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Mini-Review

Hibernation: the immune system at rest?

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RECEIVED MARCH 29, 2010; REVISED MAY 12, 2010; ACCEPTED MAY 12, 2010. DOI: 10.1189/jlb.0310174

ABSTRACT

Mammalian hibernation consists of torpor phases when metabolism is severely depressed, and T_b can reach as low as approximately –2°C, interrupted by euthermic arousal phases. Hibernation affects the function of the innate and the adaptive immune systems. Torpor drastically reduces numbers of all types of circulating leukocytes. In addition, other changes have been noted, such as lower complement levels, diminished response to LPS, phagocytotic capacity, cytokine production, lymphocyte proliferation, and antibody production. Hibernation may therefore increase infection risk, as illustrated by the currently emerging WNS in hibernating bats. Unraveling the pathways that result in reduced immune function during hibernation will enhance our understanding of immunologic responses during extreme physiological changes in mammals. ***J. Leukoc. Biol.*** **88: 619–624; 2010.**

Introduction

Endothermic animals can cope with periods of scarce food supply and increased energy demands by entering into a metabolically depressed state known as torpor. Periods of torpor are interspersed by euthermic periods that generally last less than 24 h, called "arousal" (**Fig. 1**). T_b are typically <10°C and can fall as low as –2°C [1, 2] during torpor and may last 6–40 days [2–4]. Although the exact mechanisms leading to the induction of torpor are unknown, it has been suggested that fasting as a result of reduced food supply leads to a decreased energy status of the cell [5]. This reduced cellular energy status can be sensed by adenosine-monophosphate kinase, which is sensitive to changes in the ratio AMP:ATP [6]. So-called silent information regulator two (Sir2) proteins (sirtuins), in turn, link the energy status to the circadian clock, oxidative stress, and metabolic fuel selection, thereby stimulating lipid metabolism and making fat stores the primary energy source during winter [5]. During torpor in the 13-lined ground squirrel (*I. tridecemlineatus*; previously called *Spermophilus tridecemlineatus* [7]), heart and respiratory rate decreases to <1% of euthermic values [8]. Although there are few studies that examine in detail the effect of hibernation on the immune system, available data indicate that hibernation decreases the function of the innate and adaptive immune system. Recently, hibernation has drawn much attention as a result of massive deaths among hibernating bats in the United States. Bat mortality during the hibernation season is associated with a fungus found on bat muzzles and wings (**Fig. 2**), which has an optimal growth temperature that is almost equal to the T_b during torpor and causes WNS. It is possible that changes in the immune system during hibernation are responsible for the devastating effect of the fungus on bat populations. Unraveling the mechanisms that regulate immune function during hibernation will enhance our knowledge of immunologic responses during low body temperatures in mammals. Such knowledge may aid in understanding the pathogenesis of WNS and may also be relevant to human medicine, as therapeutic hypothermia is sometimes used in trauma situations and during brain/cardiac surgery [9]. In this review, we summarize the literature about immune function during hibernation of mammals.

TORPOR INDUCES LEUKOPENIA

One of the most striking and pronounced changes in the immune system of torpid hibernators is the reduction in numbers of circulating leukocytes, which drop by ~90% during torpor in all hibernating mammals studied so far, including the European hamster (*Cricetus cricetus*) [10], the hedgehog (*Erinaceus europeaus L.*) [11], the European ground squirrel (*Spermophilus citellus*) [12], the Arctic ground squirrel (*Urocitellus parryii*) [13], and the 13-lined ground squirrel [8, 14]. The leukopenia is reported to affect granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes, and the ~10% remaining leukocytes in the blood during torpor are mainly neutrophils (90%) and lymphocytes (9%) [15]. During arousal, the number of neutrophils and monocytes increases rapidly to summer (euthermic) levels, whereas num-

Abbreviations: HA=hemagglutinin(s), IEL=intra-epithelial lymphocyte(s), I/R=ischemia/reperfusion, LBP=LPS-binding protein, LPL=lamina propria, PAMP=pathogen-associated molecular pattern, PHA=phytohemagglutinin, RRBC=rabbit RBC, T_b=minimal body temperature(s), WNS=white nose syndrome

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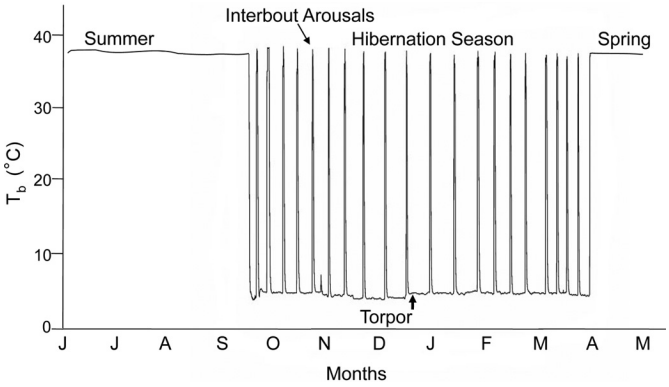

Figure 1. Body temperature trace of a 13-lined ground squirrel (*Ictidomys tridecemlineatus*) housed in the lab at an ambient temperature of 4°C. Hibernation started in mid-September and consists of torpor bouts with a body temperature of ~6°C, interspersed by short euthermic arousal periods.

bers of lymphocytes increase to only ~50% of summer values [11]. The absence of circulating neutrophils and monocytes may result in significantly reduced acute inflammatory responses and a defective clearance of microbes, and the reduction in lymphocytes will strongly affect immune surveillance, leading to impaired cellular and humoral immune responses.

NEUTROPENIA INVOLVES PRIMARILY MATURE NEUTROPHILS

The decrease in numbers of circulating neutrophils is caused mainly by a decrease in numbers of mature neutrophils: Numbers of circulating neutrophils with a filament-shaped nucleus (mature neutrophils) decrease approximately sixfold, and numbers of circulating neutrophils with a rod-shaped nucleus (immature neutrophils; neutrophilic band cells) decrease ~1.5-fold during torpor [11]. Given the short lifespan of neutrophils, the drop in mature neutrophils might be caused by apoptosis. The more likely explanation is, however, that the drop in the number of circulating neutrophils reflects their retention at specific locations, such as the lungs, liver, and spleen, where the number of leukocytes is increased during torpor [16, 17]. During arousal, the number of circulating, mature neutrophils rises [11], which could reflect release of sequestrated neutrophils or release of newly formed cells from the bone marrow, and bone marrow of hibernating squirrels contains fewer early-stage neutrophils (myeloblasts and neutrophilic promyelocytes) but significantly more late-stage neutrophils (neutrophilic metamyelocytes) without significant changes in the number of intermediate-stage neutrophils (neutrophilic myelocytes) [15]. The rapid rise in neutrophil numbers upon arousal might be a result of increased release from the bone marrow. Release of newly formed cells would augment the number of circulating neutrophilic band cells, and as these cells mature after being released, it leads indirectly to a higher number of circulating, mature neutrophils. The fact that protein production is severely limited in hibernating golden-mantled ground squirrels (*Callospermophilis lateralis*) below 18°C [18] might suggest that maturation of cells is slowed down during torpor. Taken together, the increased number of circulating, mature neutrophils is the result of (increased) maturation of neutrophilic band cells or release of retained, mature neutrophils.

LYMPHOCYTE GENERATION IS REDUCED DURING HIBERNATION

As mentioned earlier, an extreme decrease is observed in the number of circulating lymphocytes during torpor. Although the number of lymphocytes increases significantly upon

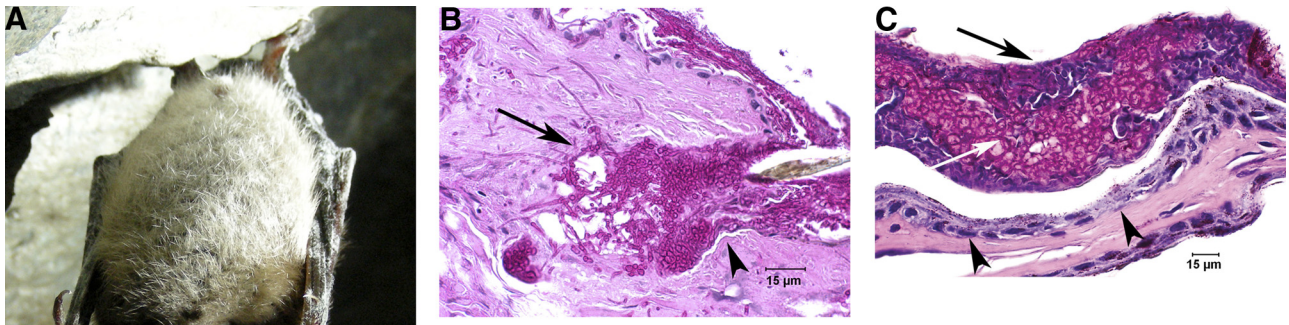

Figure 2. A little brown bat affected by WNS (A) with signs of inflammation absent in winter (B) but present in spring (C). As can be seen on the photograph (A), the fungus is mainly growing on the wings and muzzle of the animal. Histopathology (Periodic acid-Schiff stain) of a bat muzzle section from a deceased animal in winter (B) revealed invasive growth of the fungus in the hair follicle (arrowhead) and invading connective tissue (arrow) without signs of inflammation. Histopathology (Periodic acid-Schiff stain) of a wing section from an animal that survived but was unable to fly in May (C) revealed invasive growth of the fungus (white arrow), presence of inflammatory cells surrounding the fungus (long arrow), and clear signs of inflammation (formation of cellular crust overlaying intact epidermis; arrowheads). The photograph of the bat was taken by Marvin Moriarty, U.S. Fish and Wildlife Services; histopathological images are reprinted with permission of the author (Carol Meteyer, National Wildlife Health Center, U.S. Geological Survey).

arousal, the number of circulating lymphocytes during arousal is only half of the number that is circulating in summer animals. Little is known about the mechanisms behind the extreme reduction in numbers of lymphocytes during torpor, but the decreased number of circulating lymphocytes in the winter season might be explained by lower production in the bone marrow and thymus. Production of T-lymphocytes in the thymus might be reduced as a result of involution of thymic tissue during autumn/winter [19], which contains almost no lymphocytes during the hibernation period [19, 20]. Thymic involution in hibernating animals differs from age-related thymic involution in that the thymus of hibernating animals regenerates in the spring/summer period [19]. It has been suggested that release of 5′-AMP from brown adipose tissue during hibernation inhibits the proliferation of lymphocytes in the thymus [21]. Whether the generation of B-lymphocytes is also altered during the winter (hibernation) season is not known, although the reduced number of cells present in bone marrow [15] is indicative that this might indeed be the case. However, only a small proportion of the lymphocytes in the peripheral blood includes recent emigrants from bone marrow and thymus, and together, with the long lifespan of naïve lymphocytes (weeks to months) [22, 23], a diminished production of lymphocytes alone cannot explain the decrease in the number of circulating lymphocytes [10–12, 14]. The lower number of circulating lymphocytes during torpor as compared with summer animals might be a result of apoptosis or retention of cells. Massive apoptosis of lymphocytes does not likely play a role, as several other studies demonstrated the presence of lymphocytes in the gut and spleen during torpor [17, 24, 25]. The rapid reappearance of lymphocytes in the blood (within a few hours) during the arousal bouts cannot be explained by new production of lymphocytes in primary lymphoid organs. We therefore speculate that lymphocytes are retained in peripheral lymphoid organs when animals reach the torpid state, followed by release upon arousal.

INCREASE IN THE NUMBER OF INTESTINAL LYMPHOCYTES DURING HIBERNATION

Inkovaara et al. [17] showed that the number of leukocytes rises in lungs and gut during torpor compared with summer and arousal. In lungs, this concerns mostly neutrophils, whereas in the gut, there appears to be mostly lymphocytes [17]. The specific site where these leukocytes reside has not been studied, but a more recent study by us addressed this issue in more detail in the gut [24]. In the 13-lined ground squirrel, the total number of IEL and lymphocytes in the LPL increased about threefold during torpor, and numbers of leukocytes per Peyer's patch increased in torpor compared with summer animals. Given that circulating T-lymphocytes in the blood are mainly TCR$\alpha\beta^+$, influx and retention of circulating T-lymphocytes into the gut-associated lymphoid tissue would thus result in an altered ratio of TCR$\alpha\beta^+$:TRC$\gamma\delta^+$. As during torpor, relative numbers of TCR$\alpha\beta^+$, TRC$\gamma\delta^+$, and the ratio TCR$\alpha\beta^+$:TRC$\gamma\delta^+$ remain constant, and the higher numbers of T-lymphocytes observed are therefore probably not a result of influx of lymphocytes from the circulation but rather a result of local expansion of cells [24]. During hibernation, the number of double-negative T-lymphocytes (i.e., CD8α^-, CD4$^-$) is increased among LPL and IEL [24]. Double-negative T-lymphocytes are able to suppress immune responses by eliminating activated CD8$^+$ T-lymphocytes via a cytolytic mechanism [26–28]. In addition, the number of B cells (defined as CD45RA$^+$ cells) is greater among LPL during the hibernation period (i.e., torpor and arousal) [24]. Furthermore, mucosal IgA levels increase in the small intestine during torpor [24]. The higher mucosal level of IgA may minimize microbial translocation during torpor by preventing bacteria from binding to epithelial cells [29, 30]. Mucosal levels of TNF-α, IFN-γ, IL-4, and IL-10 in the small intestine increase during hibernation, and thus, the enhanced secretion of IgA could result from the higher levels of mucosal IL-4 and IL-10, which stimulate IgA synthesis and secretion [29, 31, 32]. Besides stimulating synthesis and secretion of IgA, IL-4 and IL-10 are able to inhibit the negative influence of TNF-α and IFN-γ on epithelial permeability via regulation of tight junction proteins [33, 34]. Although ionic epithelial permeability of the gut is increased during hibernation [35], absorptive and secretory functions are maintained [35–38].

FASTING MAY ALSO CONTRIBUTE TO THE IMMUNOSUPPRESSIVE STATE DURING HIBERNATION

Various hibernators exhibit different eating behavior during the winter season: The 13-lined ground squirrel (*I. tridecemlineatus*) fasts for prolonged periods (5–8 months) [24], and the Syrian hamster (*Mesocricetus auratus)* eats occasionally during arousals [39]. Prolonged fasting and hibernation are known to affect microbial composition of the intestines of Syrian hamsters [39]. As gut microbes play a major role in intestinal immune function [40], we speculate that changes in microbiota lead to immunological alterations. For example, fasting during the hibernation period (i.e., torpor and arousal) might account for the decreased cytokine production of macrophages [25]. Walrand et al. [41] demonstrated in nonhibernating animals (rats; *Rattus norvegicus*) that fasting reduces TNF-α-production in response to LPS in vitro in peritoneal macrophages, and refeeding reverses TNF-α production to normal levels. Fasting alters the phosphorylation state of macrophages in mice [42], which could affect intracellular signaling pathways that lead to transcriptional activation of cytokine production. Taken together, the reduced spontaneous production of cytokines by macrophages during the prehibernation (October) and hibernation states [25] might be, at least in part, a result of fasting during these periods.

THE INNATE IMMUNE FUNCTION IS REDUCED DURING TORPOR

Microbes and microbial products trigger the secretion of proinflammatory cytokines, such as TNF-α and IL-1β, by binding of PAMPs to pattern recognition receptors, such as TLRs

on (or in) cells of the innate immune system. An important PAMP is LPS, which forms a complex with the LBP, normally present in plasma [43]. The LPS-LBP complex binds to CD14, which forms a dimer with TLR4, resulting in activation of the cell and production of cytokines such as TNF-α, IL-1β, and IFN-α [43]. In the golden-mantled ground squirrel, the number of splenic macrophages that bind LPS is not affected by torpor/arousal cycles during hibernation [44]. However, in arctic ground squirrel peritoneal macrophages cultured at 37°C, spontaneous TNF-α production is decreased significantly during the prehibernation period (October) and torpor but is restored toward summer values during arousal [25]. A subsequent in vivo study demonstrated that i.p. injection of LPS in golden-mantled ground squirrels does not induce fever during torpor [45]. Moreover, intracerebroventricular injection of PGE1, an important signaling molecule that induces fever during infection, does induce arousal and fever in torpid animals. Thus, the absence of a febrile response to LPS in vivo is probably a result of a diminished capacity of (peritoneal) macrophages to produce cytokines such as the aforementioned TNF-α, rather than central (brain) changes leading to a diminished capacity to induce fever [45]. Together, the findings indicate that binding of LPS is not altered during torpor but that the capacity to induce subsequent cytokine production might be reduced. Yet, it is unknown on which level the signaling cascade of the innate immune function is altered by torpor.

One of the most important effector mechanisms of the (innate) immune system is phagocytosis. Although direct evidence for changes in phagocytotic function during hibernation is lacking, some insight comes from experiments examining phagocytosis of carbon particles by Kupffer cells in livers from ground squirrels. Typically, Kupffer cell activation occurs after liver cold I/R and can contribute to I/R damage. Although 72 h of cold storage had no effect on phagocytosis of carbon particles in livers derived from torpid ground squirrels, it did increase phagocytosis in livers harvested from summer ground squirrels [46]. The observation that activation of Kupffer cells did not occur in livers derived from torpid squirrels may be a result of a lower number of phagocytosing Kupffer cells present during hibernation or to a reduction in the phagocytosing capacity of individual Kupffer cells. Further evidence that hibernation may dampen I/R-induced immune activation comes from an in vivo model of intestinal warm I/R injury, in which myeloperoxidase activity (a marker of neutrophil infiltration) was elevated after I/R in summer squirrels but not in aroused hibernators [47].

The efficiency of phagocytosis can be facilitated by opsonization with complement. The phagocytic capacity and complement activity in the blood of golden-mantled ground squirrels are diminished during torpor but restored to summer levels upon arousal [48]. The decrease in complement activity was paralleled by a reduced expression of C3 mRNA in liver [48]. Thus, levels of C3 (and possibly other complement factors) appear to be lower during torpor, which explains the diminished complement activity in this state.

IN VIVO ANTIBODY RESPONSES ARE REDUCED BY LOW T_B

The capacity for antibody production during hibernation was examined by measuring antibody production against RRBCs or SRBCs, which were used as particulate antigens. Formation of antibody-producing (plaque-forming) cells in the spleen and formation of circulating immune HA are delayed for the duration of torpor (up to 40 days) after injection of SRBCs and RRBCs, respectively, into torpid ground squirrels (*S. citellus*) [3, 49]. Following arousal from torpor, the HA titer rises rapidly in response to antigens injected prior to torpor, and maximum values were observed after 7 days [3]. In addition, forced (non-natural) hypothermia of summer squirrels delays the formation of immune HA [3]. Thus, the reduced capacity to form antibody-producing cells seems to be secondary to low T_b, rather than being induced by temperature-independent processes.

T-LYMPHOCYTE FUNCTION IS REDUCED DURING TORPOR

In addition to a reduced capacity to induce a humoral immune response, cellular immune responses are affected by hibernation. The fact that skin allografts transplanted into torpid 13-lined ground squirrels are not rejected until the end of the hibernation season (in spring) [50] clearly demonstrates a decreased cell-mediated adaptive response throughout hibernation. The spontaneous and Con A-induced proliferation of T-lymphocytes derived from arctic ground squirrels in the prehibernation period (October) and in torpor is about half of the proliferative capacity of T-lymphocytes derived from summer squirrels and aroused hibernators [25]. Further, circulating mononuclear cells (i.e., lymphocytes/monocytes) derived from animals in torpor produce less IFN-γ after stimulation with the T-lymphocyte-specific mitogen PHA at 37°C, which is restored in cells derived from aroused hibernators [51]. As numbers of lymphocytes [11, 12] and IFN-γ production are higher during arousal [51], the decreased IFN-γ production in response to PHA stimulation during torpor is probably a result of a diminished number of T-lymphocytes present.

LESSONS LEARNED FROM THE WNS IN BATS

The consequences of the changes in immune function during the annual hibernation cycle are not clear, although the extreme immune-suppressed state during torpor is probably beneficial in terms of energy conservation. As most microbes do not proliferate well at low temperatures, the chance of getting infected seems to be rather small and outweighs the benefits of immunosuppression. However, the risk of a diminished immune function during the hibernation season may play a role in WNS (Fig. 2), a recently described syndrome that is associated with the deaths of millions of bats infected with the fungus *Geomyces destructans,* (a.o., *Myotis* spp., *Eptesicus fuscus, Perimyotis subflavus*) [52]. This psychrophilic (cold-loving) fungus grows optimally at temperatures between 8°C and 14°C (the

temperature of the caves where the animals hibernate). If ongoing studies confirm a causative relationship between the fungus and bat mortality, this would represent a serious threat, as the immunosuppressed state during torpor likely compromises the ability of hibernating bats to combat the infection. TNF plays a crucial role in the initiation of the (innate) immune response with subsequent influx of leukocytes as a defense against fungal growth [53]. As mentioned above, hibernation (torpor) not only results in a reduction of the number of circulating leukocytes [12] but also affects the production of TNF (and other cytokines) by leukocytes [25]. Although little is known about the immune system of (hibernating) bats, histopathological analyses of infected tissue show no signs of inflammation (Fig. 2), reflecting the absence of neutrophils during torpor and possibly of TNF as well [54].

CONCLUSIONS

The limited data that are currently available indicate that hibernation has significant effects on the function of the immune system. Although there is some evidence that immune function is seasonally regulated, independent of the metabolic state during the hibernation season, it is clear that there is a particularly substantial decline in immune function during torpor. The function of the innate immune system is diminished primarily by a reduced number of circulating neutrophils and monocytes during torpor, which might be secondary to a lowered T_b. The diminished capacity to induce a humoral response during torpor might also be secondary to a lowered T_b, as it is also observed in hypothermic summer squirrels. Cellular immunity, on the other hand, is affected by hibernation (i.e., season) and temperature. First, intrinsic changes reducing proliferative capacity of lymphocytes are observed during euthermia, before hibernation, and during torpor. Second, the extreme lymphopenia during torpor diminishes the capacity to induce a cellular response (i.e., IFN production) in vivo. Although the scarce data that are currently available indicate that the function of the immune system is reduced significantly during torpor, the mechanisms leading to this immunodeficient state are poorly understood. Temperature also deserves further attention as a regulating factor in the mammalian immune system, as temperature affects immune function in nonhibernating species. Hypothermia in rats limits inflammation and reduces the number of infiltrating leukocytes in the brain following traumatic brain injury [55, 56]. Although clinical studies yielded conflicting results regarding the effect of hypothermia on the immune system, it has been shown to decrease cytokine release and delay the interaction between leukocytes and vessel walls following cardiac surgery [57, 58]. Whether the mechanisms underlying the hibernation-associated immunosuppression are secondary to lower body temperatures or are actively induced unraveling the underlying signaling pathways will not only expand fundamental knowledge about the functioning of mammalian immune systems but also will enhance our ability to translate this information into biomedical applications, such as better use of clinical hypothermia and development of therapies for disorders in which immunological imbalance and/or inflammation play important roles.

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KEY WORDS:
torpor · immunosuppression · white nose syndrome · metabolism · hypothermia