Exercise Stress and Murine Natural Killer Cell Function (43131)

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Abstract. **Male C3He mice were trained to run on a treadmill (final speed, slope, and duration of 30 m/min, 8 degrees, 30 min/day, 5 days/week, respectively) for 10 weeks or they remained sedentary. At the end of the training program, half of the mice were sacrificed and half were given a single bout of exercise to exhaustion (50% stepwise increases in final running speed for 2-min intervals). Splenic catecholamine concentrations, splenic natural killer cell cytolytic activity against YAC-1 tumor targets, and frequency of asialo GM_1 (a murine natural killer cell surface glycolipid)-positive splenocytes were assessed. Exhaustive exercise in both trained and untrained mice reduced the *in vitro* killing of tumor targets by splenic natural killer cells relative to killing by splenocytes from mice which did not undergo the acute exercise bout ($P < 0.05$). The frequency of asialo GM_1-positive splenocytes was also reduced in the exhaustively exercised animals ($P < 0.05$). Training alone, without the additional stress of exhaustive exercise, reduced the frequency of asialo GM_1-positive splenocytes relative to a sedentary condition ($P < 0.05$), but did not compromise natural killer cell cytolytic activity against the tumor targets. Splenic epinephrine concentrations in the exhaustively exercised animals were elevated 3- to 5-fold above the concentrations observed in trained and sedentary mice. These results suggest that a single, acute exercise bout reduces the capacity of splenic natural killer cells to kill tumor targets *in vitro* and that training enhances splenic natural killer cell cytolytic activity, on a per cell basis, against tumor targets.**
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Stress is a term used to describe biologic responses to difficult situations (1). Many aspects of host resistance are affected by stress. For example, stress impairs lymphocyte proliferation to antigens and mitogens, and decreases plasma immunoglobulin concentrations and macrophage reactivity (2, 3). The mechanisms by which stress interacts with the immune system are unclear, although a neuroendocrine connection has been proposed (4, 5). Experimental evidence suggests that inability of the host to adapt to stress may result in increased susceptibility to disease, including neoplasia (6).

Natural killer (NK) cells are believed to have an important role *in vivo* in surveillance of, and resistance to, cancer (7). Natural killer cells are large, granular, major histocompatibility complex-independent non-T, non-B lymphocytes which can spontaneously lyse tumor targets (7). Murine strains which are high NK responders are more resistant to the initiation and promotion of tumor cell lines than are low NK responders (8, 9). In humans, an increased incidence of cancer is often associated with NK cell deficiencies (10, 11).

Currently, there is considerable research interest in the effects of exercise stress on the immune system (12). Exercise stress affects immune parameters such as the proliferative response of lymphocytes to mitogen stimulation (13–15) and the distribution of lymphocyte subsets in humans (16–19) and in animals (20, 21). A number of clinical studies have demonstrated an immediate and transient increase in the frequency of NK cells in peripheral blood following acute submaximal or maximal physical work (22, 23). Natural killer cell lysis of conventional tumor targets (YAC-1, K562) is increased immediately after acute exercise (24–26) and depressed 2 hr after acute physical work (27, 28). The initial enhancement appears to be greater in trained subjects compared with untrained subjects (29). A recent report documented higher NK cell activity and number in trained men compared with untrained men (30). Whether this postexercise increase in NK cell cytotoxicity reflects numerical shifts in NK number

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due to "wash-out" and demargination consequent to increased blood flow with exercise or recruitment from tissue reservoirs is not presently known. Similarly, the impact of training (apart from immediate exercise effects) on NK cell parameters have not been well studied.

The purpose of this study was 2-fold. First, we wanted to determine the impact of training on natural killer cell immune parameters since there is some evidence that training reduces the growth of tumors *in vivo* (31) and natural killer cells are an important defense mechanism against cancer (9, 32). Second, we asked whether training synergized with or antagonized the reduction in natural killer cell function observed following an acute exercise bout. The spleen was selected as the target lymphoid organ since it is highly susceptible to "stress" effects and, after the blood, is the organ exhibiting the highest NK cell activity.

Methods

Animals. Fifty male C3He mice (Charles River, St. Constant, Quebec, Canada), age 35 days and initially weighing 20.4–25.7 g were maintained on a 12-hr light/12 hr dark cycle and under thermoneutral temperatures (28 ± 1°C). The mice were housed in groups of five and were allowed *ad libitum* access to food (Ralston Purina rodent chow) and tap water. The animals were weighed weekly and were examined regularly for signs of disease and injury. All animals were acclimated to the laboratory conditions for 2 weeks prior to the experimental treatment.

Treatment Groups. The mice in each cage were randomly assigned to one of five treatment conditions. The exercise protocol, similar to that previously established in our laboratory (19, 20), consisted of training on a motorized treadmill (Collins Treadmill, Braintree, MA) for 10 weeks. An initial accommodation period of 2 weeks (build up from 12 to 30 m/min, at a 0-degree slope, with a duration increasing from 12 to 30 min/day, 5 days/week) was followed by 10 weeks of training at 30 m/min, with a final grade of 8 degrees; the mice were run for 5 consecutive days/week. When necessary, mice were gently prodded with a test tube brush. We considered this method of encouraging the mice to run to be less stressful than the electric shock treatment often described in the literature.

One group of mice (TR) was trained according to the protocol described above and was sacrificed 72 hr following the final training session. This end point was used because it is long enough to reduce possible confounding effects of exercise, but short enough so that the effects of training are still evident. A second group of mice was trained as described above (TR + EX), allowed to rest for 72 hr after the final training session, and subsequently exercised to exhaustion. The protocol for the exhaustive run consisted of a treadmill run beginning at 15 m/min, 0-degree slope with increases of slope (2 degrees) and/or speed (2 m/min) every 2 min. The criterion for exhaustion was refusal to run at the set speed and slope despite gentle prodding with a test tube brush. Final treadmill speed and slope for this group were 32 m/min and 12 degrees, respectively. These mice were sacrificed 30 min after the exhaustive run. A third group of mice (C) was exposed to the noise and novelty of the treadmill 30 min a day, 5 days a week for 12 weeks without actual exercise. A fourth group of mice (C + EX) was treated in the same manner as C for 12 weeks, following which the animals were exercised to exhaustion following the procedure described above. Final treadmill speed and slope for this group were 30 m/min and 10 degrees, respectively. A fifth group of mice served as naive controls (NC) and received no treatment other than routine care and weekly weighing; this control group was used to separate out background stress (handling, novelty, etc.) which the mice in the treatment groups may have experienced.

All mice were sacrificed by cervical dislocation between 8 and 11 AM. Spleens were immediately weighed and a tissue sample was frozen at −80°C for subsequent catecholamine determinations. Bilateral quadriceps femoris muscles were removed, immediately placed in liquid nitrogen, and stored at −80°C for succinate dehydrogenase (SDH) analysis.

Spleen Cell Suspensions. The spleen cells were dispersed into petri dishes by disrupting the outer membrane and pressing the spleen through fine gauze fabric with a syringe. The spleen cell preparations were initially suspended in 1.5 ml of medium (RPMI 1640; Gibco Canada, Burlington, Ontario) supplemented with 2 m*M* L-glutamine, 10% heat-inactivated fetal bovine serum, 50 units of penicillin/ml, 2.5×10^{-5} *M* 2-mercaptoethanol, and 10 m*M* Hepes buffer. Following removal of debris, the cells were washed with Hanks' balanced salt solution (Gibco Canada), centrifuged at 300*g* for 10 min, and then treated with 1 ml of 0.16 *M* NH_3HCl for 3 min in order to lyse the red blood cells. Following two additional washes with Hanks' balanced salt solution, the cells were resuspended in complete medium and counted in a hemocytometer using Turk's solution as a diluent. The spleen cells were suspended to a concentration of 1.5×10^7/ml for use in the cytotoxicity assay and to 2.0×10^7/ml for indirect immunofluorescence staining.

Target Cells. Target cells for the cytotoxicity assay were YAC-1, a murine T cell lymphoma, obtained from Dr. A. Croy, Biomedical Sciences, University of Guelph, and from the American Type Culture Collection, Rockville, MD (33). These cells were maintained in culture in medium at a starting concentration of $1–2 \times 10^5$/ml in 10 ml of medium which was changed daily. Cell viability, determined by trypan blue exclusion staining, was >90%. On assay days, the cells were centrifuged (300*g* for 10 min) and resuspended to a

concentration of 1×10^7/ml. Five hundred microliters of the cell suspension were incubated with 500 μl of 1.0 mCi/ml ^{51}Cr (New England Nuclear Research Products, Dupont Canada Inc., Pointe Claire, Quebec) for 90 min at 37°C in a 5% CO_2 atmosphere. The cells were washed twice in 5 ml of medium, resuspended to a concentration of 1×10^5/ml, and kept on ice until plating.

Cytotoxicty Assay. A standard ^{51}Cr release assay was carried out (34). Splenocytes were seeded into 96-well V-bottomed microtiter plates (Linbro, Flow Laboratories, McLean, VA) to give effector to target (E:T) ratios of 150:1, 75:1, 37.5:1, and 18.25:1. Each E:T ratio was carried out in triplicate. One-hundred microliters of ^{51}Cr-labeled YAC-1 lymphoma target cells were added to each well and the final volume was adjusted to 200 μl. The plates were centrifuged at 100*g* for 1 min and subsequently incubated for 4 to 5 hr at 37°C in a 5% CO_2 atmosphere. Following the incubation, the plates were centrifuged at 400*g* for 10 min, and 100 μl of the supernatant from each well were sampled for radioactivity. The amount of radioactivity from each well was determined by counting in a gamma counter (Beckman Gamma 5500). Total release was determined by counting the amount of radioactivity present in 50 μl of the labeled YAC cell suspension and spontaneous release determined by incubation of 100 μl of target cells with 100 μl of medium. The percentage of specific ^{51}Cr release was calculated using the following formula:

$$\% \text{ lysis} = \frac{(\text{cpm experimental release}) - (\text{cpm spontaneous release})}{(\text{cpm total release}) - (\text{cpm spontaneous release})}$$

Immunofluorescence. The frequency of splenocytes positive for the NK marker asialo GM_1 ($ASGM_1$) (35) was determined by indirect immunofluorescence. Fifty microliters of cell suspension (1×10^6 cells) were incubated with 5 μl of rabbit $ASGM_1$ (Cedarlane Laboratories, Hornby, Ontario) in 45 μl of counting medium (RPMI 1640, 2% fetal bovine serum, and 0.1% sodium azide) for 45 min at 4°C. The cells were washed once with 1 ml of counting medium and subsequently incubated with 100 μl of a 10 μg/ml solution of goat anti-rabbit IgG complexed to fluorescein isothiocyanate (Cedarlane Laboratories) for a further 45 min at 4°C. Following a wash with 1 ml of counting medium, the cells were resuspended in 2 g% paraformaldehyde and stored in the dark at 4°C. The lymphocytes were scored for immunofluorescence by counting a minimum of 200 cells within 2 weeks of staining.

Biochemical Analyses. Training was verified by determining the levels of SDH activity in quadriceps femoris muscle using the method of Costill *et al.* (36) as previously described by our laboratory (15, 20). Splenic tissues were analyzed for catecholamines following homogenization and extraction with alumina. The extracted samples were washed with distilled water, resuspended in perchloric acid, and catecholamine concentrations determined by reverse phase ion-pair high-performance liquid chromatography. Splenic and muscle tissues were homogenized and analyzed for protein content using the Lowry method (37).

Statistical Analysis. The data were analyzed by analysis of variance using the general linear models procedure (38). Differences between means of treatment groups for mouse weights, spleen weights, NK cell frequencies, and percentage of specific lysis were determined post hoc with the Student-Newman-Keuls multiple range test. Differences for SDH and catecholamine concentrations between trained and untrained animals were determined by one-way analysis of variance. Statistical significance was set at a probability (P) value of 0.05.

Results

Effects of Training and Exhaustive Exercise. Treadmill training for 12 weeks did not result in significant differences in final body weights between trained and untrained mice (30.2 ± 0.7 g vs 29.2 ± 0.3 g, respectively). There was, however, a significant effect of training on spleen weights; splenic wet weights from trained mice were significantly heavier (0.102 ± 0.002 g) than those from untrained mice (0.088 ± 0.005 g) ($F_{1,63} = 6.64$, $P < 0.01$).

A significant effect of training ($F_{1,44} = 9.72$, $P < 0.005$) was demonstrated by the higher levels of SDH in quadriceps femoris muscle of trained mice (111.1 ± 8.1 μ*M* NADH/g protein/min) compared with that of untrained mice (72.3 ± 3.0 μ*M* NADH/g protein/min). These results are consistent with those reported previously from our laboratory (14, 15, 20). Evidence of a training effect was also demonstrated in the length of time necessary to run the mice to exhaustion. Untrained mice (C + EX group) were run to exhaustion in 64 ± 2 min compared with 86 ± 2 min for the trained mice (TR group) ($P < 0.001$).

Results of the analyses of splenic tissue for catecholamines are presented in Table I. There were no significant effects of treatment on splenic concentrations of norepinephrine. In contrast, exhaustive exercise resulted in significantly higher concentrations of splenic epinephrine ($F_{4,31} = 56.40$, $P < 0.0001$) in both trained and untrained mice compared with tissue from mice in other treatment groups.

NK Cell Cytotoxicity. The results of the ^{51}Cr release assay for all E:T ratios are presented in Figure 1. The nature of the results were similar for all E:T ratios; therefore, only the results for an E:T ratio of 150:1 will

Table I. Splenic Catecholamine Concentrations for Mice as a Function of Exercise Treatment[a]

Treatment groups	Epinephrine (pg/mg protein)	Norepinephrine (pg/mg protein)
Naive control (NC)	10.4 ± 1.2b (8)	624.3 ± 75.7b (8)
Control (C)	10.9 ± 1.3b (9)	499.5 ± 55.2b (9)
Control + exhaust (C + EX)	33.0 ± 3.0c (8)	650.8 ± 87.6b (8)
Trained (TR)	11.7 ± 1.2b (8)	570.5 ± 106.9b (8)
Trained + exhaust (TR + EX)	54.0 ± 2.2d (8)	870.3 ± 56.2b (8)

[a] The values are mean ± SE. The numbers in parentheses are the number of mice per treatment. Values with different letters are significantly different ($P < 0.05$) as determined by the Student-Newman-Keuls multiple range test.

Table II. Frequencies (%) of Splenocytes Positive for Asialo GM_1 as a Function of Exercise Treatment[a]

Treatment groups	Frequency (%)
NC	14.8 ± 1.4b (10)
C	15.5 ± 0.9b (10)
C + EX	12.0 ± 1.2c (10)
TR	9.5 ± 0.8c (9)
TR + EX	11.0 ± 0.7c (10)

[a] The values are mean ± SE. The numbers in parentheses are the number of mice per treatment. Values with different letters are significantly different ($P < 0.05$) as determined by the Student-Newman-Keuls multiple range test. Frequency refers to the percentage of $ASGM_1$-positive splenocytes determined by fluorescent microscopy of 200 cells per subject.

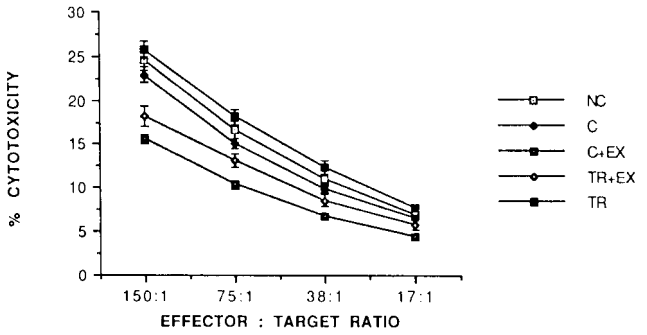

Figure 1. Effects of exercise treatment on NK cell cytotoxic activity at various effector to target ratios. Percentage of cytotoxicity refers to the percentage of YAC-1 target cells killed in a standard ^{51}Cr release assay. NC, naive controls; C, sham exercise control; C + EX, control plus exhaustive bout of exercise; TR + EX, trained (10 weeks of treadmill exercise plus exhaustive bout of exercise); TR, trained (10 weeks of treadmill exercise). Each point represents the mean ± SE.

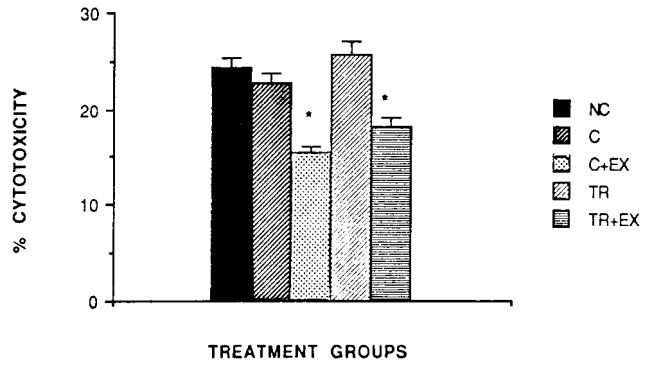

Figure 2. Cytotoxic activity of murine splenocytes at an effector to target ratio of 150:1 as a function of exercise treatment. Percentage of cytotoxicity refers to the percentage of YAC-1 target cells killed in a standard ^{51}Cr release assay. Each bar represents the mean ± SE. Treatment means are significantly different for exhaustively exercised mice (TR + EX; C + EX) compared with both trained (TR) and untrained (C and NC) mice, * $P < 0.05$.

be discussed. The results for an E:T ratio of 150:1 are presented in Figure 2. A significant effect of exercise treatment ($F_{4,44} = 20.65$, $P < 0.0001$) was noted; mean values for NK cell cytolytic activity for splenocytes from acutely exercised mice (both TR + EX and C + EX) were significantly lower than for the other groups ($P < 0.05$). NK cell cytolytic activity of splenocytes from trained mice was not different from the sedentary control groups (NC and C).

NK Cell Frequencies. The results of the indirect immunofluorescence staining are presented in Table II. There was a significant effect of exercise treatment ($F_{4,44} = 5.46$, $P < 0.001$); mean percentages of cells positive for $ASGM_1$ were significantly lower for groups of mice which received exercise treatment, irrespective of whether the exercise was acute or chronic in nature ($P < 0.05$).

Discussion

The present results show that acute exhaustive exercise in mice reduced the *in vitro* capacity of splenic NK cells to lyse tumor targets. This detrimental effect of exhaustive exercise occurred in both trained and untrained mice. Training prior to a single session of exhaustive exercise conferred only marginal protection against the reduction in NK cell killing ability observed in untrained, exhaustively exercised animals. The mechanisms accounting for this reduced NK cell lysis of tumor targets are likely to be complex. Plasma and tissue concentrations of several hormones, such as catecholamines (epinephrine and norepinephrine), pituitary hormones (ACTH and β-endorphin), and prostaglandins, are modified by exercise (39) and these hormones affect the behavior of lymphoid cells. Epinephrine, for example, has both stimulatory and inhibitory effects on NK cell cytotoxicity depending upon whether epinephrine is administered *in vivo* (40) or *in vitro* (24) and the physiologic concentration of this hormone. Glucocorticoids reduce NK cell cytotoxicity at physiologic (10^{-6} M) to suprapharmacologic (10^{-4} M) concentrations by inhibiting adhesion of the NK effector cells to the tumor targets (41). In this present study, splenic tissue concentrations of

epinephrine were significantly elevated in the acutely exercised mice and we have previously reported an elevation in corticosterone levels in this strain (15). To determine whether the elevated catecholamines and glucocorticoids were directly involved in the observed reduction in splenic NK cytolytic activity, it would be necessary to test whether inhibition or abrogation of the exercise induced hormone responses enhance NK cell cytotoxicity (e.g., by chemical sympathectomy).

Not surprisingly, the effects of acute exercise stress on the cytolytic capabilities of murine splenocytes are similar to those reported for other acute stressors in which activation of the pituitary-adrenal axis occurs. Mice exposed to conditions such as rotation stress (42), electric shock (43), and surgical stress (44) exhibit decreased NK cell cytotoxicity. Although factors such as novelty and unpredictability may be involved in the reduced NK cell cytotoxicity seen in acutely exercised mice, prior acclimation of 12 weeks to the treadmill environment tends to mitigate against such psychologic factors playing a major role. Nevertheless, it is possible that untrained mice given the exhaustive exercise protocol were experiencing greater psychologic stress than the trained mice.

The changes observed in splenic NK cell cytotoxicity after acute exhaustive exercise may reflect concurrent reductions in the number of NK cells available for killing. Exhaustively exercised mice had reduced frequencies of $ASGM_1^+$ splenocytes relative to sedentary controls which were not given this protocol. The reduction in the frequency of $ASGM_1^+$ splenocytes is consistent with the well-documented observation of increased frequencies of NK cells in the circulation after maximal and submaximal work in man (16, 25, 45). An important caveat must be considered, however; although the $ASGM_1$ glycolipid is found predominantly on NK cells (in the mouse), it is not an exclusive NK cell marker. For example, a small proportion (2%) of splenic macrophages are $ASGM_1$ positive (46). Thus, without additional concurrent NK cell enumeration procedures, it is not possible to conclude that the reduced frequency of $ASGM_1^+$ splenocytes was definitively synonymous with a reduction in NK cells. Although the physiologic significance of $ASGM_1$ is not definitively known, there is some evidence that expression of this surface marker is indicative of a $G_0 \rightarrow G_1$ transition in the cell cycle and early activation events leading ultimately to the induction of immune effector functions (47). Thus, changes in the frequency of $ASGM_1$ cells which occur with exercise and training could have important implications for the generation of natural immune effector mechanisms.

An important observation in this study was that 10 weeks of treadmill training, independent of acute exercise effects, resulted in a small enhancement of splenic NK cell lysis of tumor targets compared with sedentary controls (25.8 ± 1.2% vs 22.9 ± 0.9%). Although this enhancement by itself was not significant, on a per cell basis (i.e., when frequency of $ASGM_1^+$ splenocytes is considered), the cytolytic activity was markedly increased in the trained mice compared with sedentary controls. This finding is in agreement with a recent study by Pedersen *et al.* (30) showing that human blood NK cell lysis of K562 tumor targets is higher in trained relative to untrained men. In contrast, Watson *et al.* (48) reported reduced *in vitro* blood NK cell lysis of tumor targets in previously untrained men undergoing a 15-week walk-jog-run training program. To our knowledge, this is the first report of training modulating NK cell activity and number in a body compartment other than the circulation.

Several possible explanations arise for the apparent increase in splenic NK cell killing of tumor targets in trained individuals. The increased NK cell cytotoxicity may be an artifact of changes in the recirculation patterns of lymphoid cells. Rat large granular lymphocytes, which mediate most endogenous natural killer cell activity, have been shown to recirculate primarily between the lungs, spleen, and circulation (49). Depending upon the extent of training-induced changes in lung blood flow, NK numbers could fluctuate accordingly in lymphoid compartments such as the spleen. Such fluctuations in NK cell numbers may not be limited to lymphoid compartmental shifts. There is some evidence (50) that exercise-induced injury to skeletal muscle, such as might occur with resisted muscle lengthening during deceleration, is associated with lymphoid and NK cell infiltration. Thus, it may be that the reduction in the percentage of $ASGM_1^+$ cells in the spleen reflects recruitment to other sites where immune effector functions are "needed." Alternatively, training may activate NK cells to kill for a longer time or may increase the sensitivity of NK cell receptors for a variety of immunopotentiating hormones and lymphokines (such as interferon). We are unaware of any studies that have compared the kinetics of NK cell lysis or receptor densities on NK cells in trained versus untrained animals or humans. Finally, it may be that the exercise-induced changes in tumor killing reflect increasing tumoricidal activity of macrophages. Macrophages were not removed from the splenic cell cultures and theoretically could be involved in the lysis of the YAC-1 lymphoma tumor targets *in vitro*. At least two factors mitigate against the enhanced killing being due to macrophage activation, however: (i) macrophages are effective at tumor cytolysis after *in vitro* activation by such agents as concanavalin A supernatants, other lectins, and lipopolysaccharide (46), and our cultures were not pretreated with these macrophage-stimulating agents and (ii) macrophage cytolytic capacity against *in vitro* tumor targets, as measured by antibody dependent

cellular cytotoxicity, is not different between exhaustively trained and untrained mice (51).

In this study, the trained mice had higher splenic wet weights than the controls. It is unlikely that this increase in spleen weight was due to immigration of other lymphocyte populations. Eight weeks of exercise training in this mouse strain was associated with a decrease in the percentage of B (Ig+) lymphocytes and no net change in total T lymphocyte percentage relative to controls (20). Rather, the higher splenic weights may be due to an influx of red blood cells to be processed for destruction. During exercise and with training, the increased metabolic demands for oxygen by skeletal muscles are met by sympathetic splenic contraction with the release of red blood cells into the circulation (i.e., the reservoir function of the spleen). After cessation of exercise stress, damaged red blood cells are shunted to the spleen for destruction and the surplus red blood cells (released with exercise) are again stored in the splenic pulp and venous sinuses.

Do exercise and training-associated changes in natural killer cell number and function have physiologic significance? For the most part, acute exercise effects on natural killer cell parameters are extremely transient (27) and are likely to be without major significance for host defense. In contrast, it is not clear what are the long-term training effects on natural killer cell mechanisms of natural immunity. Limited research in rodents suggests that moderate exercise training increases resistance to experimental tumors (31, 52, 53). Such observations tend to suggest that training might upregulate antineoplastic natural immune mechanisms, such as NK cell killing capacity. However, such causal links among exercise training, natural killer cell function, and tumor growth have not been established clinically or experimentally. Clearly, further work is required to assess the interaction among these factors.

Results of this experiment demonstrate that exercise modifies murine splenic natural killer cell lysis of tumor targets and natural killer cell number. The direction (reduction or enhancement) of these effects is dependent upon whether the exercise is acute or chronic. These results raise important questions about physiologic homeostasis in the immune system and the role of exercise in host resistance to cancer.

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