Developmental and Comparative Immunology 35 (2011) 421–430

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

The little brown bat, *M. lucifugus*, displays a highly diverse V_H, D_H and J_H repertoire but little evidence of somatic hypermutation

Sara Bratsch[a], Nancy Wertz[b], Kathryn Chaloner[c], Thomas H. Kunz[d], John E. Butler[b,*]

[a] Department of Biology, University of Wisconsin–River Falls, River Falls, WI, United States
[b] Department of Microbiology, University of Iowa Carver, College of Medicine, 3550 BSB, 51 Newton Road, Iowa City, IA 52242, United States
[c] Department of Biostatistics, University of Iowa College of Public Health, Iowa City, IA, United States
[d] Center for Ecology and Conservation Biology, Department of Biology, Boston University, Boston, MA, United States

ARTICLE INFO

Article history:
Received 9 April 2010
Received in revised form 2 June 2010
Accepted 4 June 2010
Available online 19 June 2010

Keywords:
Heavy chain variable region
Repertoire
Somatic hypermutation
Bats
Phylogeny

ABSTRACT

Myotis lucifugus populations in Northeastern US are being decimated by a fungal disease. Since almost nothing is known about the immune system of bats, we are characterizing the immunoglobulin genes of bats. We show that *M. lucifugus* has a diverse V_H gene repertoire comprised of five of the seven human V_H gene families and an estimated 236 V_H3 genes. 95% of these germline VH3 genes differ in FR3. A comparison of 67 expressed V_H3 genes with 75 germline V_H3 genes revealed a mutation frequency similar to fetal piglets never exposed to environmental antigens. Analysis of CDR3 regions identified at least 13 putative J_H segments and a large D_H repertoire. The low mutation frequency, highly diverse V_H, D_H, and J_H germline repertoire suggests that this species may rely more on combinatorial and junctional diversity than on somatic hypermutation raising questions about the ability of *M. lucifugus* to respond rapidly to emerging pathogens.

1. Introduction

Our laboratory became interested in bats because little was known about the bat immune system, despite the fact that they comprise 20% of all mammals, play major roles as insectivores and pollinators and are endangered in the USA by an emerging fungal disease called white-nose syndrome (WNS; Kunz and Lumsden, 2003; Simmons, 2005). Megabats serve as vectors for various human pandemic viral diseases (Calisher et al., 2006). *Myotis lucifugus* is a small insectivorous bat with a range extending from central Alaska to the southern edges of Mexico. *M. lucifugus* can carry rabies (less than 1%), histoplasmosis and, of course, WNS. WNS was first documented in a cave near Albany New York in 2006. WNS has been found in Connecticut, Massachusetts, New York, Vermont, Pennsylvania, New Jersey, New Hampshire, West Virginia, and Virginia. This disease is named after the white fungal growth that appears on muzzles, ears, and wing membranes of hibernating bats. The fungus has been identified as being a new species of *Geomyces*, called *Geomyces destructans* (*G. destructans*) although *G. destructans* has not yet been proven to be the cause of death of the bats (Blehert et al., 2009; Gargas et al., 2009). WNS has a very high mortality rate ranging from 78 to 97% in North America and is not limited to *M. lucifugus*, but this species seems especially vulnerable.

In a separate study we show that bats from four diverse taxa transcribe IgM, IgD, IgE, IgA and multiple IgG subclasses; this is the canonical pattern of eutherian mammals, not that of birds (Butler et al., unpublished data). This was an important observation because it revealed that adaptation to flight by a mammal did not seem to alter the canonical mammalian organization of the heavy chain genome. However, that phylogenetic study generated a panel of >70 VDJ transcripts that contained 67 different V_H3 family gene sequences from *M. lucifugus*. Given that these transcripts came from an adult, this diversity could be due to somatic hypermutation (SHM), somatic gene conversion (SGC) or a diverse germline V_H repertoire or a combination of all three. SGC, used by birds, is rare among mammals except rabbits (Becker and Knight, 1990) but since some consider bats to be primitive eutherian mammals (Hill and Smith, 1984) they may provide another example of SGC.

The heavy chain variable region genome (V_H or IGHV) encodes the antibody-binding site which determines antibody specificity for antigens. The size and diversity of the V_H genome and how it is used to generate the antibody repertoire vary among mammals. In mice and rabbits the genome is relatively large, e.g. BALB/c mice have ~100 functional V_H genes plus many pseudogenes that represent 14 different V_H gene families (Das et al., 2008; Honjo and Matsuda, 1995). By contrast, the domestic swine and certain other artiodactyls have a small genomic repertoire of <30 V_H genes, often

* Corresponding author. Tel.: +1 319 335 7776; fax: +1 319 335 9006.
E-mail address: john-butler@uiowa.edu (J.E. Butler).

0145-305X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2010.06.004

entirely members of the ancestral V_H3 family (Butler, 1997, 2006; Schroeder et al., 1990). The manner in which mammals mount adaptive immune responses to pathogens can depend in part on the organization and diversity of the heavy chain variable region genome but also on mechanisms like SHM and SGC. To characterize the mechanism used by bats, we pursued our observation of a diverse expressed V_H3 repertoire by *M. lucifugus* by examining the size and character of the germline V_H repertoire of this bat species. Thus we generated a library of germline V_H3 family genes from the same bat from which the diverse transcripts of V_H3 genes were recovered. Data presented here are the first effort to address the origin of the heavy chain variable region repertoire expressed by this insectivorous bat. Our data reveal a V_H repertoire encoded by homologs of five of the seven human V_H gene families, including approximately 236 germline V_H3 genes, and extensive J_H and D_H diversity. Surprisingly we found little evidence of SHM or evidence of SGC. This observation raises can be interpreted to mean that bats generate their heavy chain variable region repertoire through combinatorial diversity, not SHM or SGC.

2. Materials and methods

2.1. Source of tissue

Tissues from *M. lucifugus* were collected from bats collected at a maturity roost in eastern Massachusetts. The tissue was placed in liquid nitrogen and shipped to Iowa on dry ice.

2.2. DNA, RNA and cDNA preparation

DNA was extracted from the liver of an adult *M. lucifugus* following the DNAzol isolation procedure (Invitrogen, Carlsbad, CA). The concentration of DNA recovered was measured using a NanoDrop 1000 spectrophotometer and diluted to a concentration of 0.2–0.3 μg/μl for PCR amplification. RNA and subsequently cDNA were prepared from the spleen of the same *M. lucifugus* bat, according to well-established procedures (Sun et al., 1994; Butler et al., 1996).

2.3. Recovery of VDJ-Cγ transcripts

VDJ-Cγ transcripts were initially recovered using porcine primer sets in a two-step hemi-nested PCR system (Suppl. Table 1). The PCR product was analyzed on an agarose electrophoretic gel (Fig. 1) and then cloned into the pCR TOPO 2.1 following the manufacturer's protocol (Invitrogen Carlsbad, CA). The transformed bacteria were then plated on Luria–Bertani agar Petri dishes containing 50 μg/ml kanamycin (LB-Kan) and incubated overnight at 37 °C. Positive clones were selected using the blue–white system. White clones were grown in about 3 ml of LB-Kan overnight. Samples were then processed using a Qiagen Quick lyses kit (Valencia, CA) and digested with *Eco*RI for 2 h to release the inserted DNA after which the product was again analyzed on an agarose electrophoretic gel. Samples showing a single nucleotide band in the 1.3 kb range (Fig. 1) were selected and sequenced in the core facility of the University of Iowa using an Applied Biosystems Model 3730 (96 capillary) DNA sequencer. Sequence analysis revealed that the V_H genes expressed with IgG (Cγ) belonged to the V_H3 family. From these sequences a bat-specific primer set was constructed which lead to the recovery of VDJ-Cγ sequences containing 67 unique V_H3 gene sequences (Suppl. Table 1). In addition, the IMGT database <http://www.imgt.org> was used to construct degenerate forward primers for the other six V_H families known to be present in the human repertoire (Suppl. Table 1). These were used to recover VDJ-Cγ transcripts belonging to other V_H families in *M. lucifugus*.

2.4. Recovery and analysis of germline V_H genes

The bat-specific V_H3 family primer was used together with a reverse primer based on the porcine V_H RSS. This generated a sequence that enabled us to prepare a bat specific V_H3 reverse RSS primer that was then employed to recover a library of unique germline V_H3 genes from adult liver. Sequences of germline genes were analyzed using Finch TV, OMIGA, SeqWeb, and NCBI Blast and germline sequences were compared to those in the IMGT database, to those in Gen Bank and to those in recent publications and to bat V_H gene sequences in VDJ-Cγ transcripts. Germline V_H3 family nucleotide and deduced amino acid sequences were also compared to those for human, mouse and swine in regard to interspecies similarity in FR1, CDR1, FR2, CDR2 and FR3 (Table 1). Mean differences were compared using *t* tests, and differences among various regions of the gene from four different mammalian species evaluated by Chi-square tests.

2.5. Estimation of the size of the V_H3 repertoire

The size of the repertoire was determined using statistical procedures based on the frequency of recovering any particular V_H3 gene. In this procedure the size of the V_H3 repertoire is denoted as *N*. *N* was estimated using statistical methods developed for estimating

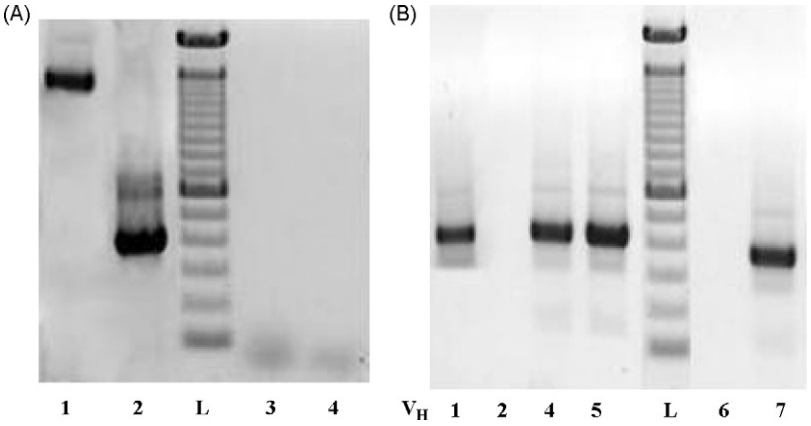

Fig. 1. Agarose gel electrophoretic analysis of PCR products containing V_H genes. (A) Lane 1: recovery of VDJ-Cγ transcripts from *M. lucifugus* using framework (FR1) primers for swine V_H genes and the conserved swine Cγ-CH2 reverse primer (Suppl. Table 1). Lane 2: recovery of bat germline V_H3 genes using the bat specific FR1 primer and the reverse primer for the RSS of *M. lucifugus*. (Suppl. Table I). L1 = 100 bp ladder. Lanes 3 and 4 = controls for lanes 1 and 2, respectively (no DNA or cDNA provided). (B) Lanes 1–7 = PCR products recovered using human FR1 primers for the different human VH families together with the porcine reverse RSS primer (Suppl. Table 1). L = 100 bp ladder Lane numbers correspond to the number of the human V_H family.