease neutrophil function (2, 18). But, what is the basal situation (normal situation) during an annual training season of cyclists? The results of the present work indicate that the phagocytic capacity of both monocytes, which are macrophage precursors, and neutrophils have a peak during May. This is when the cyclists attain their greatest level of physical preparation, and it seems that this also occurs at the level of the phagocytic response that is found to be stimulated at this time of the year when it would be able to prevent pathogen attack and therefore defend the sportsperson against infection. These results agree with those found for elite women basketball players, who present a greater phagocytic capacity in their neutrophils midway through their training period relative to sedentary-lifestyle individuals (13). Therefore, it is possible that the capacity of phagocytic response might be regarded as a good "indicator of a sportsperson's immunological status" with respect to the greater or lesser susceptibility to infection. In fact, some workers have suggested that a transitory decline in the capacity of phagocytic response that sportspeople may
Occasionally undergoing could increase their susceptibility to infection (19).

The influence of exercise on the immune system is due to not only the exercise itself but also the stress induced by the exercise, and thus the influence depends on the degree of stress experienced, with elite sportsmen and sportswomen being subjected to greater physical and psychological stress. The main modulators in stress are catecholamines and glucocorticoids, and both types of hormones are released during exercise (18). Studies in our laboratory have shown that the increased phagocytosis of murine macrophages induced by acute intense exercise is mediated by glucocorticoids (7, 16). There has also been observed a greater basal functional capacity of elite sportsperson’s neutrophils together with higher plasma cortisol levels relative to the sedentary population midway through a training season (13). But the question now is what happens over the course of a training season. The results of the present work did not show any clear correlation between the variations found in the phagocytic response and the cortisol levels. Only in November were significantly higher cortisol values observed together with low levels of the phagocytic function so that we cannot as yet establish any clear conclusion in this regard.

Catecholamines have classically been regarded as immunosuppressors, especially with respect to lymphoid function (11). Their effects on phagocytosis, however, are not clear: whereas it has been reported that β-agonist isoproterenol inhibits phagocytosis of human monocytes (3), there has also been reported a stimulation of phagocytic function of chicken and murine macrophages by norepinephrine (1, 15); and whereas high concentrations of catecholamines decrease the phagocytic capacity of neutrophils (24), low concentrations are able to increase rat neutrophil function (21). With respect to norepinephrine, in the present work we observed fundamentally a decline in May, in parallel with the greater monocyte and neutrophil phagocytic capacity. One might hypothesize that a transitory decline in norepinephrine levels may eliminate an immunosuppressory effect and allow a greater functional capacity of the phagocytes. Indeed, it has been noted that chemical sympathectomy results in an increase of macrophage activity (26). In any case, the clearest result found in the present work is the correlation between the phagocytosis and epinephrine peaks observed in the months from March to July, above all with respect to the monocytes. On the basis of this correlation, epinephrine might be regarded as a good “neuroendocrinial marker of the status of the phagocytic function” of sportspersons during their training season. It seems logical that epinephrine levels should be high at moments of greatest psychological stress of the sportsperson (when there are the most competitive events), with the nonspecific function of the phagocytes also being adapted to these requirements. But this does not necessarily mean that this neuromodulator is directly responsible for the increase in phagocytosis, although some studies have suggested that epinephrine might mediate the stimulation of certain facets of the nonspecific immune response, such as natural killer activity, after exercise (9).

In summary, we may conclude from the results obtained in the present work that 1) the cyclists presented a greater basal phagocytic capacity during the months May to July, a period that coincides with the greatest number and highest level of competitive events in which they participate; and 2) during May, there was a rise in the plasma epinephrine levels, with a very high correlation with phagocytic levels.

The results suggest that the phagocytic capacity together with the blood catecholamine levels might be a good “neuroimmunendocrinial marker of the status of sportsperson’s immune response.” It becomes necessary, however, to unify criteria in evaluating granulocyte function, because there is a great deal of variabilility in the results obtained by different workers, a fact that may be attributed to many factors, including the age and sex of the participants (20).

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