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Hibernation is associated with depression of T-cell independent humoral immune responses in the 13-lined ground squirrel

Hjalmar R. Bouma [a,*], Robert H. Henning [a], Frans G.M. Kroese [c], Hannah V. Carey [b]

[a] Department of Clinical Pharmacology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[b] Department of Comparative Biosciences, University of Wisconsin School of Veterinary Medicine, United States
[c] Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, University of Groningen, The Netherlands

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ABSTRACT

Mammalian hibernation consists of periods of low metabolism and body temperature (torpor), interspersed by euthermic arousal periods. The function of both the innate and adaptive immune system is suppressed during hibernation. In this study, we analyzed the humoral adaptive immune response to a T-cell independent (TI-2) and a T-cell dependent (TD) antigen. Thirteen-lined ground squirrels were immunized in summer or during hibernation with either a TI-2 or TD antigen on day 0 and day 14. Blood was drawn on day 0, 7, 14, 21 and 28. Both types of antigens induced a significant rise in antibody titer in summer animals. Much to our surprise, however, only immunization with the TD antigen, and not with the TI-2 antigen induced a humoral response in hibernators. Flow cytometric analysis of CD4 (helper T-lymphocytes), CD8 (cytotoxic T-lymphocytes) and CD45RA (B-lymphocytes) in blood, spleen and lymph nodes ruled out massive apoptosis as explanation of the absent TI humoral response during hibernation. Rather, reduced TI-2 stimulation of B-lymphocytes, possibly due to lowered serum complement during torpor, may explain the reduced antibody production in response to a TI-2 antigen. These results demonstrate that hibernation diminishes the capacity to induce a TI-2 humoral immune response, while the capacity to induce a humoral response to a TD antigen is maintained.

1. Introduction

Mammalian hibernation consists of periods of torpor that last several days to weeks and are characterized by lowered metabolism and body temperature, which typically reaches ±0–4 °C (Kenagy et al., 1989). These torpor bouts are interspersed by shorter (euthermic) periods called arousals (Carey et al., 2003; Storey, 1997; van Breukelen and Martin, 2002). The repetitive cycles of cooling and rewarming that occur during hibernation do not lead to gross signs of organ injury (Arendt et al., 2003; Fleck and Carey, 2005; Sandovici et al., 2004; Talaei et al., 2011; Zancanaro et al., 1999), despite the fact that such extreme conditions can induce apoptosis or necrosis in non-hibernating animals (Aslami and Juffermans, 2010; Boutilier, 2001; Hochachka, 1986; Storey, 2010). One of the adaptations that hibernators might use to resist tissue damage during torpor is the reversible suppression of the immune system. Immune suppression during torpor is characterized by leukopenia (Bouma et al., 2010b, 2011; Frerichs et al., 1994; Reznik et al., 1975; Spurrier and Dawe, 1973; Suomalainen and Rosokivi, 1973), a reduced complement level (Maniero, 2002) and absence of an innate immune response to injection of lipopolysaccharide (LPS) (Prendergast et al., 2002). In addition, specific suppression of the adaptive immune system is illustrated by extremely delayed rejection of skin allografts (Shivatcheva, 1988) and a reduced production of antibodies during torpor and arousal (Burton and Reichman, 1999; Sidky et al., 1972) (reviewed in (Bouma et al., 2010a)). Thus, hibernation reduces the capacity of the innate, cellular and humoral immune function. We showed previously that low body temperature reduces the number of circulating lymphocytes during torpor due to a lowered sphingosine-1-phosphate plasma level (Bouma et al., 2011). Further, we demonstrated that neutropenia during torpor is due to margination of cells secondary to low body temperature (unpublished data, submitted). To date, the mechanisms that underlie the reduced humoral immune function during hibernation have not been clarified.

A reduced humoral immune function in hibernation was first demonstrated by the absence of antibody production of spleen cells from torpid Syrian hamsters (*Mesocricetus auratus*) in response to *in vitro* incubation at 37 °C with sheep red blood cells (SRBCs), in contrast to spleen cells derived from euthermic hamsters (Sidky and Auerbach, 1968). This finding is in line with

* Corresponding author. Address: Department of Clinical Pharmacology, University Medical Center Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. Tel.: +31 50 363 2810; fax: +31 50 363 2812.
E-mail address: h.r.bouma@umcg.nl (H.R. Bouma).

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observations *in vivo*. Formation of plaque forming cells in the spleen following intraperitoneal (i.p.) injection of SRBCs in hibernating 13-lined ground squirrels (*Ictidomys tridecemlineatus*) is delayed for the duration of torpor (up to 40 days) (Jaroslow and Serrell, 1972). Further, secondary antigen challenge using hen egg-white lysozyme (HEL) leads to lower antibody titers in hibernating Turkish hamsters (*Mesocricetus brandti*) as compared to euthermic animals (Burton and Reichman, 1999). A humoral immune response induced by a T-cell dependent (TD) antigen such as ovalbumin or HEL depends on proper B-lymphocyte function and the presence of an adequate co-stimulatory signals provided by CD4+ T-lymphocytes through T-lymphocytes by CD40L-CD40 binding and cytokine production (Abbas and Lichtman, 2003; Noelle et al., 1992). In the case of a T-cell independent type 2 (TI-2) antigen, such as the carbohydrate ficoll, stimulation of B-lymphocytes occurs primarily by the antigen itself, potentially augmented by complement through the complement receptor 2 (CR2, CD21) that is expressed on B-lymphocytes as part of the B-cell coreceptor complex (Abbas and Lichtman, 2003; Dempsey et al., 1996; Frank and Atkinson, 2001). In the work presented here we assessed the capacity to induce a humoral immune response to both a TD antigen and a TI-2 antigen during hibernation. Summer active and winter hibernating 13-lined ground squirrels were primarily and secondarily immunized with these two types of antigen. We determined the effect on the hibernation pattern and the induction of a humoral immune response by measuring the antibody titer following injection. This experiment allowed us to differentiate between the capacity to mount a humoral immune response to a TD antigen and a TI-2 antigen during hibernation.

2. Materials and methods

2.1. Animals

Adult and juvenile (born spring of the same year) 13-lined ground squirrels of both sexes were obtained by capture in the Madison area. After capture, ivermectin (0.02 mg/kg, delivered orally) was given to all squirrels as a de-worming agent. Animals were housed individually in plastic cages with paper nesting material and free access to food (rat chow and sunflower seeds) and water. Summer animals ($n = 16$) were injected with antigens in July and euthanized at the end of the protocol on day 28 (see "Immunization Protocol" below). Animals allocated to the hibernating groups were moved to a dark room maintained at 4 °C in mid-late September of the same year. Food and water were removed once a squirrel began to show regular torpor cycles, on average within 2 weeks of entering the cold room. Hibernation patterns were monitored by placing saw dust on the animals and daily inspections for signs of (previous) movement. Hibernating animals were immunized after their third torpor bout in the fully aroused state, which was induced by moving them from the cold room into the warm room. Animals were transported back into the cold room after immunization, to allow the animals to go into torpor again. At the end of the protocol (see "Immunization protocol" below), animals were euthanized by isoflurane anesthesia (3% in O_2) followed by decapitation upon arousal. Blood (100 μl) was collected in EDTA-coated tubes for flow cytometric analysis. The remainder was collected in heparin-coated tubes and was separated by centrifugation (10 min, 800*g*) and plasma was snap-frozen in liquid nitrogen and stored at −80 °C. The spleen was removed and divided into two parts: one half was snap-frozen in liquid nitrogen and stored at −80 °C for quantitative PCR (qPCR) analysis. The other half of the spleen as well as the cervical lymph nodes were used for isolation of lymphocytes for flow cytometric analysis.

2.2. Immunization protocol

Animals were immunized at day 0 and again at day 14 by i.p. injection of either 50 μg of 4-Hydroxy-3-nitrophenylacetic-Amino-EthylCarboxyMethyl-FICOLL (NP-AECM-FICOLL; Biosearch Technologies, Novato, USA) in 300 μl phosphate buffered saline (PBS; Sigma–Aldrich, Zwijndrecht, The Netherlands) to stimulate a TI-2 humoral immune response ($n = 16$) (adapted from (Shih et al., 2002)) or with 100 μg NP-ovalbumin (Biosearch Technologies, Novato, USA) adsorbed to 2 mg alum (Thermo Scientific, Rockford, USA) in 300 μl PBS to induce a TD humoral immune response ($n = 15$) (adapted from (Randolph et al., 1999; Vora et al., 2009)). Immunization was performed by i.p. injection as this is relatively easy to perform and therefore, minimizes injection errors. Blood (~50 μl) was drawn into a heparin-coated glass capillary tube after punctuation of the pedal vein by a 27G needle under brief anesthesia (isoflurane 0.5–5% in O_2) at days 0 (before immunization), 7, 14 and 21. The final blood sample was drawn at day 28 upon euthanization of the animals. Blood samples were centrifuged immediately upon collection (10 min, 800*g*), separated into plasma and pellet and snap-frozen in liquid nitrogen followed by storage at −80 °C. Succeeding each blood draw an equal volume of saline (0.9% NaCl) was injected i.p. to prevent dehydration of the animals.

2.3. Flow cytometry

Directly after euthanization of the animals, lymphocytes were isolated from lymph nodes and spleen by cutting these organs into small pieces and washing with 0.9% NaCl on a 100 μm cell strainer (BD Biosciences, San Jose, California). Next, following centrifugation (10 min, 800*g*), cells were resuspended in 3 ml flow cytometry buffer, which consisted of PBS supplemented with 2% v/v heat-inactivated newborn calf serum (NCS; Sigma–Aldrich, Zwijndrecht, The Netherlands) and 0.1% w/v sodium azide. This cell suspension was then pipetted on top of 3 ml Lympholyte solution (Cedarlane Labs, Ontario, Canada) in a 15 ml tube and centrifuged (20 min, 800*g*). Lymphocytes from blood were isolated by incubating 100 μl whole blood with 500 μl erythrocyte lysis buffer (8.26 g NH_4Cl, 1 g $KHCO_3$ and 0.037 g EDTA in 1 L H_2O) for 15 min on ice, followed by the addition of 1 ml PBS and centrifugation (10 min, 800*g*). After centrifugation of isolated lymphocytes derived from blood, cervical lymph nodes or spleen, the pelleted cells were resuspended in 100 μl flow cytometry buffer supplemented with the following antibodies at the appropriate dilution: APC-conjugated mouse anti-rat CD4 (BioLegend, San Diego, USA), FITC-conjugated mouse anti-rat CD8 (1:25, BD Biosciences, San Jose, California) and PE – conjugated mouse anti-rat CD45RA (BD Biosciences, San Jose, California). Next, cells were incubated for 1 h on ice in the dark, followed by washing using an equal volume of flow cytometry buffer, centrifugation (10 min, 800*g*) and resuspension in 200 μl flow cytometry buffer prior to analysis on a FACS-Calibur (BD Biosciences, San Jose, California).

2.4. Elisa

NP-specific immunoglobulins were determined by coating Immunolon 4HBX ELISA plates (Thermo Scientific, Rockford, USA) with 100 μl PBS containing 1 μg NP-KLH (Biosearch Technologies, Novato, USA) at 4 °C overnight. Plates were then washed with PBS supplemented with 0.05% v/v Tween-20 and blocked with 3% Probumin (Millipore, Amsterdam, The Netherlands) in PBS for 2 h at room temperature (RT). After washing, plates were incubated with 50 μl of plasma diluted 1:5000 in blocking buffer (2 h, RT), washed and incubated with 100 μl (1:1000) of either polyclonal goat anti-rat IgG:HRP (Thermo Scientific, Rockford, USA) or polyclonal goat anti-rat IgM:HRP (AbD Serotec, Kidlington, UK) (1 h,