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## Bronchial epithelial damage after a half-marathon in nonasthmatic amateur runners

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**Chimenti L, Morici G, Paternò A, Santagata R, Bonanno A, Profita M, Riccobono L, Bellia V, Bonsignore MR.** Bronchial epithelial damage after a half-marathon in nonasthmatic amateur runners. *Am J Physiol Lung Cell Mol Physiol* 298: L857–L862, 2010. First published April 2, 2010; doi:10.1152/ajplung.00053.2010.—High neutrophil counts in induced sputum have been found in nonasthmatic amateur runners at rest and after a marathon, but the pathogenesis of airway neutrophilia in athletes is still poorly understood. Bronchial epithelial damage may occur during intense exercise, as suggested by investigations conducted in endurance-trained mice and competitive human athletes studied under resting conditions. To gain further information on airway changes acutely induced by exercise, airway cell composition, apoptosis, IL-8 concentration in induced sputum, and serum CC-16 level were measured in 15 male amateur runners at rest (baseline) and shortly after a half-marathon. Different from results obtained after a marathon, neutrophil absolute counts were unchanged, whereas bronchial epithelial cell absolute counts and their apoptosis increased significantly ( $P < 0.01$ ). IL-8 in induced sputum supernatants almost doubled postrace compared with baseline ( $P < 0.01$ ) and correlated positively with bronchial epithelial cell absolute counts ( $R^2 = 0.373$ ,  $P < 0.01$ ). Serum CC-16 significantly increased after all races ( $P < 0.01$ ). These data show mild bronchial epithelial cell injury acutely induced by intense endurance exercise in humans, extending to large airways the data obtained in peripheral airways of endurance-trained mice. Therefore, neutrophil influx into the airways of athletes may be secondary to bronchial epithelial damage associated with intense exercise.

neutrophils; endurance exercise; apoptosis; bronchial epithelial cells; inflammatory mediators

THERE IS INCREASING EVIDENCE that habitual training is associated with mild airway inflammation in athletes of different endurance sports performed in cold or temperate environments (3, 4, 21, 23, 32). Airway inflammation in endurance athletes shows some peculiar features, since it may not be associated with bronchial hyperreactivity, postexercise respiratory symptoms (3, 21), or evidence of cell activation after exercise (3, 4, 23).

In runners, we (3) previously reported high neutrophil counts in induced sputum at rest and a further increase shortly after a marathon. Similarly, young competitive rowers and swimmers showed predominance of neutrophils in induced sputum both at rest and after exercise (4, 23). A trend toward increased airway neutrophil counts was also found in runners

studied the day after a half-marathon (7). The mechanism responsible for exercise-induced neutrophil migration into the airways has not been elucidated. Damage of airway epithelium may occur during exercise due to osmotic changes associated with insufficient conditioning of inspired air. The osmotic changes would activate bronchial epithelial cells, with subsequent release of inflammatory mediators and airway neutrophilia (1). In vitro, bronchial epithelial cells exposed to hyperosmolar medium released chemotactic factors for neutrophils such as IL-8 (14), suggesting a possible role of bronchial epithelial cells in the recruitment of inflammatory cells.

Exercise-induced bronchial epithelial damage has been documented in animal models. In mice, endurance training caused increased inflammatory cells and damage of bronchial epithelium in small airways (8). Bronchial epithelial cells damage also occurred in horses after exercise while breathing cold air (10). In dogs challenged with repeated insufflation of dry and cold air, loss of ciliated epithelium and airway remodeling occurred (11). In humans, however, direct evidence of damage of airway epithelium acutely induced by exercise is still missing. In young competitive rowers, we found that bronchial epithelial cell counts in induced sputum tended to increase after supramaximal exercise of 3- to 4-min duration (23). In addition, evidence of bronchial epithelial damage has been reported in competitive swimmers studied under resting conditions (5), but negative data were reported after exercise (4). Furthermore, bronchial epithelial cell counts in induced sputum were always found to be low in marathon runners either at rest or after exercise (3, 7).

Since our previous data suggested that bronchial epithelial cell damage may be an early event during intense exercise (23), we hypothesized that induced sputum samples collected after a running race shorter than a marathon, i.e., half-marathon, might provide evidence of bronchial epithelial damage. To this aim, we assessed airway cell composition and the concentration of inflammatory mediators such as IL-8, known to mediate recruitment of neutrophils during airway inflammation, and Clara Cell protein 16 (CC-16), a marker of increased lung permeability, in nonasthmatic runners before and shortly after three half-marathons held in Palermo, Italy, at different times of the year (October, May, and November). Environmental factors (temperature and humidity) and concentrations of airborne pollutants [ozone and particulate matter with a diameter of  $<10 \mu\text{m}$  ( $\text{PM}_{10}$ )] were also analyzed to evaluate their possible role in exercise-induced changes in airway cell composition and release of inflammatory mediators.

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## MATERIALS AND METHODS

**Subjects and study design.** Fifteen healthy, nonsmoking amateur male runners (average training volume  $85 \pm 26$  km/wk) were studied. No subject used anti-inflammatory agents before or during the study or reported a diagnosis of asthma, asthma-like symptoms, or habitual use of  $\beta_2$ -agonists. No subject reported symptoms of current or recent (i.e., in the 4 wk preceding the study) upper respiratory tract infections (2) or upper respiratory illness (31). The study was approved by the local Ethical Committee of University of Palermo; all subjects gave written informed consent.

Subjects were studied before and after 3 different half-marathons held at different times of the year. This study design was necessary, as only a limited number of samples can be obtained and processed in a field study. Baseline data were obtained 3 days before the race,  $\sim 18$  h after training; postrace samples were obtained  $\sim 2$  h after completion of the half-marathon (October 2002,  $n = 5$ , mean finishing time:  $81.8 \pm 8.4$  min; May 2007,  $n = 8$ , mean finishing time:  $88.3 \pm 8.3$  min; November 2007,  $n = 7$ , mean finishing time:  $96.5 \pm 13.4$  min). Five subjects ran 2 races (1 subject ran October and May races, 2 subjects ran October and November races, 2 subjects ran May and November races). All races started at 9 AM on Sunday, and car traffic was not allowed in the circuit.

**Air quality and environmental data collection.** Data on temperature and humidity and air pollutants ( $PM_{10}$  and ozone) were obtained by the environmental agency Azienda Municipalizzata Igiene Ambientale for the week preceding the race and the day of the race as previously described (7).

**Pulmonary function measurements and blood samples.** Standard bronchoprovocation tests with methacholine up to the concentration of 25 mg/ml were obtained at study entry and were negative in all cases (data not shown). Lung function [forced expiratory volume in 1 s ( $FEV_1$ ) percentage of predicted; forced vital capacity (FVC) percentage of predicted] was assessed by spirometry (22) before and after sputum induction (Biomedin, Padova, Italy). A 20-ml blood sample was drawn from the antecubital vein for preparation of serum aliquots and stored at  $-20^\circ\text{C}$  for subsequent analyses.

**Induced sputum production and processing.** Sputum induction and processing were according to Fahy et al. (13) as previously reported (3). After washing the oral cavity with saline, subjects were exposed to a hypertonic (5%) aerosol (ultrasonic nebulizer; Fisonex; Fisons Italmichimi SpA, Rome, Italy). Sputum samples were homogenized by adding an equal volume of 0.1% dithiothreitol saline and centrifuged. Supernatants were frozen at  $-20^\circ\text{C}$ . Cell pellets were resuspended in 2% human serum albumin saline solution; total cell counts and viability were assessed with a standard hemocytometer and trypan blue exclusion, respectively. Cells were then cytocentrifuged (Cytospin 2; Shandon, Runcorn, United Kingdom) and stained (Diff-Quick; Merz-Dade, Dudinggen, Switzerland). At least 400 cells/slide were counted. Squamous cell counts were subtracted from total cell counts, and differential counts were expressed as corrected percentages.

**Detection of apoptosis.** Apoptosis of bronchial epithelial cells was assessed in situ on slides fixed with paraformaldehyde-lysine-perio-

date (PLP)-sucrose by indirect TdT-mediated dUTP nick end labeling (TUNEL; In Situ Cell Death Detection Kit; Roche Diagnostics, Basel, Switzerland). All cytopsins were lightly stained with hematoxylin and read at  $\times 40$  magnification. At least 400 cells/slide were counted.

**Biochemical analysis on serum and sputum supernatants.** Serum CC-16 concentrations were measured (Human Clara Cell Protein ELISA; BioVendor). Sputum supernatants were analyzed for IL-8 (IL-8 ELISA kit; Amersham).

**Statistical analysis.** Results are reported as means  $\pm$  SD. Two-way ANOVA was used for data analysis with baseline and postrace conditions and time of the year as independent variables; Bonferroni correction was used for post hoc comparisons. Simple linear regression was used to analyze the relationships between IL-8 concentration and bronchial epithelial cell absolute counts (StatView 5.0.1; Abacus Concepts, Berkeley, CA). Significance was at  $P < 0.05$ .

## RESULTS

**Air quality data.** Table 1 reports the average environmental conditions and concentrations of main pollutants recorded during the weeks of study and on the day of the races. The largest difference in outdoors temperature was  $\sim 10^\circ\text{C}$  between October or May and November, the observed range reflecting that the week preceding the race and the day of the race in November were rainy and cold as also shown by air humidity values. The highest mean values of ozone and  $PM_{10}$  were recorded in May and October, reflecting the highest temperatures, but at all times, pollutant concentrations were below the hourly limits and alarm thresholds currently used in the European Union. Furthermore, no main difference was observed between the levels of pollutants recorded during the weeks of sample collection and on the days of competition.

**Clinical data and pulmonary function.** Average baseline lung function in athletes was normal ( $FEV_1$ :  $4.0 \pm 0.4$  l,  $106.6 \pm 11.7\%$  predicted; FVC:  $4.9 \pm 0.6$  l,  $108.9 \pm 12.0\%$  predicted) and did not vary after the race ( $FEV_1$ :  $4.0 \pm 0.6$  l; FVC:  $4.9 \pm 0.5$  l). No subject reported respiratory symptoms at baseline or after race. Sputum induction did not cause symptoms or affect spirometry.

**Airway cells.** Average cell viability was  $80.3 \pm 12.5\%$  without significant differences among races or between baseline and postrace conditions. Mean induced sputum cellularity did not show variations among races or between baseline and postrace conditions (October total cells, baseline:  $0.9 \pm 0.5 \times 10^6/\text{ml}$ , race:  $1.3 \pm 1.6 \times 10^6/\text{ml}$ ; May total cells, baseline:  $0.5 \pm 0.2 \times 10^6/\text{ml}$ , race:  $0.8 \pm 0.3 \times 10^6/\text{ml}$ ; November total cells, baseline:  $0.8 \pm 0.4 \times 10^6/\text{ml}$ , race:  $0.9 \pm 0.3 \times 10^6/\text{ml}$ ). Absolute and differential cell counts in induced sputum are shown in Fig. 1 and Table 2, respectively.

Table 1. Average environmental conditions and pollutant concentrations recorded during the week of sample collection and on the day of the race

|                                 | October                 |                 | May                     |                         | November         |                  |
|---------------------------------|-------------------------|-----------------|-------------------------|-------------------------|------------------|------------------|
|                                 | Week                    | Race            | Week                    | Race                    | Week             | Race             |
| Ozone, $\mu\text{g}/\text{m}^3$ | $63.5 \pm 32.3$         | $70.4 \pm 20.5$ | $72.6 \pm 22.7\ddagger$ | $102.6 \pm 2.0\ddagger$ | $48.5 \pm 17.1$  | $43.5 \pm 0.5$   |
| $PM_{10}$ , $\mu\text{m}^3$     | $37.4 \pm 15.5\ddagger$ | $20.4 \pm 1.9$  | $33.5 \pm 7.4\ddagger$  | $27.6 \pm 5.2\ddagger$  | $22.6 \pm 6.8$   | $11.4 \pm 0.4$   |
| T, $^\circ\text{C}$             | $20.7 \pm 1.9$          | $19.3 \pm 1.2$  | $20.5 \pm 1.6$          | $20.5 \pm 0.7$          | $12.8 \pm 2.1^*$ | $9.8 \pm 0.3^*$  |
| Humidity, %                     | $55.6 \pm 11.3$         | $59.5 \pm 7.4$  | $56.8 \pm 11.1$         | $57.6 \pm 4.0$          | $64.2 \pm 9.4^*$ | $78.3 \pm 1.1^*$ |

Values are means  $\pm$  SD. \* $P < 0.001$  for differences between November and October or May measurements;  $\ddagger P < 0.0001$  for differences between May and October or November;  $\ddagger P < 0.05$  between October or May and November. Hourly limits for pollutants were  $120 \text{ mg}/\text{m}^3$  for ozone and  $50 \text{ mg}/\text{m}^3$  for particulate matter with a diameter of  $< 10 \mu\text{m}$  ( $PM_{10}$ ); alarm threshold was  $240 \text{ mg}/\text{m}^3$  for ozone. T, temperature.

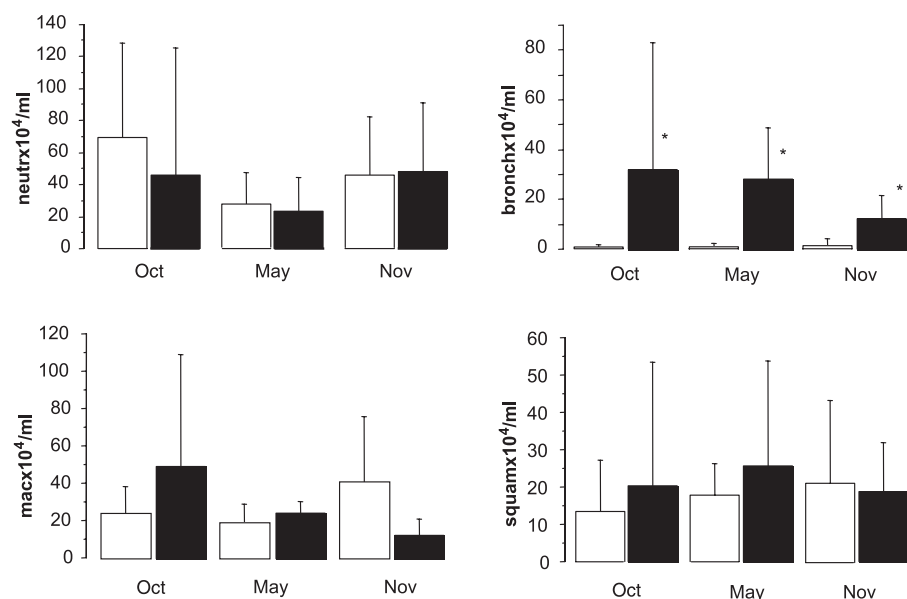


Fig. 1. Absolute cell counts in induced sputum at baseline (empty bars) and after race (filled bars) at the time points of the study. Bronchial epithelial cells (bronch) significantly increased after each race ( $*P < 0.01$ ), whereas neutrophils (neutr) were unchanged. Oct, October; Nov, November; mac, macrophages; squam, squamous cells.

Bronchial epithelial cell absolute and differential counts increased significantly after all races ( $P < 0.01$ ); neutrophil differential counts were unchanged or tended to decrease after the race ( $P < 0.05$ ). No difference between baseline and postrace conditions was found for neutrophil absolute counts and macrophage absolute and differential counts at any time points (Table 2). The statistical analysis was repeated using data from 1 race per subject ( $n = 15$ ), and results did not appreciably change.

No correlation was found between pollutants levels, humidity, temperature, and cell counts or inflammatory mediator concentrations in induced sputum.

**Apoptosis.** Figure 2, top, shows the appearance of induced sputum cells and their apoptosis at baseline (Fig. 2, A and C) and after the half-marathon (Fig. 2, B and D); Fig. 2, bottom bar graphs report the mean data regarding apoptosis of induced sputum cells. Apoptotic cells expressed as percentage of total cells increased after all races ( $P < 0.001$ ; Fig. 2, left graph). Apoptosis of bronchial epithelial cells as percentage of total bronchial epithelial cell counts was undetectable at baseline and increased significantly after all races ( $P < 0.01$ ; Fig. 2, middle graph). The contribution of bronchial epithelial cells to total apoptosis increased after all competitions and accounted for the majority of apoptotic cells ( $P < 0.01$ ; Fig. 2, right graph). Apoptosis of other cell types was very low and did not show major changes at any time points (data not shown). Apoptotic cells did not correlate with pollutant exposure levels or temperature/air humidity at any time points.

**Inflammatory mediators in sputum.** IL-8 concentration in sputum supernatant was low at baseline, increased slightly after the race ( $P < 0.01$ ; Fig. 3A), and correlated with postrace bronchial epithelial absolute cell counts ( $R^2 = 0.373$  postrace,  $P < 0.01$ ; Fig. 3B). No correlation was found between the concentration of IL-8 and apoptosis of bronchial epithelial cells (data not shown).

**Analyses on blood.** As a marker of pulmonary peripheral damage, CC-16 was measured in serum. CC-16 levels were in the normal range in all subjects at rest and increased significantly from baseline to postrace condition ( $P < 0.01$ ; Fig. 3C).

## DISCUSSION

To our knowledge, this is the first study in human nonasthmatic amateur runners reporting a significant and reproducible increase in bronchial epithelial cell counts in induced sputum after a half-marathon. Our results suggest that endurance exercise causes bronchial epithelial cell damage associated with increased apoptosis and increased IL-8 concentration in induced sputum. Therefore, it is possible that the increased neutrophil counts found in induced sputum of endurance athletes is at least partly secondary to epithelial cell damage occurring early during exercise.

Findings in experimental models point to bronchial epithelial cells as an important target of exercise-induced airway changes. Hyperventilation with cold and dry air in dogs (11), exercise in cold air in horses (10), and endurance training in

Table 2. Differential cell counts in induced sputum of runners

|              | October      |              | May          |              | November    |              |
|--------------|--------------|--------------|--------------|--------------|-------------|--------------|
|              | Baseline     | Race         | Baseline     | Race         | Baseline    | Race         |
| %Squamous    | 15.9 ± 11.1  | 14.4 ± 7.3   | 38.4 ± 20.5  | 28.5 ± 21.7  | 25.3 ± 19.7 | 27.3 ± 19.8  |
| %Macrophages | 33.4 ± 25.0  | 39.4 ± 28.8  | 40.2 ± 20.2  | 32.8 ± 11.4  | 48.7 ± 26.6 | 19.1 ± 14.1  |
| %Neutrophils | 65.3 ± 24.3* | 32.4 ± 24.8  | 52.2 ± 23.7* | 26.4 ± 12.7  | 47.4 ± 27.2 | 55.3 ± 24.5  |
| %BEC         | 0.5 ± 0.8    | 27.7 ± 21.5* | 1.7 ± 1.6    | 33.3 ± 21.8* | 1.5 ± 2.7   | 22.6 ± 26.9* |

Values are means ± SD. Bronchial epithelial cells (BEC) significantly increased after each race ( $*P < 0.01$ ), whereas neutrophils were unchanged or tended to decrease ( $P < 0.05$ ).



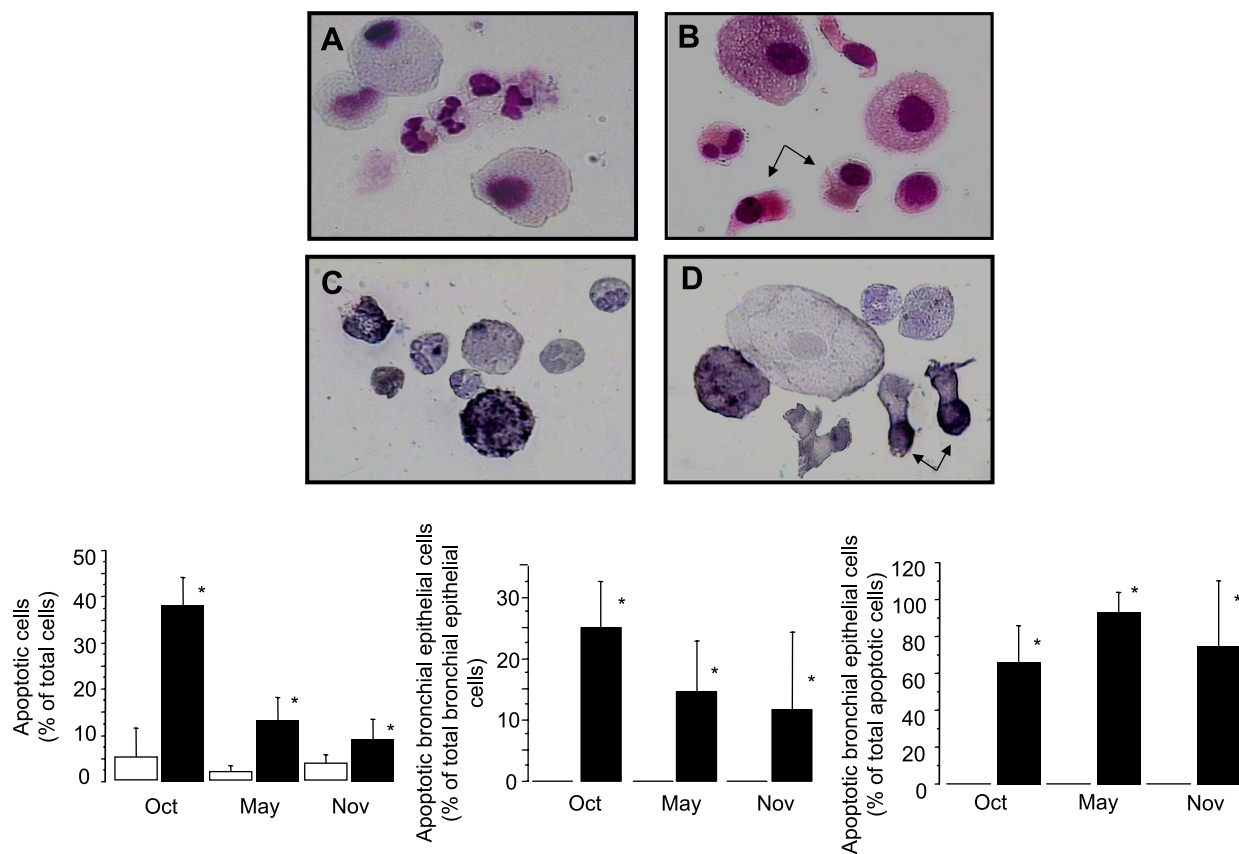


Fig. 2. Induced sputum samples obtained after the half-marathon (*B–D*) and at baseline (*A–C*). *A* and *B*: Diff-Quick staining; *C* and *D*: TdT-mediated dUTP nick end labeling (TUNEL) staining. Original magnification,  $\times 40$ . Bronchial epithelial cells and their apoptosis can be detected after race (*B* and *D* arrows, respectively). Bar graphs shows apoptosis in induced sputum cells. Total apoptosis significantly increased after all races ( $*P < 0.001$ ; *left graph*). Apoptosis of bronchial epithelial cells was undetectable at baseline and increased after each race in induced sputum ( $*P < 0.01$ ; *middle graph*). The contribution of bronchial epithelial cells to total apoptosis increased after all competitions and accounted for the majority of apoptotic cells ( $*P < 0.01$ ; *right graph*).

mice (8) were shown to damage bronchial epithelium in large and peripheral airways. In addition, bronchial epithelial cells in induced sputum tended to increase after all-out rowing, which requires a very high ventilation, suggesting a possible role of epithelial shear stress (28). Besides functioning as a barrier against environmental toxins and injury, bronchial epithelial cells modulate inflammation and play a crucial role for the normal immune response. Bronchial epithelial cells can release a broad variety of inflammatory mediators (cytokines, chemokines, lipids, and peptides) that enhance the recruitment of leukocytes into the lungs (20). Mediators released by bronchial epithelial cells and infiltrating leukocytes in turn may affect the differentiation of airway epithelium and cell death processes of epithelial cells (33).

Respiratory water loss commonly occurs during exercise due to insufficient conditioning of inspired air. Bronchial epithelial cell damage caused by endurance exercise may be secondary to osmotic changes with decreased bronchial lining fluid level during hyperventilation or to cooling-rewarming of the airways (1). Moreover, IL-8, a chemotactic factor known to mediate recruitment of neutrophils into the airways during inflammation, is released by cultured bronchial epithelial cells after repeated stretch (9, 29, 34), suggesting an additional potential mechanism by which exercise could modulate bronchial epithelial cell function.

In animal models, neutrophil influx into the airways required some time (at least 2 h) to develop after airway challenge with dry and cold air (11). When induced sputum in runners was sampled after a marathon lasting  $\sim 3.5$  h, bronchial epithelial cells were absent, and neutrophils were the predominant cell type. Induced sputum samples collected the morning after a half-marathon showed a slight increase in neutrophils compared with resting conditions (7). IL-8 concentration in induced sputum supernatants doubled after the half-marathon and was positively correlated with bronchial epithelial cell absolute counts. We speculate that the increase in neutrophils found in large airways of athletes after prolonged exercise (3) or the morning posttrace (7) might be at least partly secondary to release of chemotactic factors, such as IL-8, by bronchial epithelial cells during early exercise.

Some evidence supports our interpretation. Bronchial epithelial cells exposed to hyperosmolar medium or airway cooling in vitro released IL-8 (14, 15). Conversely, IL-8 concentration in sputum supernatants collected on the morning after a half-marathon was low (7), suggesting a transient exercise-induced inflammatory activation. In runners, increased IL-8 in induced sputum collected at rest was found during a competitive period, but no correlations were found with sputum cells counts (12). Finally, runners habitually training outdoors are exposed to particulate matter, such as diesel fuel, which could

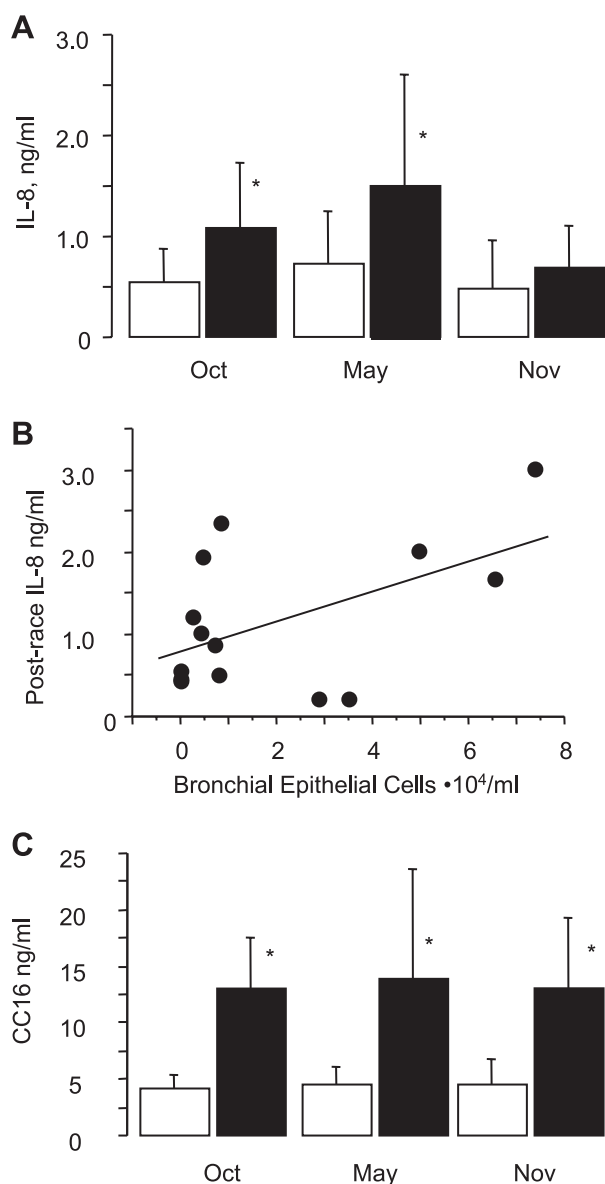


Fig. 3. Inflammatory mediators in induced sputum and serum samples. A: IL-8 concentrations significantly increased post-race ( $*P < 0.01$ ). B: IL-8 was positively correlated with bronchial epithelial cell absolute counts post-race. C: serum CC-16 concentration significantly increased after each race ( $*P < 0.01$ ).

be an additional stimulus to attract neutrophils (27), but we found no correlation between IL-8 and mean air pollutant levels.

Because apoptosis plays an important role in limiting inflammatory and immunogenic responses (16, 33), we hypothesized that apoptosis of bronchial epithelial cells may at least partly account for the limited inflammation found in large airways of nonasthmatic amateur runners (3). Up to 25% of bronchial epithelial cells after a half-marathon were apoptotic. Apoptosis of bronchiolar epithelium also occurred in endurance-trained mice (8). A recent study from our laboratory (7) showed low bronchial epithelial cell counts in induced sputum of runners at rest but increased apoptosis the morning after a half-marathon or shorter competitions. Therefore, apoptosis of bronchial epithelial cells may be a major mechanism physio-

logically limiting inflammatory activation in the airways of athletes.

Increased bronchial epithelial cells in induced sputum have been found in elite swimmers (5), but sampling was limited to resting conditions; in addition, interpretation of these results is complicated by the effects of exposure to chlorine compounds in swimmers (35). In our studies in amateur runners, bronchial epithelial cell counts at rest were always found to be very low (3, 4, 23). We cannot exclude that the level of training/performance may affect bronchial epithelial cell counts in athletes. Another study on the effects of eucapnic voluntary hyperventilation in nonasthmatic athletes reported unchanged neutrophil counts (30), but this model may be insufficient to fully mimic the effects of prolonged exercise on the airways. Additional studies are necessary to further explore the role of training status and/or type of test on airway cells in athletes.

Recent data based on lung-derived proteins measured in serum or urine suggest that pulmonary epithelial permeability may increase after intense exercise (18). In more detail, CC-16 is a 16-kDa protein with anti-inflammatory function released by Clara cells (18) and used as a marker of distal lung epithelial damage, especially in the early stage of toxic lung injury by air pollutants (19). CC-16 was found to be increased also during (6) or shortly after exercise (24). We found significantly increased serum levels of CC-16 in post-race samples. Our (7) previous study found that CC-16 levels did not correlate with air pollutant levels and were normal in samples collected the morning after a half-marathon. Thus intense exercise appears to transiently increase epithelial permeability.

It is possible that the observed exercise-induced proinflammatory changes in amateur runners may chronically affect respiratory health. Unfortunately, there are no large studies on this topic, whereas most of the current literature debates on the role of exercise-related inflammation and bronchial hyperreactivity in athletes. Our data were obtained in nonasthmatic athletes, with normal bronchial provocation tests, and could be considered as representative of normal airway responses to acute exercise in trained subjects.

Our study has several points of strength. We studied small groups of athletes in three distinct running competitions and under different environmental conditions. This design was necessary to increase the number of observations, since field studies on airway cells cannot be performed in large samples. The results were similar in all experiments, irrespective of environmental conditions. More importantly, we studied our runners both at rest and shortly after exercise and were able to identify transient effects of exercise, such as the increase of bronchial epithelial cells and their apoptosis or increased IL-8 and CC-16. Unfortunately, repeated studies in the same subjects after exercise are not recommended, since the procedure itself may affect induced sputum composition and specifically the number of neutrophils and eosinophils (36).

In conclusion, in half-marathon runners, we found increased bronchial epithelial cell counts and IL-8 concentration in induced sputum collected shortly after exercise, different from results obtained after a marathon showing increased numbers of neutrophils in the airways. These data are in line with previous findings on peripheral airways obtained in a mouse model (8) and suggest that neutrophil influx may be secondary to mild bronchial epithelial damage caused by intense exercise.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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