Exercise-induced changes to in vitro T-lymphocyte mitogen responses using CFSE

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Green, Katherine J., and David G. Rowbottom. Exercise-induced changes to in vitro T-lymphocyte mitogen responses using CFSE. J Appl Physiol 95: 57–63, 2003.—Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling of lymphocyte populations can provide unique insights into cell function at rest and with exercise, due to its ability to quantify cell division on an individual cell basis. This study aimed to characterize the effect of acute, intense exercise on T-lymphocyte function. Well-trained endurance runners completed 60 min of treadmill running at 95% of individual anaerobic threshold. Blood samples were collected before exercise; after 30 and 60 min of exercise; and after 30, 60, and 90 min of recovery. Isolated peripheral blood mononuclear cells were labeled with CFSE and cultured with or without mitogen (phytohemagglutinin). After culture, cell suspensions were labeled with CD3 (allophycocyanin) and CD8 (phycoerythrin), and expansion rates and cell death rates were calculated for each sample, as well as mitosis rates for each cell generation. Exercise was associated with a 60% decrease in cell expansion in both CD4 and CD8 cell types from before exercise to midexercise (P < 0.05). The significant decrease in expansion rate in the midexercise samples for both cell types was mirrored by a 65% increase in cell death (P < 0.05) in both cell types at that sample point. Exercise had no effect on the mitosis rate of either CD4 or CD8 cells in any cell generation (generations 0–3). This study indicates that 1 h of intense exercise affects in vitro T-lymphocyte function. These data suggest, for the first time, that exercise decreases cell expansion rate via an increase in cell death of both CD4 and CD8 T lymphocytes, rather than a decrease in mitosis.

fluorescent cell tracking; cell division; mitogen-induced proliferation; high-intensity exercise; immune function

FOR MANY YEARS, EXERCISE IMMUNOLOGISTS have tried to assess whether an acute exercise bout influences individual T-lymphocyte function. The aspect of T-lymphocyte function most commonly assessed is mitogen-induced proliferation. Should a decrease in the proliferative capabilities of T lymphocytes occur, it might explain the observed high incidence of infections in elite athletes (15, 17). Despite many studies employing a range of exercise protocols and methods for assessing T-lymphocyte proliferation, there remains no clear repeatable observation regarding individual T-lymphocyte function postexercise.

Previous studies using standard lymphocyte proliferation assays, such as incorporation of [3H]thymidine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction have reported significant reductions in T-lymphocyte responses to mitogen after medium- and long-duration intense exercise (24). However, interpretation of the data from these studies is difficult, because results are strongly influenced by the number and type of cells in different samples. [3H]thymidine incorporation and MTT reduction assays use a constant number of peripheral blood mononuclear cells (PBMC) or amount of whole blood from all samples. Yet mitogens stimulate various cell types differentially, and exercise induces a large increase in natural killer (NK) cells relative to T cells (33, 35). Because NK cells do not divide in response to mitogen (37), any increase in the relative proportion of NK cells in a given number of PBMC or amount of whole blood will result in fewer cells in postexercise samples capable of responding to mitogen. Therefore, the resulting reduction in postexercise proliferation responses compared with preexercise is potentially due to exercise-induced alterations in cell numbers rather than a reduction in cell function per se.

In recognition of this problem, several researchers have corrected proliferation data to reflect the number of responding cells. This post hoc approach has resulted in the elimination of the decline in mitogen responsiveness after short-duration intense and moderate exercise (23, 26, 28) but not after longer duration intense exercise (8, 21, 27). This suggests that impaired T-lymphocyte proliferation may occur with longer duration intense exercise. However, the problem remains that this simple correction of proliferation data per responding cell is indirect and does not necessarily reflect individual cell function. An additional difficulty associated with adjustment of proliferative responses is the unknown influence of mitogens on individual lymphocyte subsets (24). At present, despite the knowledge that exercise can differentially affect the number of CD4 and CD8 cells in the circulation, there have been few investigations aimed at determin-

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ing the function of these subsets independently. To understand exercise effects on T-lymphocyte function, it is necessary to employ techniques capable of providing direct insight into individual cell function of the different T-lymphocyte subsets.

Recent advances in flow cytometry have provided a means to use fluorescent dyes to track individual cell division and thus allow independent examination of individual cells. Carboxyfluorescein succinimidyl ester (CFSE) is such an intracellular dye. The dye is taken up by lymphocytes, and membrane-impermeable intracellular fluorescent dye-protein conjugates are formed in labeled cells. These conjugates are retained throughout mitosis, and one-half of the fluorescent label is inherited by each daughter cell after division. With the use of this characteristic of the dye, individual cell division may be tracked by using a flow cytometry histogram plot (16). The division history of labeled lymphocytes and the frequency distribution of cells in successive generations can, therefore, be determined. Since Lyons and Parish (16) first described the use of CFSE in a flow-cytometric method for determining cell division, it has been used to assess cell function in vitro and in vivo (3, 7, 32). Using CFSE to monitor cell division has several advantages over other methods. Important to exercise immunology is that individual lymphocyte subsets can be distinguished by further immunophenotyping, and their rates of division quantified separately. Therefore, CFSE labeling of lymphocyte populations can provide unique insights into cell function during and after exercise. Using the CFSE technique, this study aimed to characterize the effect of exercise on the proliferative capacities of the T-lymphocyte subsets of CD4 and CD8 cells.

METHODS

Subjects

Eight well-trained male runners who met the inclusion criteria of a 2-yr training history and a current 10-km race time of ≤36 min were recruited for this study. Subjects were informed of the purpose and risks of participation in the study before written consent was obtained. The University Human Research Ethics Committee of the Queensland University of Technology approved this study.

Maximal Incremental Exercise Test

Subjects completed a continuous incremental running test on a motorized treadmill (14 k/h, 1% increase in grade each minute) to volitional exhaustion. Breath-by-breath gas analysis was conducted on expired pulmonary gases throughout the test (CPX/D mobile cart system, Medical Graphics, St. Paul, MN). Maximal O2 consumption (VO2 max) was defined as the single highest 5-s average value attained during the test, coinciding with a respiratory exchange ratio >1.10. Ventilatory threshold was determined from a graph of the ventilation-to-oxygen consumption (VO2) ratio plotted against time. Threshold was defined as the inflection point of the ventilation-VO2 curve and was identified independently by two researchers (39).

Exercise Trial

Subjects reported to the laboratory at 5:30 AM, having remained from exercise for the previous 24 h and having only consumed water since midnight. They were fitted with an indwelling venous cannula, through which blood samples were drawn. After a 10-min rest, a blood sample [5:45 AM; time 1 (T1)] was taken. Subjects completed a standardized warm up consisting of 5-min slow-speed running followed by stretching. At 6:00 AM, subjects began running, and for the entire test the speed and gradient of the treadmill remained constant. All subjects ran at 14 k/h, and the gradient was adjusted individually to elicit a VO2 equivalent to 95% ventilatory threshold, based on the incremental maximal test. Metabolic and heart rate measures were made in the last 5 min of exercise before blood samples were drawn, to ensure that subjects were maintaining the appropriate workload. After 30 min of exercise [6:30 AM; time 2 (T2)], subjects momentarily stopped running and sat down to permit a blood sample to be taken. Subjects resumed running immediately after the blood sample was taken and typically did not stop running for longer than 1–2 min. Blood samples were taken immediately after exercise [7:00 AM; time 3 (T3)] and 30 min [7:30 AM; time 4 (T4)], 60 min [8:00 AM; time 5 (T5)], and 90 min [8:30 AM; time 6 (T6)] after exercise. During the recovery period, subjects were seated quietly in the laboratory. During the entire testing session, subjects only consumed water.

Lymphocyte Separation

Whole blood was collected into sodium heparin tubes (8-ml Vacutainer; Becton Dickinson, Lane Cove, NSW, Australia), diluted with an equal volume of PBS, then layered over Ficoll-paque (Sigma, St. Louis, MO) density gradient separation solution, and centrifuged at 300 g for 20 min at room temperature. The mononuclear cell layer (PBMC) was removed and washed twice in RPMI-1640 medium (Sigma) supplemented with 2 mM glutamine (Gln; Sigma) and gentamycin (Sigma). Cell viability and cell counts were assessed by Trypan blue exclusion, and then cells were labeled with CFSE.

CFSE Labeling of Lymphocytes and Culture

CFSE was stored frozen in a 1 mM stock solution until ready for use. A pellet of 1 × 10⁷ cells was resuspended in 0.5 ml PBS; 0.5 ml of CFSE labeling solution (10 μM) was added to the cell suspension to yield a final CFSE concentration of 5 μM. Afterward, 1-min samples were diluted with PBS and centrifuged at 400 g for 10 min before being resuspended in culture medium (RPMI-1640, 2 mM glutamine, 10% FCS) to yield a final cell concentration of 8 × 10⁶ cells/l. Two hundred microliters of cell suspension were cultured with either 5 μg/ml phytohemagglutinin (PHA) (stimulated) or RPMI (unstimulated). Cultures were incubated at 37°C in a 5% CO₂ incubator (Columbia Scientific, Lower Hutt, New Zealand) for 72 h in Falcon tubes (Becton Dickinson).

Flow Cytometry

After culture, cell suspensions were labeled with CD3 (allophycocyanin), CD8 (phycoerythrin), and Viaprobe (7-aminoactinomycin D) for 15 min in the dark at room temperature. Samples were then washed with 3 ml PBS and resuspended in 200 μl paraformaldehyde solution (2%) and placed in Trucount tubes (Becton Dickinson). Surface marker and CFSE analysis was conducted by using a FACSCalibur flow cytometer (Becton Dickinson). The same forward- and side-scatter parameters were used for each trial as established for human peripheral leucocytes. Standard gating procedures were used to select
mainly lymphocytes and to differentiate between labeled and unlabeled cells. Viable lymphocytes were identified by using Viaprobe staining and forward- and side-scatter parameters. Fluorescent staining was used to further characterize T lymphocytes (CD3) and lymphocyte subsets CD3"CD8" and CD3"CD8" (CD3"CD4"). Data were analyzed by using FlowJo version 3.3 software (Treestar, San Carlos, CA).

Assessment of Lymphocyte Expansion

The absolute number of cells in each sample was calculated by determining the ratio of gated lymphocytes or lymphoblasts to gated Trucount beads, multiplied by the known number of beads in the Trucount tube. The increase in parent (G0) cells from unstimulated to PHA-stimulated samples can be attributed to cells undergoing mitosis or cell death. If it is assumed that no cell death occurs, all missing G0 cells must be assumed to have undergone mitosis or cell death. The same assumption can be made for each generation. There was considerable overestimation of cell numbers in the final generation, if the assumption of no cell death was used. Iterative processes were then used to change the assumed cell death rate until all “missing” cells could be accounted for by mitosis or cell death. The mitosis rate for each generation was then calculated as the percentage of cells estimated to have undergone mitosis. The cell death rate was the percentage of cells estimated to have undergone cell death.

Blood Cortisol

Cortisol was measured by using a commercial radioimmunoassay kit (Coat-A-Count, Bio-Mediq DPC Pty, Doncaster, Victoria, Australia). Because cortisol is known to have a significant diurnal variation, subjects reported to the laboratory under the same conditions as the exercise trial, and resting blood samples were taken at time points corresponding to those during exercise (i.e., 5:45 AM, T1; 6:30 AM, T2; 7:00 AM, T3; 7:30 AM, T4; 8:00 AM, T5; 8:30 AM, T6). These samples were only analyzed for serum cortisol to allow comparison of diurnal and exercise-induced changes.

Preexercise T-lymphocyte Function

Preexercise blood samples were collected from all eight subjects recruited into the study. In addition to the focus on the effect of exercise, this study included separate analysis on these samples to provide further characterization of T-lymphocyte function with the use of the CFSE technique.

Statistical Analysis

All data are expressed as means (±SD) throughout and in all graphical presentations. For all of the preexercise observations, a one-way ANOVA with repeated measures was carried out with a main effect of cell type (CD4 and CD8) for expansion and apoptosis; a two-way ANOVA with repeated measures on both factors was used to consider the factors of generation (G0–G3) and cell type for mitosis rates. For the exercise and control data, to accommodate the repeated-measures design and avoid exclusion of one subject with partially complete data over time, two missing data points (control trials T5 and T6) were filled with mean values calculated for that variable at that sample point from other subjects’ data. In the case of expansion and apoptosis rates, a two-way ANOVA with repeated-measures design was conducted. Main effects of time (T1–T6 sample points) and cell type (CD4 and CD8) were fitted, as well as the interaction between time and cell type. For mitosis rates, the factors of generation (G0–G3) and cell type were considered. For cortisol, a two-way ANOVA was conducted with the factors of condition (control and exercise) and time. Pearson product-moment correlation coefficient was carried out among cortisol, cell death rates, and cell numbers; for each variable, individual time points as well as averaged data were used. Statistical significance level was set at P < 0.05; Cohen’s statistical power was calculated to be 72–82%. Due to the low number of subjects, post hoc analysis was not possible. To aid interpretation of the data effect, size was calculated as the difference between means divided by the mean SD of the two samples.

RESULTS

Exercise Trials

Despite eight subjects being recruited into the study, only six subjects (age: 29 ± 7 yr, height: 1.78 ± 0.04 m, weight: 67 ± 4 kg) successfully completed the exercise.
All eight subjects donated a resting blood sample, and eight samples were used in the assessment of preexercise T-lymphocyte function. Only the six subjects who completed the exercise challenge were included in the analysis of exercise effects on T-lymphocyte function. During the incremental maximal test, the mean \( \dot{V}_O_2 \) peak value was 70 ± 2 ml·kg\(^{-1}\)·min\(^{-1}\), and mean maximum heart rate was 190 ± 8 beats/min. The exercise trial was completed at 95% of individual anaerobic threshold, equivalent to 83 ± 3% of \( \dot{V}_O_2 \) max. Mean heart rate during the exercise trial was 166 ± 7 beats/min.

**Preexercise T-lymphocyte Function**

There were several significant observations made regarding the different behavior of CD4 and CD8 subsets within the assay system. The mitosis rates, cell death rates, and the overall expansion of the CD4 and CD8 cell populations were significantly different. In early cell generations (G1 and G2), CD8 cells had higher mitosis rates than CD4 cells, yet there was a significant decline in mitosis rates of both cell types over successive generations (and time in culture) (Fig. 2). CD4 cells underwent greater amounts of cell death compared with CD8 cells in all samples (29 ± 4 vs. 6 ± 4%). The combination of lower mitosis and higher cell death of CD4 cells produced a significantly lower cellular expansion of CD4 cells than CD8 cells. Comparison of the cell types within the combined cell culture indicated that, when the overall expansion of the T-cell population was considered, 70% of the newly formed cells were CD8 and 30% were CD4 cells (Fig. 3).

**Exercise Effects**

**Lymphocyte expansion.** Exercise significantly reduced the amount of lymphocyte expansion of both CD4 and CD8 cells. Cellular expansion decreased between T1 and T2 from 2.48 ± 0.59 to 1.56 ± 0.31 for CD8 cells (effect size 1.03) and from 1.17 ± 0.22 to 0.75 ± 0.12 for CD4 cells (effect size 0.82). The expansion of the CD8 cell population was larger than for CD4 cells in all samples (Fig. 4).

**Lymphocyte mitosis.** The percentage of CD4 or CD8 cells undergoing mitosis in each generation was not affected by exercise. There was no difference between T1 and T2 in any cell generation for either CD4 or CD8 cells: G0 (effect size 0.12), G1 (effect size 0.31), G2 (effect size 0.02), and G3 (effect size 0.96). As seen in resting samples, the mitosis rates of CD4 and CD8 cells declined in each successive cell generation and were higher in CD8 cells compared with CD4 cells.

**Lymphocyte cell death.** Exercise had a significant effect on the percentage of dead cells in the cell culture. Compared with the preexercise sample (T1), there was a significantly higher cell death rate in cells in the exercise sample (T2). Cell death increased between T1 and T2 from 32 ± 5 to 42 ± 3% in CD4 cells (effect size 1.07) and from 17 ± 9 to 25 ± 6% for CD8 cells (effect size 0.42). CD4 cells exhibited higher cell death rates than CD8 cells during all exercise samples (Fig. 5). There were no significant correlations between the number of cells in circulation and cell death rates.
**Serum cortisol.** There was no significant difference in the cortisol concentration between the exercise and control condition at any time point. There was a significant main effect of time for both conditions as cortisol decreased significantly from T1 to T6 sampling points (Fig. 6). There was no correlation between cortisol concentration and cell death at any time point.

**DISCUSSION**

This study confirms previous observations that T-lymphocyte function is reduced after medium-duration, high-intensity acute exercise (8, 21, 27). Furthermore, our data suggest that the overall reduced expansion of the cell population in postexercise compared with preexercise samples was due to an increase in cell death (apoptosis), rather than a decrease in the capacity of the cells to divide (mitosis).

In common proliferation assays, such as [3H]thymidine incorporation, a constant number of PBMC is used. However, because of changes in the relative proportion of different cell subsets during exercise, there is a decrease in the proportion of mitogen-responsive cell types postexercise (33, 35). Due to the potential for observed changes in cell function to be an artifact of changes in the number of mitogen-responsive cells, interpretation of results from mitogen-induced proliferation assays after exercise is difficult (18, 34). Several researchers have made mathematical corrections to adjust for differences in the number of responding cells in postexercise samples. To date, the understanding of the effect of exercise on cell function has been limited by the use of techniques incapable of directly determining individual cell function. The major advantage of the technique used in the present study is that it is a direct measure of individual cell function and differentiates between CD4 and CD8 cells. Furthermore, cellular expansion can be broken down into its two composite events: mitosis and cell death.

**Lymphocyte Expansion**

Previous studies using similar moderate-duration, intense-exercise protocols [60 min at 75% \( \dot{V} \text{O}_{2 \text{max}} \) (21) and 45 min at 80% \( \dot{V} \text{O}_{2 \text{max}} \) (27)] have demonstrated a reduction in total lymphocyte response to mitogens. The data in this present study confirm these prior findings and extend the observations to report that both CD4 and CD8 cell expansions were significantly lower in the midexercise sample. Moreover, we independently determined the absolute numbers of CD4 and CD8 cells in each sample. We found that exercise reduced the expansion of CD4 cells to less than one (1.0) at T2 and T3 (Fig. 4). An expansion of less than one indicates fewer cells in the PHA-stimulated sample compared with the unstimulated sample. Although there was evidence of cellular mitosis, it can be assumed that the cell death rate was greater than the mitosis rate. Furthermore, our analysis of the expansion of the total lymphocyte population has indicated that, in both resting and exercise samples, CD8 cells undergo greater expansion than CD4 cells. These data suggest that previous studies using PHA have predominantly measured CD8 expansion (Fig. 3).

**Lymphocyte Mitosis**

It has often been assumed that a decrease in proliferation after exercise is the product of a diminished response to mitogen, resulting from reduced mitosis. However, the greater sensitivity of the present approach suggests that exercise does not reduce the propensity of cells to undergo mitosis. Using CFSE has allowed the calculation of the percentage of cells undergoing mitosis in each sample and in each cell generation. Data from this study suggest that further exploration of how exercise affects other features of cell function (namely apoptosis) may be more important. The lack of any exercise effect on mitosis is consistent with our earlier observations regarding the expression...
of the early activation marker CD69 on the surface of CD4 and CD8 lymphocytes. We have previously reported that exercise does not alter the proportion of cells expressing activation markers in response to the mitogen PHA, signifying no effect on the propensity of cells to divide (6). Paradoxically, we have found a significantly higher expression of CD69 on CD4 cells compared with CD8 cells. However, in this study, we noted a significantly higher rate of mitosis in CD8 cells compared with CD4 cells. It should also be noted that the percentage of parent (G0) cells undergoing mitosis (50%) is substantially less than the percentage that has been previously reported to express CD69 (80–95%) under similar assay conditions (2, 19). Based on data from the present study and others (10), CD69 expression may not be a reliable predictor of mitosis.

**Lymphocyte Cell Death**

Several studies have used exercise-induced DNA damage as a marker of apoptosis in leukocytes (14, 20, 29, 30). The qualitative techniques, such as DNA electrophoresis used in these studies, have described morphological changes (late apoptotic events), but have not quantified apoptosis. Methods for quantifying apoptosis, such as the use of the apoptosis marker Fas ligand (CD95) or phosphatidylserine exposure (annexin V) have rarely been used in exercise studies. Of the few studies that have measured the expression of apoptosis markers after exercise, these studies have simply calculated the percentage of apoptotic cells before and after exercise. In mice splenocytes, exercise has not been reported to increase the expression of apoptosis markers (9), whereas in humans the expression of the CD95-receptor expression on lymphocytes has been shown to be upregulated (22). One principle difference between prior studies and the present study is that we have quantified the effect of exercise on the apoptotic activity of lymphocytes in culture after exposure to the mitogen PHA.

Cell death in culture may be the result of necrotic or apoptotic cell death. However, we observed no exercise-associated increase in the amount of necrotic cell death (as assessed by use of Viaprobe) and have concluded that the loss of cells in culture was, therefore, due to the occurrence of apoptosis. The increase in apoptosis rates of cultures after exercise and the higher rates of apoptosis in CD4 cells compared with CD8 cells may be related to activation-induced cell death (AICD) in response to the mitogen PHA, or apoptosis induced by some other mechanism. Although data from the present study do not clearly differentiate mechanisms, we have previously noted considerable spontaneous death and AICD in our culture system (unpublished observations), so it is likely to be a combination of both mechanisms. Spontaneous cell death may be related to the culture media or other assay conditions (13), and, if AICD is occurring, it is likely that Fas (CD95) is responsible (1, 12, 36). Our observation that CD4 cells are more susceptible to cell death than CD8 cells is in agreement with previous studies (4). It is also possible that CD4 subsets T-helper type 1 (Th1) and type 2 (Th2) undergo Fas-induced apoptosis differentially; some results have indicated that Th2 are more resistant to apoptosis after signaling through CD3/T-cell receptor (36). Whereas this study did not investigate the mechanism behind the cell death observed, future studies should examine whether the exercise-induced increase in cell death in cell cultures is spontaneous or AICD.

Further explanation as to why cell death is increased with exercise may come from the following possible mechanisms. First, exercise-induced lymphocytosis is a result of an influx of lymphocytes from marginalized pools into the circulation and coincides with the higher cell death rates in culture in the midexercise sample (T2). This amplified lymphocyte pool may contain lymphocyte populations that are intrinsically more susceptible to cell death. This possibility is supported by reports that lymphocytosis is associated with an increased proportion of activated phenotypes (5) and that previously activated or memory T lymphocytes are more susceptible to apoptosis (31). Furthermore, the observation that exhaustive exercise is associated with an increase in the expression of Fas (CD95 receptor) would support this mechanism (22). In this present study, however, we did not observe any significant correlations between circulating cell numbers and cell death rates. Alternatively, exercise or in vivo modifiers released during exercise may have a direct effect on the apoptotic activity of cells. There are several reports that indicate that glucocorticoids are capable of inducing apoptosis of lymphocytes (11) and that cells may become more sensitive to glucocorticoids after exercise (38). Although we found no changes in cortisol, the possibility exists that the duration of our exercise challenge was insufficient to elicit an elevated cortisol response. Furthermore, our low subject numbers have placed limitations on our correlation analyses. To investigate this potential mechanism, future studies may benefit by employing greater subject numbers and exercise protocols of longer duration. Nevertheless, we observed an increase in cell death during exercise compared with the control trial. It has been suggested that carbohydrate ingestion can reduce the cortisol response to exercise and lessen perturbations in immune cell numbers and function (25); this may be one possible method for further study of this mechanism.

**Conclusions**

This report is the first to quantify separately the effect of exercise on the response of CD4 and CD8 cells to the mitogen PHA. In the present study, expansion of these cell populations declined after exercise, without any corresponding impairment in the mitotic ability of the cells. In addition, we observed significantly higher expansion of the CD8 cell population compared with CD4 cells. The reduction in expansion appeared to be related to an exercise-associated increase in the rate of cell death of both CD4 and CD8 T lymphocytes. These data suggest that the effect of exercise in impairing in vitro cell function is principally mediated by an in-
crease in the percentage of cells that undergo cell death. Further studies are required to investigate the mechanism behind the exercise-associated increase in cell death of lymphocytes and the relevance of this observation to infection rates in elite athlete groups.

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