G-CSF, but not corticosterone, mediates circulating neutrophilia induced by febrile-range hyperthermia

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FEVER IS GENERALLY REGARDED as a protective response to infection (23). Retrospective clinical studies demonstrate an association between the presence of fever and reduced length of viral illnesses (12, 13, 30, 41) or improved survival in bacterial infections (1, 7, 18, 29, 43). However, this association is lost in patients with higher disease acuity (15). In two retrospective clinical studies of patients with bacteremia and sepsis, treatment with the antipyretic agent, acetaminophen, was associated with improved survival, although actual core temperatures were not mentioned in the report (25, 26). Studies in animal models in which febrile-range hyperthermia (FRH) was achieved by increasing ambient temperature generally support a protective effect of the temperature increase itself during infections (2–5, 9, 20, 24, 39, 42). However, our own recent studies of FRH in experimental infections suggest that the site of infection is an important factor in determining the ultimate effect of FRH on survival in the infected host. Whereas FRH improved survival in experimental Klebsiella pneumoniae peritonitis by accelerating pathogen clearance (20), the same warming protocol tended to worsen survival in K. pneumoniae pneumonia, despite accelerating clearance of bacteria from the lungs (36). When inflammation was initiated in the lungs in the absence of a replicating pathogen, as occurs in a treated pneumonia (36) or in response to hyperoxia (14), coexposure to FRH decreased survival by augmenting collateral tissue injury. Such pulmonary injury is characterized by loss of endothelial barrier function (14, 36) and bronchiolar epithelial necrosis (36).

In each of these models of lung injury and infection, exposure to FRH enhanced intrapulmonary neutrophil accumulation. We have identified two mechanisms that contribute to the enhanced neutrophil accumulation in the presence of FRH (core temperature 39.5°C). Exposure to FRH causes an expansion of the circulating neutrophil pool (14) and augments expression of the ELR+ CXC chemokines, a class of neutrophil chemoattractants, and the neutrophil growth factor and activator, granulocyte-macrophage colony-stimulating factor (GM-CSF), at sites of infections or injury (14, 36). Interestingly, while FRH was not sufficient to induce expression of GM-CSF or the ELR+ CXC chemokines in the absence of an exogenous proinflammatory stimulus, exposure to FRH alone for 24 h stimulated a doubling of peripheral neutrophil counts and a 7.3-fold increase in circulating levels of the neutrophil growth factor, granulocyte colony-stimulating factor (G-CSF) (14). While we showed that neutralization of G-CSF abrogated the FRH-induced neutrophilia stimulated by 24-h exposure to FRH, we did not evaluate the potential contribution of neutrophil demargination or the adrenal glucocorticoid response to FRH-induced neutrophilia, each of which has previously been demonstrated to accompany exercise-induced hyperthermia (6).

In this study, we analyzed the kinetics of FRH-induced neutrophilia and expression of circulating corticosterone and

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G-CSF in a conscious hyperthermic mouse model. We show that neutralization of G-CSF, but not pharmacological blockade of glucocorticoid receptors, abrogates FRH-induced neutrophilia. Finally, we show that epinephrine-induced neutrophil demargination is preserved in the FRH-exposed mice, suggesting that FRH does not cause peripheral neutrophilia by stimulating neutrophil demargination.

MATERIALS AND METHODS

Animal exposure and temperature monitoring. Eight- to ten-week-old male outbred CD-1 mice, weighing 25–30 g, were purchased from Harlan-Sprague (Indianapolis, IN), housed in the Baltimore Veterans Affairs Medical Center animal facility under the supervision of a full-time veterinarian, and used within 4 wk of delivery. Mice were adapted to standard plastic cages for at least 4 days before the study. To avoid the influence of diurnal cycling, all experiments were started at approximately the same time each day (between 8:00 AM and 10:00 AM). One week before each experiment, sentinel mice were implanted with telemetric intraperitoneal temperature sensors (Mini Mitter, 1.05-g in vivo temperature sensor, model 100–0035).

Cages containing three to four mice were placed in modified infant isolates with temperature set to 24°C to maintain euthermia or 34–34.5°C to maintain FRH. In some experiments, core temperature of one sentinel mouse per group was continuously monitored using the Mini Mitter Automated Data Acquisition System. Except for the ambient temperature, handling of the euthermic and hyperthermic mice was identical. All procedures were approved by the University of Maryland, Baltimore Animal Care and Use Committee.

Measurement of circulating neutrophil count. At selected times, groups of mice were anesthetized by 20-s exposure to isoflurane and euthanized by exsanguination via cardiac puncture followed by cervical dislocation. Total leukocyte count in heparinized blood was determined manually using a hemacytometer after erythrocyte lysis in 2.2% acetic acid. The differential leukocyte count was manually determined using morphological criteria in Diff-Quik-stained cyto-preparations after erythrocyte lysis with Tris-buffered ammonium chloride. The absolute neutrophil count was calculated as the product of total leukocyte count and the fraction of total leukocytes comprising neutrophils. Plasma was stored at −80°C for analysis of G-CSF concentration and corticosterone levels.

G-CSF neutralization and glucocorticoid receptor blockade. In experiments to determine whether G-CSF was required for FRH-induced neutrophilia, mice were treated with 2-mg rabbit anti-G-CSF (40) administered intraperitoneally just before beginning a 36-h exposure to FRH. Sham-treated control mice received nonimmune rabbit immunoglobulin. Additional euthermic mice were treated with anti-G-CSF or were sham treated and were housed at 24°C for 36 h. Mice were euthanized, blood was collected by cardiac puncture, and the neutrophils were manually counted. To determine whether endogenous glucocorticoid was required for FRH-induced neutrophilia, mice were treated with 50 mg/kg mifepristone (Sigma, St. Louis, MO) suspended in 100 μl PBS and delivered by gavage immediately before either treatment with 15 mg/kg sc dexamethasone (Sigma) in saline and euthanized 24 h later, or 36-h exposure to FRH. Sham-treated mice received 100 μl PBS via gavage. Sham dexamethasone-treated controls received 50 μl sc saline. Heparinized blood was collected via cardiac puncture during euthanasia, and cell counts were performed manually, as described above.

The residual potential for neutrophil demargination in mice exposed to FRH for 36 h and euthermic controls was determined by analyzing the capacity of epinephrine to increase the peripheral neutrophil count. Peripheral neutrophil counts were measured before and 1 h after administration of 2.5 mg/kg epinephrine bitartrate (Sigma) dissolved in 0.2 ml ip PBS. Immediately before epinephrine administration, 70 μl of blood were collected from the retroorbital plexus. Both groups of mice were housed at 24°C for 1 h and then euthanized to obtain heparinized blood via cardiac puncture. Total peripheral neutrophil count was analyzed as described above.

Measurement of plasma G-CSF and corticosterone. G-CSF was measured in the University of Maryland Cytokine Core Laboratory using a standard two-antibody ELISA with commercial antibody pair and recombinant standard from R&D (Minneapolis, MN), as previously described (20). The lower detection limit was 15 pg/ml. Corticosterone was measured by radioimmunoassay using a kit from MP Biomedical (Irvine, CA), which had a lower detection limit of 10 ng/ml.

Statistical analysis. All data are presented as means ± SE. Differences between two groups were tested using an unpaired Student t-test. Differences among more than two groups were tested by a Fisher protected least squares difference test applied to a one-way ANOVA. Differences between pre- and postepinephrine neutrophil counts were analyzed by a paired Student’s t-test.

RESULTS

FRH stimulates expansion of the circulating polymorphonuclear neutrophil pool. As our laboratory previously reported (14, 20), because mice have a limited capacity to eliminate heat, increasing the murine environment from 24°C to 34°C was sufficient to cause a rapid increase in core temperature from normal basal temperature (36.5–37°C) to a temperature in the upper febrile range (39.5–40°C) (Fig. 1A). For the remainder of this study, we will refer to mice housed at 24°C as euthermic and those housed at 34°C as hyperthermic. Our laboratory has previously reported that this level of hyperthermia was well tolerated for up to 2 wk (14).

Groups of mice were sequentially euthanized, and total peripheral polymorphonuclear neutrophil (Fig. 1B) and lymphocyte (Fig. 1C) counts were determined (Fig. 1B). Exposure to FRH caused an increase in peripheral neutrophil, evident after 24-h exposure, and peaking after 36-h exposure at 3.6-fold baseline levels. In the same mice, exposure to FRH caused a parallel 69% reduction in the peripheral lymphocyte count. By 42 h of continued exposure to FRH, the lymphopenia resolved, and the neutrophilia decreased by one-third compared with peak levels at 36 h.

FRH stimulates corticosterone and G-CSF expression. We previously demonstrated that exposure to FRH for 24 h stimulates the appearance of circulating G-CSF, a neutrophil mitogen and survival factor (14). However, exposure to hyperthermia has also been reported to activate an adrenocorticoid response (8), which may also stimulate peripheral neutrophilia (10). To begin to determine the potential contribution of these two mediators to FRH-induced neutrophilia, we analyzed circulating levels of corticosterone (Fig. 2A) and G-CSF (Fig. 2B) in mice exposed to FRH for up to 42 h. Such an exposure triggered an increase in circulating corticosterone from 82 ± 12 pg/ml at baseline to a peak of 267 ± 30 pg/ml after 30-h FRH exposure and subsequent return to baseline levels by 42 h of continuous exposure to FRH. In the same mice, plasma levels of G-CSF increased from 2.87 ± 2.87 pg/ml at baseline to 19.6 ± 19.6 pg/ml after 24 h of FRH exposure and 117 ± 56 pg/ml after 36 h, before becoming undetectable after 42-h continuous exposure to FRH.

To determine whether a shorter exposure to FRH was sufficient to induce maximal peripheral neutrophilia, we exposed mice to FRH for 24 h, then returned the animals to a 24°C environment for an additional 12 h before analyzing...
peripheral neutrophil counts and plasma G-CSF concentration. This treatment caused a more modest 60% increase in peripheral neutrophil count (2.09 \pm 0.59 \times 10^6/ml) and a 3.3-fold increase in G-CSF levels (6.5 \pm 0.71 pg/ml) than did continuous 36-h exposure to FRH (see Figs. 1B and 2B).

G-CSF neutralization, but not glucocorticoid receptor blockade, abrogates FRH-induced neutrophilia. To determine whether the observed activation of the adrenocorticotropin response was required for FRH-induced neutrophilia, we analyzed the capacity of the pharmacological glucocorticoid antagonist mifepristone to attenuate neutrophilia in mice exposed to FRH. To confirm the efficacy of this agent, we showed that pretreatment with 50 mg/kg mifepristone administered as a gavaged slurry blocked the neutrophilia induced by an exogenous glucocorticoid, dexamethasone (Fig. 3A). However, the same mifepristone treatment protocol failed to block FRH-induced neutrophilia (Fig. 3A).

Likewise, to determine whether the augmented G-CSF expression induced by FRH contributed to FRH-induced neutrophilia, the effect of G-CSF neutralization was measured in mice exposed to FRH for 36 h and in euthermic controls (Fig. 3B). Mice were treated with rabbit anti-mouse G-CSF antibody or sham-treated with nonimmune rabbit immunoglobulin just before exposure to FRH was initiated. Compared with sham-treated controls, treatment with anti-G-CSF antibody completely blocked the peripheral neutrophilia in FRH-exposed mice. Interestingly, immunoneutralization of G-CSF reduced the peripheral neutrophil count below baseline levels in the hyperthermic but not in the euthermic mice.

Epinephrine-induced neutrophil demargination is preserved in FRH-exposed mice. To determine whether neutrophil demargination contributes to the peripheral neutrophilia stimulated by exposure to FRH, we analyzed the effect of epinephrine, an agent known to cause substantial neutrophil demargination (32), on peripheral neutrophil counts in euthermic mice and mice exposed to FRH for 36 h (Fig. 4). Treatment with 2.5 mg/kg ip epinephrine increased absolute peripheral neutrophil counts 2.2-fold (1.35 \pm 0.21 to 2.94 \pm 0.68 \times 10^6/ml) in the euthermic mice and stimulated a similar 1.8-fold increase (3.97 \pm 0.39 to 7.21 \pm 0.53 \times 10^6/ml) in mice exposed to FRH for 36 h, suggesting that the proportion of marginated neutrophils was not significantly altered by FRH, despite a
threefold increase in peripheral neutrophil counts in these experiments.

**DISCUSSION**

We previously showed that increasing core temperature from basal to febrile levels augments neutrophil delivery to injured or inflamed tissues by stimulating peripheral neutrophilia and increasing expression of neutrophil chemoattractants. In murine models of pulmonary oxygen toxicity and gram-negative pneumonia, increasing core temperature by ~3°C augments pulmonary expression of the ELR\(^+\) CXC chemokine family of neutrophil chemoattractants. While enhanced expression of the chemokine genes required coexposure to a proinflammatory stimulus, expansion of the circulating neutrophil pool occurred in mice exposed to FRH without exogenous proinflammatory stimuli. In our laboratory’s previous study (14), we showed that 24-h sustained exposure to FRH caused a 2-fold increase in the peripheral neutrophil counts and a 7.3-fold increase in plasma levels of the neutrophil growth factor, G-CSF. While we showed that immunoneutralization of G-CSF blocked the peripheral neutrophilia caused by 24-h exposure to FRH, we did not analyze the potential contribution of two additional mechanisms known to increase peripheral neutrophil counts, glucocorticoid-induced neutrophil production and catecholamine-induced demargination. In the present study, we extended our previous results by showing that the peripheral neutrophil pool continues to expand during 36-h sustained exposure to FRH before returning to baseline levels after 42 h. Such exposure to this level of hyperthermia is common in febrile illnesses, in individuals with impaired thermoregulation, or in those in extreme environments.

Such an increase in peripheral neutrophil count might result from increased release of neutrophils from the bone marrow and/or through neutrophil demargination into the circulating pool. In our previous study of pulmonary oxygen toxicity, expansion of the circulating neutrophil pool occurred, despite a profound increase in neutrophil accumulation in the hyperthermic, hyperoxic lung (14). Because the lung is one of the major sites of neutrophil margination (19), the coincidence of increased peripheral neutrophilia and intrapulmonary neutrophil accumulation pointed to increased generation of new neutrophils rather than demargination as the major source of excess circulating neutrophils in FRH-exposed mice. In the present study, we utilized exogenous epinephrine at a dose that has been shown to cause neutrophil demargination in mice (32) to determine whether mice exposed to FRH had depleted their marginated neutrophil pool. Treatment with epinephrine caused similar approximately twofold increases in peripheral absolute neutrophil counts in hyperthermic and euthermic mice, indicating that neutrophil demargination does not significantly contribute to the peripheral neutrophilia present after 36-h exposure to FRH. In fact, the absolute marginated neutrophil pool was almost threefold greater in the warmer mice, suggesting that hyperthermia and subsequent exposure to either endogenous or exogenous catecholamines may cause even greater peripheral neutrophilia. This process may be especially relevant to the intensive care setting in which exogenous catecholamines are frequently used to support cardiovascular function in febrile patients.
A number of studies in humans show that exposure to hyperthermia for up to 3 h caused by external warming or vigorous exercise in warm environments stimulates, at most, a modest increase in circulating levels of endogenous corticosteroids (reviewed by Ref. 6), which might provide a proximal stimulus for peripheral neutrophilia (16). In the present study, we found that sustained exposure to FRH for 30 h stimulated an increase in circulating levels of corticosterone, the timing of which was consistent with a potential role in driving late (36 h) peripheral neutrophilia. However, glucocorticoid receptor blockade with mifepristone failed to reduce the increase in absolute neutrophil count. To demonstrate that mifepristone was effective in blocking glucocorticoid-induced peripheral neutrophilia, we analyzed its effects on the peripheral neutrophil expansion stimulated by treatment with the exogenous glucocorticoid dexamethasone. Dexamethasone induced a similar level of peripheral neutrophilia as did exposure to FRH, and the dexamethasone-induced neutrophilia was completely blocked by treatment with mifepristone.

Mukae et al. (33) reported that administration of exogenous recombinant G-CSF to rabbits increased proliferation of neutrophil precursors and reduced transit time in the bone marrow, leading to peripheral neutrophilia that peaked 12 h after G-CSF administration. These pharmacodynamics are consistent with the observations in the present study in which appearance of increased circulating G-CSF, within 24 h of initiating exposure to FRH, preceded peak peripheral neutrophilia by 12 h. To further analyze the contribution of G-CSF to FRH-induced peripheral neutrophilia, we showed that immunoneutralization with a polyclonal anti-G-CSF antibody previously shown to block IL-17-induced neutrophilia (40), but not an equivalent dose of nonimmune antibody, completely blocked peripheral neutrophilia in mice exposed to FRH for 36 h. Collectively, these data suggest that the increase in circulating G-CSF levels, but not the endogenous glucocorticoid response, drives the late peripheral neutrophilia induced by sustained exposure to FRH. We emphasize that we have focused our study on sustained neutrophil expansion caused by persistent exposure to FRH. These data do not exclude contributions of catecholamine-induced demargination and endogenous glucocorticoids to early neutrophilia stimulated by shorter exposures to hyperthermia, as implicated in a number of correlative studies reviewed by Brenner et al. (6). In fact, the complementary actions of catecholamines, glucocorticoids, and G-CSF may stimulate an early and sustained expansion of the circulating neutrophil pool during febrile illnesses.

While we showed that exposing mice to FRH causes profound increases in circulating G-CSF levels, we have not yet determined whether FRH directly induces G-CSF expression rather than stimulates expression of one or more proximal inducers of G-CSF. Our laboratory has previously reported that exposing murine fibroblast, type II pneumocyte, macrophage, or endothelial cell lines to 39.5°C in vitro fails to induce expression of G-CSF (14), suggesting that the induction of G-CSF by FRH in vivo may be indirect. Expression of G-CSF can be activated by several proinflammatory cytokines, including TNF-α, IL-1β, IL-17, IL-18, GM-CSF, and interferon-γ (27, 28, 34, 38, 40). Ostberg et al. (35) reported that exposing mice to a similar warming protocol as used in the present study, 39.8°C for 6 h, did not induce detectable levels of circulating TNF-α or IL-1β. In our own laboratory, we found that exposing mice to 40°C for 6 h failed to stimulate detectable levels of circulating IL-1β or TNF-α (21, 22), and exposure to 39.5°C for 24 h failed to induce circulating GM-CSF. However, we cannot exclude a contribution of delayed or spatially restricted TNF-α, IL-1β, or GM-CSF expression in causing the increase in circulating G-CSF levels in FRH-exposed animals. Furthermore, to the best of our knowledge, the effect of FRH on IL-17, IL-18, or interferon-γ expression has not yet been determined.

In addition to its effects as a neutrophil growth and survival factor (33), G-CSF also has been reported to increase PMN degranulation, secretory vesicle mobilization, and surface CD11b expression (11) and to augment superoxide generation (45) and chemotaxis (44). Hierholzer et al. (17) reported that intratracheal instillation of recombinant G-CSF was sufficient to cause neutrophil recruitment and lung injury in the rat. Furthermore, in our murine models of pulmonary oxygen toxicity (14) and intratracheal endotoxin challenge (36), exposure to FRH augmented generation of the ELR⁺ CXC chemokines KC, LPS-induced CXC chemokine, and macrophage inflammatory protein-2, as well as GM-CSF, each of which can also increase the cytotoxic potential of neutrophils (31, 44). Rosenspire et al. (37) recently reported that exposure to FRH directly increases release of reactive oxygen intermediates and nitric oxide in human neutrophils in vitro. Thus FRH may not only increase the size of the circulating neutrophil pool but may also prime these cells for accelerated recruitment and enhanced cytotoxic potential. As we have previously reported, such a mechanism has the capacity to improve survival during infection by accelerating pathogen clearance (20), but may also lead to potentially lethal collateral tissue injury, as we have shown in the models of pulmonary oxygen toxicity (14) and bacterial pneumonia (36).

In summary, we have shown that sustained exposure to temperatures within the febrile range is sufficient to stimulate expression of circulating G-CSF and expand both the circulating and margined neutrophil pools. Taken together with the established neutrophil priming effects of FRH and our previous reports about FRH augmenting expression of the neutrophil activating cytokines, KC, LPS-induced CXC chemokine, macrophage inflammatory protein-2, and GM-CSF, we propose that the temperature elevation during a sustained fever increases the potential for neutrophil recruitment and activation. This process may improve survival during infections by accelerating pathogen clearance, but, in some situations, it also has the potential to reduce survival by causing collateral tissue injury. The ultimate effect of this process on survival is determined by a balance between these effects and the context in which it occurs.

GRANTS

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