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Chronic exercise stress in mice depresses splenic T lymphocyte mitogenesis *in vitro*

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SUMMARY

This study investigated changes in functional response to splenic T lymphocytes of mitogens following acute and chronic exposure to endurance exercise. Splenic T cell response *in vitro* to concanavalin A (Con A) and the total number of lymphocytes per spleen were compared between mice assigned to the following treatment conditions: (a) exercise training (EX) by treadmill running (28 m/min, 8° slope for 30 min, 5 times per week for 4 weeks preceded by 2 weeks of endurance build-up), (b) exercise training as above followed by a single, acute bout of exercise to exhaustion (EX + AC) (35 m/min, 8° slope, 30 min to 2 h duration) (c) exposure to the novel environment for 6 weeks without exercise (control), and (d) exposure to the novel environment as in (c) followed by a single, acute bout of exercise to exhaustion. Treadmill running for 6 weeks significantly enhanced succinate dehydrogenase activity in skeletal muscle compared to the sedentary, control condition, and was broadly interpreted as indicative of a training effect. EX mice had significantly reduced splenic lymphocyte proliferative responses to optimal and supraoptimal concentrations of Con A compared with control animals. Incorporation of [^{3}H]thymidine into splenic lymphocytes from EX + AC mice was the most markedly depressed. Total number of lymphocytes per spleen was significantly lower in EX compared with control mice. These results suggests that chronic exercise challenge in mice is associated with T lymphocyte hyporesponsiveness in the secondary lymphoid organs, such as the spleen.

Keywords exercise training treadmill running mice spleen concanavalin A succinate dehydrogenase

INTRODUCTION

In recent years, there have been several reports on the immunological effects of acute exercise stress. In response to an acute exercise challenge, peripheral blood T lymphocytes taken from untrained adults show reduced responsiveness *in vitro* to polyclonal mitogens, such as concanavalin A (Con A) (Eskola *et al.*, 1978; Hedfors, Holm & Ohnell, 1976; Landmann *et al.*, 1984). Lymphocytosis due primarily to an expansion of circulating B cells also characterizes the response of sedentary individuals to an acute sport stress. The mechanisms underlying these immunological effects have not been clearly established, although stress-induced elevations in plasma epinephrine concentrations may be involved. Exercise acutely increases beta-adrenergic receptor density on mononuclear cells (Burman *et al.*, 1985) and beta-adrenergic activation depresses T lymphocyte blastogenesis *in*

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vitro (Crary *et al.*, 1983). The effect of chronic exercise challenge and physical conditioning on lymphocyte function is more equivocal. Peripheral blood T lymphocyte proliferation to mitogens (Watson *et al.*, 1986) and K cell activation (Hanson & Flaherty, 1981) have been reported to be enhanced in physically trained subjects following an exercise challenge at work intensities of 70 to 85% of $\dot{V}O_2$max. In contrast, thymic weights of Swiss ICR mice that were forced to swim to exhaustion for 9 consecutive days were reduced compared with rested animals (Reyes, Lerner & Ho, 1981). These findings raise the possibility that lymphoid tissues and peripheral blood lymphocytes respond differentially to chronic exercise stress.

To date, there are no data on secondary lymphoid tissue lymphocyte function during either acute or chronic exercise stress. Our studies were therefore designed to determine the direction of the effect of physical training and of acute exercise challenge on splenic T lymphocyte number and function.

MATERIALS AND METHODS

Animals. Sixty-two male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA), aged 12 weeks and weighing 16·2–17·2 g, were housed in groups of five in standard plastic cages with sawdust bedding, on a 12 h light/12 h dark cycle and at a thermoneutral ambient temperature (28 ± 1°C). Food (Ralston Purina rodent chow) and tap water were freely available to all animals throughout the study. Mice were inspected periodically for evidence of clinical disease and were excluded from the study if one or more of the following signs were present: palpable tumours, skin ulcerations, diarrhoea, cataracts, or gross changes in coat texture. Three mice were eliminated from the study through disease or death. Mice were weighed weekly (Ohaus Triple Beam Balance) over the course of the study.

Treatment groups. Mice were randomly assigned to one of five treatment conditions for the duration of the study. Group 1 ($n = 13$) had regular exercise training (EX) for 6 weeks on a treadmill, consisting of 2 weeks build-up (12 m/min to 26 m/min speed, and from 12 min to 30 min/day) and 4 weeks of training (28 m/min, 8° slope, 30 min, 5 consecutive days per week). These mice were killed 30 min after completing the final training run. Group 2 ($n = 13$) had exercise for 6 weeks (as above), followed by a 48 h rest period (EX + RE) after which the mice were killed. Group 3 ($n = 13$) had exercise as in groups 1 and 2, and then a 72 h rest period, followed by acute, exhausting exercise (35 m/min, 8° slope) with the exhaustion criterion set at continued refusal to run at the prescribed speed with repeated physical prodding (EX + AC). A minimum of 30 min exercise was attained in all cases. Mice were killed 30 min after the acute exercise bout. Group 4 ($n = 10$) was the control group (C) and received no treatment other than sham exposure to the novel environment for 6 weeks (i.e. being placed in the stationary treadmill laneway and exposed to noise and vibrations without actually running). Group 5 ($n = 10$) received the same treatment as the controls for 6 weeks followed by an acute, exhausting exercise challenge (C + AC) as in group 3. The mice were then killed 30 min after the exercise bout.

Succinate dehydrogenase. Quadriceps femoris muscle was excised from each animal at the end of the study after the mice were killed by cervical dislocation. The muscle was immediately frozen in liquid nitrogen and stored at −80°C for subsequent determination of succinate dehydrogenase (SDH) activity based upon the method of Costill *et al.* (1976); changes in the activity of SDH, an oxidative enzyme, were interpreted broadly as indicative of a training effect (Vihko, Saliminen & Rantamaki, 1979). Frozen muscle samples were homogenized (0·17 M phosphate buffer, 10% BSA, 0·35 μl 2-mercaptoethanol), 10 μl samples incubated at 37°C for 10 min, and 100 μl of reaction mixture (0·3 M phosphate buffer, 1·5 M sodium succinate, 10% BSA, 0·15% phenazine methosulphate) were added. Following incubation (37°C for 30 min), 200 μl of 1 mol/l NaOH were added together with 100 μl of brombenzol and the mixture was centrifuged (1000 *g*). The supernatant was decanted and fluorometric values obtained after addition of a reaction mixture containing 1 M hydrazine, 200 mM EDTA, and 100 mM NAD+. Thereafter, a second enzymatic reaction was initiated by adding 100 μl of fumerase and 100 μl of malate dehydrogenase. Fluorometric determination of NADH concentration was made after 2 h. The protein content of

each muscle homogenate was determined by the Lowry method as modified by Schacterle & Pollack (1973).

Splenic lymphocytes. Spleens were aseptically removed, gently teased apart with sterile forceps and 26 gauge needles, and the cells dispersed into 2 ml of RPMI-1640 supplemented with 2 mM L-glutamine, 5% heat-inactivated fetal bovine serum, 50 U penicillin/ml, and $2{\cdot}5 \times 10^{-5}$ M mercaptoethanol (herein called medium). The cells were washed once in medium, the erythrocytes lysed by brief exposure to hypotonic NaCl, dead cells and cellular debris allowed to agglutinate, and the splenocytes resuspended in medium (Mishell & Shiigi, 1980). The total number of splenic mononuclear cells was determined by counting in a haemocytometer using Turk's solution as a diluent. Cell viability was assessed by trypan blue staining and the final concentration of nucleated cells adjusted to 1×10^7 cells/ml in medium.

Mitogenesis. Lymphocytes (100 μl) were seeded in triplicate into 96-well flat-bottomed microtitre plates (Flow Labs, Mississuaga, Ontario, Canada) and polyclonal T cell proliferation was induced with 2·5 μg/ml or 4·0 μg/ml of Con A (Sigma Chemical Co., St Louis, MO, USA). These concentrations of Con A were shown to be optimal and supraoptimal, respectively, by dose response studies of murine splenic lymphocyte blastogenesis. Control cultures received an equal volume of medium (40 μl). The cell cultures were incubated for 68 h at 37°C in a humidified 5% CO_2–95% air atmosphere, pulsed for 6 h with 0·5 μCi of [methyl ^{3}H]thymidine ([^{3}H]TdR) (2·0 Ci/mmol specific activity; Amersham Canada Ltd, Oakville, Ontario, Canada), and harvested onto glass fibre filters using a semi-automated multiple microculture harvester (Skatron A.S., Lierbyen, Norway). Radiolabelled thymidine incorporation into splenic lymphocytes was measured by liquid scintillation spectrometry (Beckman LS 1801 Beta Counter) using a commercially available cocktail (Beckman HP/b, Beckman Instruments Canada, Toronto, Ontario, Canada). Incorporation of [^{3}H]TdR was expressed as the average ct/min of triplicate samples.

Statistical analysis. Lymphocyte proliferation data were analysed by a repeated measures analysis of variance (ANOVA) model with unequal n, using treatment group as the between-subjects factor and Con A level as the within-subjects factor. The nature of the treatments implied the use of four pre-planned contrasts (EX vs C; EX vs EX+RE; EX+RE vs EX+AC; C vs C+AC). Total splenic lymphocyte number and succinate dehydrogenase activity were analysed by one-way analysis of variance and the same contrasts as in the repeated measures ANOVA. A P value of 0·05 was accepted as statistically significant.

RESULTS

Training effect. Results of the exercise treatment on SDH activity in murine quadriceps femoris skeletal muscle are presented in Table 1. The main effect of treatment was significant

Table 1. Succinate dehydrogenase (SDH) activity in quadriceps femoris muscle of C57BL/6J mice following exercise training

Treatment group	n	SDH activity* (μmoles/g protein/min)
EX	13	45·1±2·2**
EX+RE	13	43·5±3·5
EX+AC	13	42·5±2·8
C+AC	10	28·5±1·5
C	10	30·0±2·4

* Group mean±s.e.m.

† $P<0{\cdot}0001$ vs C; see text for details of statistical results.

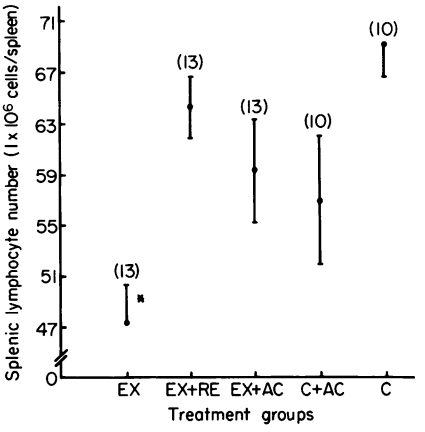

Fig. 1. Total number of lymphocytes per spleen in exercised and control (sedentary) mice. Values are group means ± s.e.m. See sample size in parenthesis. See text for explanation of treatment groups. * $P < 0{\cdot}0001$ vs C group.

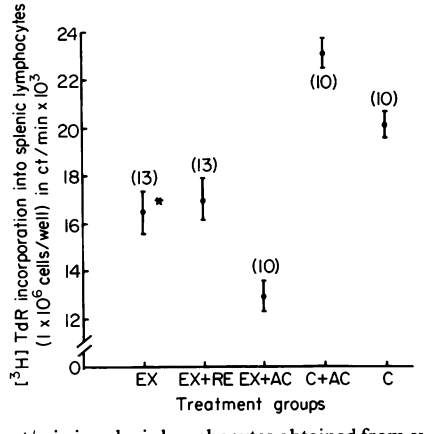

Fig. 2. [³H] TdR incorporation in ct/min in splenic lymphocytes obtained from exercised and control (sedentary) mice. Con A was added to the cell cultures (1×10^6 cells/well) in a final concentration of 2·5 μg/ml. Values are group means ± 1 s.e.m. * $P < 0{\cdot}0004$ vs C group. See text for details of the statistical analysis.

($F(4,107) = 8{\cdot}72$, $P < 0{\cdot}01$) with EX animals having a higher SDH activity than C animals ($P < 0{\cdot}0001$). SDH activity was virtually unchanged in the EX + RE and EX + AC compared with the EX group. Similarly, acute, exhausting exercise in the control mice (C + AC group) did not significantly affect mean SDH activity compared with the values obtained for the controls that were not exposed to the acute stress (C group). Final body weights of trained mice did not differ significantly from final body weights of untrained mice (22·1 ± 0·58 vs 20·9 ± 0·54 g).

Lymphocyte number per spleen. Figure 1 shows the average total number of splenic mononuclear cells per spleen of EX, EX + RE, EX + AC, C, and C + AC groups. There was a significant main effect of treatment ($F(4,107) = 5{\cdot}59$, $P < 0{\cdot}01$) with EX mice having a markedly lower number of lymphocytes per spleen compared with C mice ($P < 0{\cdot}0001$). In general, rest tended to normalize lymphocyte number in the chronically exercise-stressed animals compared with the nonrested, exercised mice ($P < 0{\cdot}0004$). Acute exercise stress in the control mice tended to marginally depress splenic lymphocyte number compared with nonstressed controls ($P < 0{\cdot}02$).

Lymphocyte mitogenesis. The effect of the various exercise treatments on splenic lymphocyte proliferation responses to Con A is shown in Fig 2 and 3. The results of the ANOVA showed a

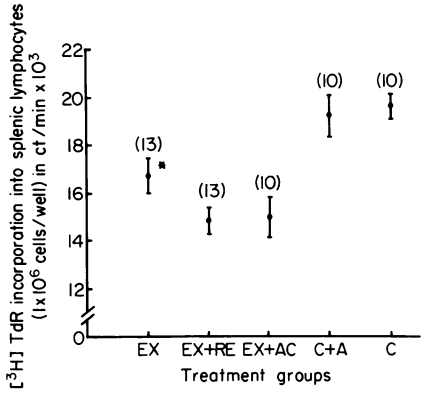

Fig. 3. [^{3}H] TdR incorporation in ct/min in splenic lymphocytes obtained from exercised and control (sedentary) mice. Con A was added to the cell cultures (1×10^6 cells/well) in a final concentration of 4·0 μg/ml. Values are group means $\pm$ s.e.m. * $P < 0{\cdot}005$ vs C group. See text for details of statistical analysis.

significant treatment effect ($P < 0.0001$), a marginally significant Con A effect ($P < 0{\cdot}05$, and a significant Con A by treatment interaction effect ($P < 0{\cdot}001$). Figure 2 shows the effect of the lower concentration (2·5 μg/ml final concentration in well) of Con A on splenic lymphocyte proliferation. Splenic lymphocytes (1×10^6 cells/well) from the chronically EX stressed mice had a reduced proliferative response to mitogen compared with the nonstressed, sedentary C mice ($P < 0{\cdot}0004$). When an acute, exhausting exercise challenge was superimposed on chronic exercise stress, a further depression in lymphocyte mitogenesis occurred ($P < 0{\cdot}0001$). Acute stress produced a significant enhancement of splenic lymphocyte proliferative capacity in the control animals.

In order to determine whether the EX effect persisted even when splenic T lymphocytes were maximally stimulated ('supraoptimal' concentration), proliferative responses were determined in the presence of 4·0 μg/ml of Con A (Fig. 3). EX mice had a significantly lower mitogenic response compared with C mice ($P < 0{\cdot}0054$), as observed at the lower mitogenic challenge. Both the control mice and the exercised mice that were given acute, exhausting exercise challenge (C + AC; EX + AC) had reduced splenic lymphocyte proliferative responses to Con A compared with their respective contrast groups, although the differences were not significant. Baseline (unstimulated) splenic lymphocyte responses (RPMI–1640) did not differ significantly among the treatment groups and ranged from 4570–7320 ct/min.

DISCUSSION

A common but unproven belief is that regular endurance exercise improves one's resistance to disease, presumably by enhancing immunological function (Godin & Shephard, 1985). Although the literature clearly demonstrates that regular endurance exercise can lead to a significant improvement in a number of indices of cardiovascular health, including heart rate at rest and during submaximal work, stroke volume of the heart, and maximal oxygen uptake (Clausen, 1977; Stromme & Ingjer, 1982), there are few reports on the effect of physical conditioning on immunological function. There are some suggestions that acute physical stress, such as exhausting exercise, with associated elevations in blood corticosteroid and catecholamine levels, may actually depress immune function and increase susceptibility to disease (Riley *et al.*, 1981).

Our results show that both chronic and acute exercise challenge in mice can modify the number and function of resident lymphocytes in the spleen. Physical training by treadmill running for 6 weeks produced a marked decline in the total number of splenocytes/spleen compared with the control condition. Chronic exercise followed by 2 days of rest ameliorated this reduction in splenic

lymphocyte number, with the total number of cells approaching that observed in the controls. Acute exercise stress in both the trained and untrained mice was also associated with lower total splenic lymphocyte numbers compared with nonstressed mice. Although the mechanisms underlying this depletion and restoration of splenic lymphocyte number are not immediately evident from our data, changes in the pattern of lymphocyte trafficking or recirculation may be involved. Lymphocytosis and leukocytosis have been reported in acutely exercise-stressed subjects (Busse *et al.*, 1980; Landmann *et al.*, 1984). Presumably a proportion of this expansion in blood lymphocyte number entails a redistribution from marginated tissue reserves such as the spleen. Autoradiography of labelled T lymphocytes should clarify this phenomenon.

The proliferative response of splenic lymphocytes to the T cell mitogen, Con A, was markedly lower in the chronically exercised mice relative to the sedentary controls. This hyporesponsiveness persisted irrespective of whether an optimal or supraoptimal concentration of stimulant was used to drive the blastogenesis. Although the absolute number of lymphocytes per spleen declined in the EX group, it is unlikely that this reduction would explain the functional hyporesponsiveness to mitogen for at least two reasons: first, all lymphocyte cultures were adjusted to an equal number of cells during the experiments and second, the hyporesponsiveness to mitogen persisted even when the total number of splenic lymphocytes returned to control values (EX+RE group). Shifts in the absolute or proportional number of T to B cells and in the T cell subsets may be involved in the exercise-related depression in tissue lymphocyte proliferative capacity.

The effect of an acute exercise stress on splenic T lymphocyte response to mitogen was bimodal, depending upon the treatment group. In trained animals, an acute exhausting exercise challenge further depressed lymphocyte mitogenesis, agreeing with earlier reports on the effects of acute stress on peripheral blood lymphocyte blastogenesis (Eskola *et al.*, 1979; Laudenlager *et al.*, 1983; Monjan & Collector, 1977). In control animals, acute exercise stress either had no apparent effect on T cell mitogenesis or was associated with an enhanced proliferative response to Con A. The reason for this lack of acute-stress effect is not known but may be related to the intensity and/or duration of physical challenge. Trained mice ran at 35 m/min for an average time of 1·5 h (range = 30 min to 2 h); untrained mice ran at the same speed for average time of 30 min to reach exhaustion. Whether a longer exposure to the exercise challenge would produce lymphocyte hyporesponsiveness to T cell mitogens remains to be determined.

The proliferative responses ot T lymphocytes to antigens and mitogens are altered in a variety of circumstances, including acute stress and chronic exposure to various drugs and hormones. For example, glucocorticoid administration (in corticosteroid sensitive species) will suppress lymphoproliferative responses (Stevenson & Fauci, 1981; Vischer, 1972). The results presented in this study show that chronic exercise stress is associated with a depression in tissue lymphocyte number and function, at least in the spleen. Whether these tissue immunological changes reflect a retrafficking of T lymphocytes from secondary lymphoid organs to the circulation (i.e., lymphocytosis of exercise; Simon, 1984) needs to be elucidated. However, these findings clearly show that immunological changes occur in lymphoid organs following chronic and acute exercise challenge; furthermore, the data point to the possibility that tissue lymphocyte changes are commensurate with but not necessarily identical to changes in blood lymphocytes with exercise stress.

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