Exercise and T-lymphocyte function: a comparison of proliferation in PBMC and NK cell-depleted PBMC culture

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Green, Katherine J., David G. Rowbottom, and Laurel T. Mackinnon. Exercise and T-lymphocyte function: a comparison of proliferation in PBMC and NK cell-depleted PBMC culture. J Appl Physiol 92: 2390–2395, 2002.—This study utilized recently developed microbead technology to remove natural killer (NK) cells from peripheral blood mononuclear cell (PBMC) preparations to determine the effect of acute exercise on T-lymphocyte function, independent of changes in lymphocyte subpopulations. Twelve well-trained male runners completed a 60-min exercise trial at 95% ventilatory threshold and a no-exercise control trial. Six blood samples were taken at each session: before exercise, midexercise, immediately after exercise, and 30, 60, and 90 min after exercise. Isolated PBMC and NK cell-depleted PBMC were stimulated with the mitogen phytohemagglutinin. Cellular proliferation was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye uptake. In the PBMC cultures, there was a significantly lower mitogen response to phytohemagglutinin in exercise compared with the control condition immediately postexercise. There were no significant differences between the control and exercise conditions in NK cell-depleted PBMC cultures or in the responses adjusted for the percentage of CD3 cells. The present findings do not support the view that T-lymphocyte function is reduced after exercise.

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SEVERAL LINES OF EVIDENCE suggest that acute, intensive exercise bouts can increase susceptibility to upper respiratory tract infection (URTI) (29). Acute exercise can alter both the number and function of the immune cells in circulation. In particular, there are many reports of exercise-induced suppression of T-lymphocyte function as measured by responsiveness to mitogen stimulation (3, 6, 20, 30, 33, 37). T lymphocytes are a heterogeneous population of cells consisting of T-helper (CD4) and T-cytotoxic (CD8) cells. The principal role of CD8 cells is cytotoxic killing of virus-infected cells, whereas CD4 cells activate other immune cells to kill intracellular targets or produce antibody (16). Because T lymphocytes play a central role in the immune response to many pathogens, understanding their role in resisting URTIs in athlete populations is important. Despite several investigations, there is still controversy as to whether intensive exercise causes a decrease in the function of individual T lymphocytes in circulation. It has been suggested that the observed reduction in mitogen-stimulated lymphocyte proliferation assays may be an artifact of changes in the number of circulating lymphocyte subsets after exercise (7, 14, 30, 36, 39).

The main problem with interpreting traditional proliferation assay data after intensive exercise is the use of a constant number of peripheral blood mononuclear cells (PBMC) in culture. Intensive exercise causes a redistribution of lymphocyte subsets in the circulation such that relative percentages of subsets are altered, in particular the proportion of natural killer (NK) cells relative to T lymphocytes (8). Because NK cells are unresponsive to phytohemagglutinin (PHA) mitogenic stimulation in culture (40), an increased relative proportion of NK cells and a corresponding reduction in T cells result in a lower percentage of the cells in culture capable of responding. In support of this, mitogen response is positively correlated with the percentage of circulating T lymphocytes postexercise (28, 36) and negatively correlated with the percentage of NK cells in these samples (7, 14).

A number of experimental approaches have been used to adjust responses for the changes in T-lymphocyte subsets, whereby T-lymphocyte proliferation is assessed independent of numerical changes. Numerical adjustment of the mitogen-stimulated proliferation to reflect the proportion of CD3+ cells appears to eliminate the decreased response in postexercise samples. Published reports that use this approach suggest that changes in the proportion of CD3+ cells can explain alterations in T-cell mitogenic response after moderate- but not high-intensity exercise (13, 23, 30). It would, therefore, appear that, at least after moderate exercise, T-lymphocyte mitogenic response might be explained by decreases in the proportion of T lymphocytes in culture. However, as increased susceptibility is...
noted after long-duration, intense exercise, further study with the use of a direct measurement of individual cell function is warranted.

Hinton et al. (14) found that, in contrast to a PBMC suspension, isolated T cells showed no decreased response to mitogenic stimulation after intensive interval exercise. The different results seen in the intact PBMC and isolated T-cell cultures suggest that the T lymphocytes themselves are not exhibiting a reduced proliferation response. The method employed by Hinton et al. used monoclonal antibodies conjugated to magnetic microbeads to separate T lymphocytes (CD4 and CD8) from a PBMC suspension. However, this technique involved direct interaction of monoclonal antibodies with the cells of interest, and it is likely that interference with the CD4 and CD8 costimulatory molecules could influence the activation status of the cells (15, 22). Furthermore, there is considerable evidence to suggest that optimal mitogenic stimulation of T lymphocytes is dependent on intimate cell contact with accessory cells, such as monocytes (5, 18).

At present, there is sufficient evidence to suggest that exercise-induced changes in lymphocyte subsets, particularly an increased fraction of NK cells, are a likely factor contributing to decreased lymphocyte proliferation. However, the problem remains that there are few experimental protocols that yield an assessment of T-lymphocyte function independent of changes in lymphocyte subsets. Following on from the method used by Hinton et al. (14), we used the same magnetic microbead technology to remove only NK cells from PBMC preparations and assessed mitogen-induced proliferation of the “untouched” T lymphocytes after 60 min of treadmill running. The hypothesis of this study was that NK cell-depleted PBMC preparations would not show any exercise-associated decrease in mitogen-stimulated proliferation.

METHODS

Subjects. Twelve well-trained male runners who met the inclusion criteria of 2-yr training history and a current 10-km race time of ≤36 min were recruited for this study. All subjects gave their written, informed consent to participate. The University Human Research Ethics Committee of the Queensland University of Technology approved this study.

Experimental procedures. Testing was conducted in the Human Performance Laboratory at the Queensland University of Technology, Brisbane, and included four separate visits: a familiarization session, a maximal incremental exercise test, an exercise trial, and a control trial. The exercise and control trials were completed in random order.

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 (Medical Graphics CPX/D mobile cart system, Medical Graphics, St. Paul, MN). Maximum O2 consumption (VO2max) was defined as the single highest 5-s average value attained during the test coinciding with a respiratory exchange ratio >1.10. Ventilatory threshold (VT) was determined from a graph of the ventilation (VE)-to-oxygen consumption (VO2) ratio (VE/VO2) plotted against time. Threshold was defined as the inflection point of the VE/VO2 curve and was identified independently by two researchers (42).

Exercise and control trials. Subjects reported to the laboratory at 5:30 AM, after having refrained 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) was taken. For the control trial, subjects rested quietly in the laboratory, and blood samples were taken at time points corresponding to the sampling times in the exercise trial. In the exercise trial, subjects completed a standardized warm-up consisting of 5-min slow-speed running followed by stretching. At 6:00 AM, subjects began 60 min of running at 95% of VT. For the entire test, the speed of the treadmill remained constant at 14 k/h, and the gradient was adjusted to elicit a VO2 equivalent to 95% VT. 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), subjects momentarily stopped running and sat down in order for a blood sample to be taken. Blood samples were taken immediately after exercise (7:00 AM) and 30 min (7:30 AM), 60 min (8:00 AM), and 90 min (8:30 AM) after exercise (Fig. 1).

During the recovery period, subjects were seated quietly in the laboratory; over the entire testing session, subjects only consumed water.

Circulating leukocyte numbers. Full blood counts (white blood cells, lymphocytes, monocytes, and neutrophils) were obtained by standard procedures from a clinical hematology laboratory with a Sysmex Se 9000 (Roche Diagnostics, Sydney, Australia).

Lymphocyte separation and culture. 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 Chemical, 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 media (Sigma Chemical) supplemented with 2 mM glutamine (Sigma Chemical) and gentamycin (Sigma Chemical). Cell viability and cell counts were assessed by trypan blue exclusion, and a portion of the cells was resuspended in culture medium (CM; RPMI-1640; 2 mM glutamine; 10% FCS) to yield a final cell concentration of 8 × 10^6 cells/l, for total PBMC proliferation. The remaining cells were resuspended in 80 μl of RPMI media for NK cell depletion.

NK cell depletion. Isolated and washed PBMC were resuspended in 80 μl of RPMI media, and 20 μl of magnetic cell separation (MACS) CD56 microbeads (Becton Dickinson) were added. Samples were incubated for 15 min at 6°C, then

![Fig. 1. The study design. Six blood samples were taken from each subject during an exercise trial and a nonexercise control trial. The exercise trial consisted of 60 min of treadmill running between 6:00 and 7:00 AM.](http://jap.physiology.org/DownloadedFrom)
washed with RPMI, and centrifuged at 300 g for 20 min. MACS MS+ separation columns (Becton Dickinson) were placed in a MiniMACS magnet (Becton Dickinson) and prepared for separation by flushing with 500 μl of RPMI. The cell pellet was resuspended in 500 μl of RPMI and passed through the magnetized column. The column was rinsed with 3 x 500 μl of RPMI, and the effluent (NK cell-depleted PBMC) was collected into tubes containing CM. Cell suspension volume was adjusted to yield a final cell concentration of 8 x 10^6 cells/ml. Previous studies have indicated that immediately postexercise coincides with the greatest increase in the proportion of NK cells in PBMC. Therefore, samples from four individuals were analyzed to determine the proportions of NK cells in PBMC. Thus, samples from four individuals were analyzed in the control condition. Samples were labeled with CD3-FITC and CD56/CD16-phycoerythrin conjugated monoclonal antibodies (Becton Dickinson). Cells were labeled with CD3-FITC and CD56/CD16-phycoerythrin conjugated monoclonal antibodies (Becton Dickinson). Cells were labeled with CD3-FITC and CD56/CD16-phycoerythrin conjugated monoclonal antibodies (Becton Dickinson). Cells were labeled with CD3-FITC and CD56/CD16-phycoerythrin conjugated monoclonal antibodies (Becton Dickinson).

Assessment of lymphocyte proliferation. Lymphocyte proliferation was determined independently for the PBMC and NK cell-depleted PBMC preparations. In a 96-well U-bottomed microtiter plate, 200 μl of each cell suspension from each sample were cultured with either RPMI media (unstimulated) or 5 μg/ml PHA (stimulated). Previous mitogen titration determined that this was the optimal concentration of PHA. Eight stimulated replicates and eight unstimulated replicates were cultured for each sample. Cultures were incubated at 37°C in a 5% CO2 incubator (Contherm Scientific, Lower Hutt, New Zealand) for 64 h before the CM was replaced with 100 μl RPMI (without FCS) containing 10% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical) and incubated for a further 6 h (24). Acid isopropanol was added (100 μl) to dissolve the MTT crystals, and the plate was read on a multiscan plate reader (LabSystems Oy, Helsinki, Finland) at wavelengths of 492 and 650 nm. The data were expressed as the absorbance at 492-nm wavelength subtracted from the absorbance at 650 nm. Results for both the PBMC and NK cell-depleted PBMC cultures are reported as means of the eight replicates expressed as a ratio between the stimulated and unstimulated samples (proliferation index). Data were also expressed as proliferation per CD3+ cell, calculated by dividing the PBMC proliferation index by the proportion of CD3+ cells present in the sample.

Statistical analysis. All data are expressed as means ± SE. For all variables, a two-way ANOVA with repeated-measures design was conducted. Main effects of time (6 sample points) and condition (exercise and control) were fitted, as well as the interaction between time and condition. Statistical significance level was set at P < 0.05. To accommodate the repeated-measures design and avoid exclusion of subjects with partially complete data over time, missing data points were filled with mean values calculated for that variable at that sample point from other subjects’ data. In instances when a significant interaction occurred between time and condition, post hoc analysis in the form of one-way ANOVA (main effect of condition) was carried out at each time point.

RESULTS

Exercise trials. All 12 subjects (age: 30 ± 7 yr, height: 1.79 ± 0.05 m, weight: 68 ± 4 kg) successfully completed the 60-min exercise trial. During the incremental maximal test, the mean VO2 max for the group was 68 ± 4 mL·kg⁻¹·min⁻¹, and mean maximum heart rate was 187 ± 10 beats/min. The exercise trial was completed at 95% of VT, which was equivalent to 84 ± 3% of VO2 max. Mean heart rate during the exercise trial was 165 ± 10 beats/min.

Circulating leukocyte numbers. There was a significant interaction between condition (exercise and control) and time for white blood cell, lymphocyte, and neutrophil but not monocyte numbers (Table 1). For white blood cells, exercise counts were significantly higher compared with control at time points 6:30, 7:00, 8:00, and 8:30 AM. Lymphocytes exhibited a typical biphasic response to exercise as counts were elevated compared with control at 6:30 and 7:00 AM and were reduced below control levels at 8:00 and 8:30 AM. Neutrophil numbers steadily increased in the exercise condition with elevated counts at 7:00, 7:30, 8:00, and 8:30 AM.

Mitogen responses to PHA. There was a significant interaction between time and condition for the mitogen response to PHA in the PBMC cultures (Fig. 2A). Response to mitogen was significantly lower in exercise compared with the control condition at 7:00 AM (immediately postexercise). There were no significant differences between the control and exercise conditions in

| Table 1. Changes in circulating leukocyte numbers over time in an exercise condition and a nonexercise control condition |
|-----------------+-----------------+-----------------+-----------------+-----------------+-----------------+-----------------+-----------------|
|                | Condition       | 5:45           | 6:30           | 7:00           | 7:30           | 8:00           | 8:30           |     |
| White blood cells | Control         | 5.87 ± 0.30    | 5.72 ± 0.35    | 5.74 ± 0.35    | 5.68 ± 0.34    | 5.64 ± 0.31    | 5.71 ± 0.26    | <0.001 |
|                  | Exercise        | 5.73 ± 0.43    | 8.40 ± 0.60*   | 8.53 ± 0.47*   | 7.24 ± 0.76    | 8.33 ± 0.82*   | 9.03 ± 0.72*   | <0.001 |
| Lymphocytes      | Control         | 2.12 ± 0.18    | 1.90 ± 0.17    | 1.92 ± 0.16    | 1.82 ± 0.16    | 1.82 ± 0.14    | 1.83 ± 0.13    | <0.001 |
|                  | Exercise        | 2.18 ± 0.26    | 3.69 ± 0.30*   | 3.34 ± 0.22*   | 1.61 ± 0.19    | 1.23 ± 0.12*   | 1.27 ± 0.12*   | <0.001 |
| Neutrophils      | Control         | 2.82 ± 0.21    | 2.94 ± 0.21    | 3.02 ± 0.21    | 3.01 ± 0.24    | 3.01 ± 0.22    | 3.04 ± 0.19    | <0.001 |
|                  | Exercise        | 2.64 ± 0.28    | 3.78 ± 0.39*   | 4.35 ± 0.49*   | 4.85 ± 0.73*   | 6.28 ± 0.83*   | 7.10 ± 0.71*   | NS    |
| Monocytes        | Control         | 0.55 ± 0.05    | 0.57 ± 0.04    | 0.55 ± 0.04    | 0.54 ± 0.03    | 0.54 ± 0.03    | 0.52 ± 0.04    | NS    |
|                  | Exercise        | 0.61 ± 0.05    | 0.65 ± 0.05    | 0.58 ± 0.03    | 0.48 ± 0.05    | 0.56 ± 0.05    | 0.59 ± 0.03    | NS    |

Values are means ± SE in units of x 10^6 cells/ml. Exercise condition was 60-min treadmill running at 95% ventilatory threshold between 6:00 and 7:00 AM. P value relates to the interaction between condition and time for each cell type. NS, not significant. *Significant difference between exercise and control condition, P < 0.05.

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NK cell-depleted PBMC cultures (Fig. 2B) or in the responses adjusted for the percentage of CD3 cells (Fig. 2C). The preexercise and postexercise NK cell-depleted PBMC preparations contained 85.7 ± 4.6 and 88.9 ± 1.5% CD3⁺ cells and 2.5 ± 0.3 and 2.5 ± 1.4% NK cells, respectively. The average proportion of CD3⁺ and NK cells in the control condition was 84.8 ± 3.9 and 2.6 ± 0.3%, respectively.

**DISCUSSION**

Despite many reports of reduced T-lymphocyte proliferation after exercise, it is now generally accepted that traditional proliferation assays that use a mixture of peripheral lymphocytes do not provide an accurate picture of the effect of exercise on T-lymphocyte function. Although there are many reports on this topic, the effects of acute exercise on individual T-lymphocyte function and the possible clinical significance remain unknown. The major reason for inconsistent results is thought to be the large increase in nonresponding NK cells in lymphocyte preparations. Therefore, in this study, we used recently developed microbead technology in a new experimental approach and removed NK cells from PBMC preparations. We were then able to determine the effect of acute exercise on the function of a constant number of T lymphocytes. The main finding of this study was that, although the proliferative response of complete PBMC was depressed after acute, high-intensity exercise, the proliferative response of NK cell-depleted PBMC preparations was not affected. In addition, when PBMC responses were adjusted per CD3 cell, no effect of exercise was observed.

At rest, T lymphocytes typically comprise ~70–75% of the total lymphocyte pool, whereas B lymphocytes and NK cells, in roughly equal proportions, comprise the remainder (12). Acute, intensive exercise is associated with an increase in circulating numbers of all lymphocyte subtypes (8–10). However, the relative proportion of T lymphocytes decreases to 65%, due mainly to an increase in the NK cell proportion to 25–30% (11, 31). Our assay system was effective in removing the majority of NK cells (<3% remaining) and thus maintaining a constant proportion of T lymphocytes in the preexercise (85.7%) and postexercise (88.9%) samples and in the control condition (84.8%). We chose specifically to remove only NK cells from PBMC for several reasons. First, of all lymphocyte subsets, NK cells have repeatedly been shown to undergo the greatest increases in cell numbers after exercise (8). Second, there are several reports that cell-cell interaction between lymphocytes and monocytes is essential for optimal PHA-induced proliferation (4, 5, 18). In addition, many studies have reported no change or very small changes in B-lymphocyte (38) and monocyte proportions after exercise.

In the only other direct experimental approach, proliferation in cultures of pure T lymphocytes was unaffected by acute, intensive interval training, despite a reduced mitogen response in standard PBMC cultures (14). One limitation of this previous study is the possible alteration in CD4 and CD8 cell responses, resulting from the attachment of monoclonal antibody to the CD4 and CD8 receptors for the purpose of T-lymphocyte purification (15, 22). In contrast, we used monoclonal antibodies to remove NK cells from PBMC preparations so that proliferative function was assessed in only the remaining “untouched” portion of cells. Despite these methodological differences, our data are in agreement with those of Hinton et al. (14), in that we found no deleterious effect of acute, intensive exercise on T-lymphocyte function.

An alternative approach in several other studies has been to use a post hoc numerical adjustment of PBMC responses to correct for the proportion of CD3⁺ cells in culture. This approach has been found to eliminate the decline in mitogen responsiveness after short-duration, intense, and moderate exercise (27, 30, 32). How-
ever, after longer duration intense exercise, such as 60 min at 75% \( \dot{V}O_2 \text{max} \) (23) and 45 min at 80% \( \dot{V}O_2 \text{max} \) (30), the correction was incomplete, and the decline in postexercise samples was still apparent. The data from the present study, however, indicate that correction for CD3 cells completely removed the effect of exercise on T-lymphocyte proliferation.

It should be noted that, in previous long-duration, intense exercise studies, the reduction in nonadjusted responses was only observed after 60 min of recovery and not immediately postexercise as is observed in the present study and in that by Hinton et al. (14). It is possible that the assay systems used in different studies may account for an alternative time point of depression in proliferative response. The present study used reduction of MTT rather than \[^3H\]thymidine incorporation as a measure of proliferation. Despite the fact that both methods have been shown to quantify cellular proliferation and to correlate well with one another (19), there are important differences between these two methods that may explain the inconsistent results. MTT assays measure expansion of the cell population by considering the total number of cells at the end of the assay relative to the number of cells placed in the culture initially. \[^3H\]thymidine incorporation, on the other hand, only quantifies the number of newly formed cells during the period of incubation with this radiolabeled DNA precursor and does not consider the number of unproliferated parent cells present in the culture at the end of the assay (34).

The observation that individual T-lymphocyte function is not affected by long-duration, high-intensity exercise is supported by recent studies of other measures of T-lymphocyte function after exercise. The expression of CD69 (an early activation marker) on the surface of T lymphocytes has been established as a good indicator of cell function (2, 21). Ronsen et al. (35) reported no effect of long-duration, intense exercise (65 min at 70% \( \dot{V}O_2 \text{max} \)) on the expression of CD69 on CD4 and CD8 cells. This is supported by unpublished observations in our laboratory with the same 60-min exercise protocol described in the present study. Conflicting observations have been made by using a short-duration incremental exercise test to exhaustion (41), in which reduced CD69 expression was reported postexercise.

Considerable debate surrounds the interpretation of mitogen proliferation assays. One of the purposes of this study was to clarify some of the conflicting literature regarding in vitro T-lymphocyte responses to exercise. An important advance in this regard is the use of experimental techniques that provide independent assessment of T-lymphocyte function. However, this experimental approach is at the expense of a culture environment that closely resembles in vivo conditions. Consequently, several researchers have advocated the use of whole blood assays in the belief that they more closely resemble the in vivo environment (25). Even though the use of whole blood may have advantages, the combination of many cell types, the number of those cells, and potential immunomodulatory factors, all of which may influence proliferation separately, make the interpretation of individual T-lymphocyte function difficult. Other challenges with the interpretation of in vitro mitogen-induced proliferation of cells from the peripheral circulation include a restricted pool of accessible cells, namely the blood compartment, and debate surrounding the relative merits of mitogen- vs. antigen-specific responses. It has been reported that antigen-specific responses are decreased for several days after exercise (1, 17), whereas reports of mitogen responsiveness indicate that they are only transiently depressed, if at all, during the hours after exercise (26). Whereas data from the present study suggest that mitogen-induced proliferation of T lymphocytes is not reduced postexercise, it remains possible that other in vivo factors may have an immunosuppressive effect on cellular function and contribute to the reported high incidence of infection in athletes.

In summary, we have observed no change in mitogen-induced proliferation response of T lymphocytes with the use of NK cell-depleted PBMC. In combination with the responses adjusted per CD3 cell, our data indicate that 60 min of running at 95% VT (85% \( \dot{V}O_2 \text{max} \)) result in no decline in lymphocyte proliferation. The present findings do not support the view that in vitro T-lymphocyte function is reduced after exercise.

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