Role of lung macrophages on susceptibility to respiratory infection following short-term moderate exercise training

E. A. Murphy,1 J. M. Davis,1 A. S. Brown,1 M. D. Carmichael,1 N. Van Rooijen,3 A. Ghaffar,2 and E. P. Mayer2

1Department of Exercise Science, Arnold School of Public Health, and 2Department of Pathology and Microbiology, School of Medicine, University of South Carolina, Columbia, South Carolina 29208; and 3Department of Cell Biology and Immunology, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands

Submitted 27 April 2004; accepted in final form 4 August 2004

Murphy, E. A., J. M. Davis, A. S. Brown, M. D. Carmichael, N. Van Rooijen, A. Ghaffar, and E. P. Mayer. Role of lung macrophages on susceptibility to respiratory infection following short-term moderate exercise training. Am J Physiol Regul Integr Comp Physiol 287: R1354–R1358, 2004. First published August 12, 2004; doi:10.1152/ajpregu.00274.2004.—Moderate exercise training is associated with a decreased risk for upper respiratory tract infection in human and animal studies, but the mechanisms have not been elucidated. Lung macrophages play an important role in resistance to respiratory infection, and moderate exercise can enhance macrophage antiviral resistance, but no studies have directly tested the role of lung macrophages in this response. This study tested the effect of lung macrophage depletion on susceptibility to infection following short-term moderate exercise training. Mice were assigned to one of four groups: exercise (Ex) and resting controls (Con) with and without clodronate encapsulated liposomes (CL2MDP-lip). Ex mice ran for 1 h on a treadmill for 6 days at 36 m/min, 8% grade. Fifteen minutes following exercise or rest on the last day of training, mice were intranasally inoculated with a standardized dose of herpes simplex virus type 1. Clodronate (Ex-CL2MDP-lip and Con-CL2MDP-lip) or PBS liposomes (Ex-PBS-lip and Con-PBS-lip) (100 μl) were intranasally administered following exercise or rest on the 4th day of training and again on the 4th day postinfection. Morbidity, mortality, and symptom severity were monitored for 21 days. Exercise decreased morbidity by 36%, mortality by 61%, and symptom severity score on days 5–7 (P < 0.05). Depletion of lung macrophages negated the effective benefits of moderate exercise. This was indicated by no differences between Ex-CL2MDP-lip and Con-PBS-lip in morbidity (89 vs. 95%), mortality (79 vs. 95%), or symptom severity score. Results indicate that lung macrophages play an important role in mediating the beneficial effects of moderate exercise on susceptibility to respiratory infection.

It has been hypothesized that moderate exercise may increase the activity of various immune cell parameters and thus decrease the risk for infection, whereas intense exercise decreases the activity of these same parameters and increases the risk of infection (2, 3, 7–9, 18, 21, 25–27, 29). Most of the infection data come from human studies using self-reported sickness logs, but recent evidence from controlled experimental studies in animals confirms the effects of both moderate (9) and exhaustive exercise (7, 8) on the risk of upper respiratory tract infection (URTI). However, the strength of evidence supporting this hypothesis continues to be questioned due to a lack of systematic studies designed to determine the precise etiology of such a relationship.

Alterations in susceptibility to infection have been associated with changes in various immune cell parameters, especially those of the innate immune system. For example, exhaustive exercise has been shown to decrease macrophage antiviral resistance (7, 8), antigen presentation (4), and antigenspecific cytokine response to induced URTI (16), as well as natural killer cell cytotoxicity (16). Moderate exercise has been associated with an increase in macrophage antiviral resistance (9), macrophage chemotaxis, adherence, oxidative metabolism and phagocytic activities (10, 30), an enhanced antigen-specific cytokine response (17), as well as increased natural killer cell activity (27, 39). However, there are no studies that directly test the specific role of any of these immune system alterations as mediators of exercise-induced changes in risk of infection.

Our laboratory’s (9) recent work has focused on lung macrophage resistance to herpes simplex virus type 1 (HSV-1) infection as a mechanism of an exercise effect on infection following intranasal inoculation with HSV-1. Macrophages act as a first line of defense in eliminating viral pathogens by inhibiting virus growth within itself, as well as to inactivate extracellular virus, suppress virus replication in adjacent cells, and destroy infected cells (22, 40). Although studies show a good association between exercise-induced alterations in macrophage resistance to HSV-1 and risk of infection following intranasal inoculation with HSV-1 (7–9), a direct test of the role of macrophages in this response has not been done. Depletion of tissue macrophages with clodronate-filled liposomes (CL2MDP-lip) has been used for this purpose in other infection models (34, 35), but not in exercise studies. The CL2MDP-lip-mediated macrophage suicide approach is especially advantageous for this purpose, because it can selectively eliminate macrophage function at various sites of the body, depending on the route of administration (35). After phagocytosis of the CL2MDP-lip, the phospholipid bilayers of the liposomes are disrupted, and clodronate is released into the cytoplasm, which causes apoptotic cell death of macrophages (36).

The purpose of this study was to test the effect of lung macrophage depletion on the benefits of repeated daily moderate exercise on susceptibility to URTI. This was done by using a murine model of exercise and respiratory infection involving intranasal inoculation with HSV-1 (2, 7–9). The...
exercise protocol consisted of 6 consecutive days of treadmill running [1 h/day at a relative intensity of ~75–90% maximal O2 consumption (V\textsubscript{O2,max})] designed to mimic a short period of moderate exercise training. Fifteen minutes following the last session of exercise or rest, mice were intranasally inoculated with a standardized dose of HSV-1 and monitored for morbidity, mortality, and symptom severity.

**METHODS**

**Mice.** Male CD-1 mice, 4 wk of age, were purchased from Harlan Sprague-Dawley Laboratories and acclimated to our facility for at least 3 days before any experimentation. Mice were purchased as pathogen-free stock, and periodic screening of sentinel mice yielded negative results for common murine viral or bacterial pathogens. Mice were housed four per cage and cared for in the animal facility at the University of South Carolina Medical School. Mice were maintained on a 12:12-h light-dark cycle in a low-stress environment (22°C, 50% humidity, low noise) and given food (Purina Chow) and water ad libitum. All experiments were performed at the end of the active dark cycle.

**Treadmill acclimation and exercise protocol.** The University’s Institutional Animal Care and Use Committee approved the protocol described. Exercise (Ex) mice [Ex-CL\textsubscript{2}MDP-lip and Ex-PBS-filled liposomes (PBS-lip)] were acclimated to the treadmill for a period of 20 min/day for 3 consecutive days at 18 m/min and 5% grade. The exercise protocol consisted of a 1-h bout of treadmill running (performed in the morning, 7 AM) for 6 consecutive days. Mice ran on the treadmill (2 per lane) at a speed of 36 m/min and a grade of 8%, which is estimated to elicit ~75–90% V\textsubscript{O2,max}, assuming a V\textsubscript{O2,max} of 173–206 ml·kg\textsuperscript{-1}·min\textsuperscript{-1} for mice (12, 31). Male CD-1 mice are capable of running for ~2.5 h at this exercise intensity in our hands. This exercise protocol was used because we have recently shown a beneficial effect of this model of short-term moderate exercise training on susceptibility to infection compared with our laboratory’s previous study (7) that showed no effect of a single 30-min session of moderate exercise on URTI, which suggests that multiple sessions of moderate exercise may be required to enhance resistance to infection in this model. Electric shock was not used in these experiments as mice readily respond to a gentle tap of the tail or hindquarters, encouraging them to maintain pace with the treadmill. Mice in the control (Con) groups (Con-CL\textsubscript{2}MDP-lip and Con-PBS-lip) remained in their cages in the treadmill room throughout the exercise bouts. These mice were exposed to similar handling and noise in an attempt to control for extraneous stresses that may be associated with the environment of the treadmill room. Con mice were deprived of food and water during the exercise sessions.

**CL\textsubscript{2}MDP-lip administration.** Clodronate was a kind gift from Roche Diagnostics (Mannheim, Germany). It was encapsulated in liposomes, as described earlier (35). CL\textsubscript{2}MDP-lip were administered to mice 2 days before infection with HSV-1 (following day 4 of exercise), as well as on day 4 postinfection, to deplete lung macrophages. Lung macrophage depletion was done via intranasal administration: mice received 100 μl of CL\textsubscript{2}MDP-lip. This procedure has been shown to eliminate almost all of lung macrophages (32). Some macrophages begin to return after a period of 5 days, but complete repopulation does not occur until around day 18 (32). Mice were briefly anesthetized by using halothane for this procedure. Macrophage-intact mice received similar doses of PBS-lip. CL\textsubscript{2}MDP-lip are engulfed by macrophages via endocytosis. After disruption of the phospholipid bilayers of the liposomes under the influence of the lysosomal phospholipases in the macrophage, clodronate, which is dissolved in the aqueous compartments between the liposomal bilayers, is released into the cell. The clodronate is accumulated intracellularly, and, after exceeding a threshold concentration, the cell is irreversibly damaged and dies by apoptosis (36).

**In vivo titration of HSV-1.** Intranasal inoculation of HSV-1 VR strain in the mouse is an established experimental model of respiratory infection (2, 7–9, 23). Although HSV-1 is not a common respiratory virus in humans, it can cause various pathological conditions in humans, such as meningoencephalitis, hepatitis, esophagitis, tracheobronchitis, and pneumonia, as well as being associated with cases of acute respiratory distress syndrome (23). The intranasal route of administration was chosen to mimic the typical route of entry for viral infection. HSV-1 was propagated on Vero cells; the cells were harvested when viral cytopathic effects were present on all cells. The cells were freeze-thawed and then centrifuged at 2,000 g for 20 min. The supernatant was divided into aliquots and stored at −70°C in medium supplemented with 10% fetal bovine serum and 2% penicillin, streptomycin, and l-glutamine. The virus titer was evaluated by a standard plaque assay and determined to be 8.2 × 10\textsuperscript{6} plaque-forming units/ml. The virus was titrated in vivo by administering 50 μl of various stock viral dilutions to additional mice in an initial experiment to determine the lethal dose. Morbidity, mortality, and symptom severity were monitored for 21 days.

**Intranasal infection with HSV-1.** On the day of the experiment, mice (n = 17–20 per group) were exposed to either control treatment or exercise for 1 h. Immediately following exercise or control treatment, mice were returned to their cages. Fifteen minutes later, mice were lightly anesthetized with halothane and inoculated intranasally with 50 μl of HSV-1 VR strain. The dose yielded an 80% mortality rate among Con mice in preliminary dose-response experiments. The actual dose of this virus was determined to be 2.1 × 10\textsuperscript{7} plaque-forming units/mouse. The pathogenesis and symptomatology of infection following intranasal inoculation of HSV have been well characterized (2, 7–9, 23). Following infection, the mice were returned to their respective cages and housed in an isolated P2 facility. All animals were monitored twice daily for a period of 21 days for signs of morbidity, mortality, and symptom severity by an investigator blinded to the treatments. Several typical symptoms of illness were included in the symptom severity scale (see Table 1), including ruffled fur; redness around the eyes, nose, or mouth; hunched back; and decreased activity. Mice that displayed any of these symptoms were considered moribund.

**Statistical analysis.** Statistical analyses were performed by using commercially available statistical packages from the SAS system (version 8.2, SAS Institute, Cary, NC) and SigmaStat (version 2.03, SigmaStat, SPSS, Chicago, IL). Differences in morbidity and mortality between groups across the 21-day postinfection period were determined by using a Lifetest Survival Analysis program in SAS (P < 0.05). Differences in symptom severity were compared by using a Kruskal-Wallis analysis of variance on ranks with Dunn’s post hoc analysis (P < 0.05).

**Table 1. Symptom severity scale for herpes simplex virus type 1 infection**

<table>
<thead>
<tr>
<th>Eyes</th>
<th>0—no signs, normal</th>
<th>1—sore red eyes</th>
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<tr>
<td>Lesions</td>
<td>0—none</td>
<td>1—lesion on head</td>
</tr>
<tr>
<td>Fur</td>
<td>0—well groomed</td>
<td>1—ruffled fur</td>
</tr>
<tr>
<td>Neurological/neuromuscular</td>
<td>0—normal movement</td>
<td>2—hunched back</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2—hindlimb paralysis</td>
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<tr>
<td></td>
<td></td>
<td>3—unresponsiveness</td>
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Animals were scored twice daily based on symptoms. Cumulative scores range from 0 to 10, based on the varying degree of symptoms of sickness, such as lesions, sore red eyes, ruffled fur, and neurological dysfunctions.
RESULTS

Morbidity. Morbidity data indicate the day on which any sickness symptom was observed. The results of the experiment showed that there were differences in morbidity across the groups over the 21-day postinfection period. Figure 1 illustrates the time course for morbidity for the four groups. Intranasal administration of HSV-1 following short-term moderate exercise training resulted in a decrease in morbidity compared with resting Con ($P = 0.0017$). Ex mice (Ex-PBS-lip) experienced only a 59% incidence in morbidity, whereas 95% of Con mice (Con-PBS-lip) exhibited symptoms of morbidity. Short-term moderate exercise clearly resulted in a decrease in morbidity over the 21-day postinfection period. Administration of CL$_2$MDP-lip to Ex mice negated the beneficial effects of short-term moderate exercise. There was no significant difference in morbidity between Ex mice receiving CL$_2$MDP-lip and Con mice receiving PBS-lip (89 vs. 95%). There was no difference between Con mice receiving PBS-lip administration and Con mice receiving CL$_2$MDP-lip (95 vs. 89%).

Symptom severity. Severity of symptoms was also recorded to better address possible differences in severity of sickness. Morbidity data simply indicate the day on which any sickness symptom was observed, whereas symptom severity data indicate the magnitude of sickness symptoms and are measured over time. Symptoms are given a score at onset, depending on the degree of severity. These easily identifiable symptoms have a similar pattern of development: earlier symptoms are weighted less than symptoms that appear later during the infection. Figure 2 shows the symptom severity score for the four groups of mice. The mean day to death across all groups was day 8; symptom severity was analyzed across groups on days before this (days 1–7). Symptom severity was significantly different across the groups over the postinfection period ($P < 0.05$). Ex-PBS-lip mice had a significantly decreased symptom severity score compared with Con-PBS-lip mice on days 5–7 ($P < 0.05$). CL$_2$MDP-lip administration in Ex mice negated the beneficial effects of exercise on reducing the symptom severity score following infection on days 5–7. Symptom severity score for Ex-CL$_2$MDP-lip mice was not significantly different from that for Con-PBS-lip and Con CL$_2$MDP-lip mice. The highest symptom severity scores recorded for each of the four groups over the 21-day postinfection period were observed on day 7.0 as follows: 0.86 ± 0.51 in Ex-PBS-lip, 3.7 ± 0.53 in Ex-CL$_2$MDP-lip, 4.6 ± 0.67 in Con-PBS-lip, and 4.0 ± 0.55 in Con-CL$_2$MDP-lip.

Mortality. Similar treatment effects were found for mortality (i.e., time to death) over the 21-day postinfection period. Figure 3 illustrates the time course in mortality across the four groups. Intranasal administration of HSV-1 following 6 days of moderate exercise resulted in a decrease in mortality ($P < 0.0001$) compared with the Con mice. Ex-PBS-lip mice showed a mortality rate of 29% over the 21 days compared with 90% in the Con-PBS-lip mice. Short-term moderate exercise clearly resulted in a decrease in mortality over the 21-day postinfection period. Administration of CL$_2$MDP-lip to Ex mice negated the beneficial effects of short-term moderate exercise. There was no significant difference in mortality between Ex mice receiving CL$_2$MDP-lip and Con mice receiving PBS-lip (79 vs. 90%). There was also no difference between Con mice receiving PBS-lip administration and Con mice receiving CL$_2$MDP-lip.
between Con mice receiving PBS-lip and Con mice receiving CL2MDP-lip (90 vs. 72%).

**DISCUSSION**

Moderate exercise has been shown to reduce susceptibility to infection, although the specific immune mechanism(s) that may contribute to this effect is largely unknown. Increases in various functions of macrophages have been associated with moderate exercise (9, 10, 30). However, the increase in macrophage function following moderate exercise has not been shown to have a direct role in reducing susceptibility to URTI. This study used an established model of exercise and respiratory infection (2, 7–9) to determine the effect of lung macrophage depletion on the benefits of short-term moderate exercise. The respiratory tract infection and pathology in this model are similar to those observed in human disease and have been used to evaluate the efficacy of antiviral drugs (14, 23). Our laboratory (9) has used it previously to show that repeated daily moderate exercise (1 h/day for 6 days) decreases susceptibility to respiratory infection that was associated with an increase in intrinsic antiviral resistance of macrophages to HSV-1. However, this is the first study to show a direct effect of lung macrophage depletion on this or any model of exercise and infection. These results suggest that lung macrophages are essential for the beneficial effects of exercise in this model of HSV-1 infection.

Macrophages have been implicated in exercise effects on infection risk due to their clear role as a first line of defense against most infectious agents (7–9). Two types of macrophage-mediated resistance mechanisms have been described, which include intrinsic and extrinsic resistance (40). Intrinsic resistance refers to the capacity of the macrophage to inhibit virus growth within the cell itself; extrinsic resistance refers to the macrophage’s ability to inactivate extracellular virus, suppress virus replication in adjacent cells, and destroy infected cells. Activated macrophages can release antiviral factors such as TNF-α and IFN-α/β. Macrophages also have the ability to produce chemokines, which can recruit and activate additional cell types to combat viral infection (37). In addition to playing a role in innate immunity, macrophages are able to process and present antigen to T lymphocytes (33); however, numerous studies have demonstrated that alveolar macrophages are poor antigen presenters compared with dendritic cells (5, 15). It is possible, however, that alveolar macrophages may influence the degree of activity of dendritic cells by releasing cytokines (24).

There is also good evidence of an association between exercise and alterations in macrophage function that support these hypothesized effects on infection risk. Recently, our laboratory (9) found a decrease in susceptibility to HSV-1 infection following 6 days of moderate daily exercise, which was associated with an increase in intrinsic antiviral resistance of macrophages to HSV-1. Kohut et al. (17), using a similar animal model of HSV-1 respiratory infection, demonstrated that moderate exercise was associated with increased production of cytokines typically released by macrophages with an enhanced cell-mediated immune response in older mice. Several investigators have reported exercise-induced increases in macrophage chemotaxis, adherence, respiratory burst, and phagocytic activities following a single bout of exercise (11, 28). Chronic exercise has also been reported to increase macrophage function. Fernandez and De la Fuente (13) reported that stimulation of phagocytic functions (chemotaxis capacity, phagocytosis capacity, and superoxide anion production) was higher for peritoneal macrophages in mice after swimming exercise for 90 min/day over a period of 20 days, compared with sedentary controls. Chronic treadmill running exercise for 16 wk in young mice increased macrophage cytolytic activity (20). Sugiuira et al. (30) reported an increase in macrophage phagocytosis, glucose consumption, superoxide anion production, and lysosomal enzyme activity following 12 wk of treadmill exercise. However, none of these studies directly tested the importance of any of these changes in macrophage function on infection.

Selective depletion of macrophages in vivo through administration of liposomes containing dichloromethylene diphosphonate was used in this study to determine the role of macrophages on the reduced susceptibility to URTI following short-term moderate exercise in mice. Depletion of macrophages by the methods described in this study has been reported by others to eliminate 75% of lung macrophages (19). Other models of HSV-1 infection using selective macrophage depletion have shown an important role of macrophages in controlling HSV-1 replication (1, 6). Cheng et al. (6) found that macrophages play an important role in restricting HSV-1 growth after corneal infection, in which macrophage-depleted mice were found to have virus titers as much as 10^5-fold higher than in non-macrophage-depleted mice. Similarly, Bauer et al. (1) found that macrophages influence the course of HSV-1 keratitis in mice and that macrophage depletion was found to influence viral replication in the cornea as well as the immune-mediated process of herpes stromal keratitis. Other models of infection have also shown an important role of macrophages in infection. Elimination of macrophages in the lungs was found to increase titers of influenza virus following infection (38). However, this study did not find the expected effect of the clodronate liposomes in the resting Con groups. This apparent discrepancy is likely due to ceiling effect resulting from the large viral dose that was given [dose that elicits 80% mortality (LD80)]. An LD80 was used in this study to allow sufficient opportunity for the hypothesized reduction in risk of infection in the Ex groups. When we use a lower viral dose (LD30) in Con mice (unpublished data), we find that macrophage depletion does significantly increase morbidity (88 vs. 54%) and mortality (50% vs. 23%) following intranasal inoculation with HSV-1.

The results of this study utilizing a well-characterized macrophage depletion technique are the first to specifically identify lung macrophages as mediators of the beneficial effects of repeated daily moderate exercise on susceptibility to respiratory infection in mice. The data provide significant additional support for the hypothesized role of lung macrophages in exercise-induced changes in resistance to infection that have been largely based on associations between changes in tissue macrophage function and disease outcomes. Other research using different strategies is needed to further characterize the primary signals that increase macrophage function during and following exercise, which of the many antiviral functions of macrophages are most important, and the extent to which macrophages work independently or via numerous interactions.
with other immune and nonimmune parameters to affect resistance to infection.

REFERENCES