The two suborders of chiropterans have the canonical heavy-chain immunoglobulin (Ig) gene repertoire of eutherian mammals

John E. Butler, Nancy Wertz, Yaofeng Zhao, Shuyi Zhang, Yonghua Bao, Sara Bratsch, Thomas H. Kunze, John O. Whitaker Jr., Tony Schountz

A Department of Microbiology, University of Iowa, Iowa City, IA, United States
B State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing, China
C School of Life Sciences, East China Normal University, Shanghai, China
D Department of Biology, University of Wisconsin-River Falls, United States
E Center for Ecology and Conservation Biology, Department of Biology, Boston University, Boston, MA, United States
F Department of Biology, Indiana State University, Terra Haute, IN, United States
G School of Biological Sciences, University of Northern Colorado, Greeley, CO, United States

ARTICLE INFO

Article history:
Received 23 July 2010
Received in revised form 24 August 2010
Accepted 25 August 2010
Available online 9 September 2010

Keywords:
Bat
Immunoglobulin genes
Phylogeny

ABSTRACT

Bats comprise 20% of all mammals, yet little is known about their immune system and virtually nothing about their immunoglobulin genes. We show that four different bat species transcribe genes encoding IgM, IgE, IgA and IgG subclasses, the latter which have diversified after speciation; the canonical pattern about their immunoglobulin genes. We show that four different bat species transcribe genes encoding IgM, IgE, IgA and IgG subclasses, the latter which have diversified after speciation; the canonical pattern of CH1, CH3 and two hinge exons; the second hinge exon was fused to CH3. IgA in all species resembles human IgA2 with the putative cysteine forming the bridge to the light chain found at position 77. Sequence comparisons yielded no evidence for a diphyletic origin of the suborders. Bats show no close similarity to another mammalian order; the strongest association was with carnivores. Data reveal that CH diversity and VDJ and CDR3 organization are similar to other eutherian mammals, although the expressed VH3 family repertoire was unusually diverse.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Among living vertebrates, bats and birds are unique in their ability to fly, and this common feature sets them apart ecologically and physiologically from other groups. Bats are in some ways the nocturnal equivalent of birds, having evolved and radiated into a diversity of forms to fill many of the same niches. Bats have successfully colonized almost every continental region on earth (except Antarctica) as well as many oceanic islands and archipelagos (Kunz, 1982; Kunz and Lumsden, 2003). Bats impact the environment in several ways. Insectivorous species suppress insect populations (Cleveland et al., 2006; Kalka et al., 2008; Williams-Guillen et al., 2008; Jones et al., 2009), and plant-visiting bats serve as pollinators and disperse seeds (Fujita and Tuttle, 1991; Shilton et al., 1999; Muscarella and Fleming, 2007; Fleming et al., 2009). Insectivorous species like the common little brown bat (Myotis lucifugus) may consume their entire body mass each night in insects (Kurta et al., 1989). Thus, they are the most important predators of nocturnal insects and consume 5–10 times more insects than swallows and flycatchers. So-called “megabats” or “flying foxes” of the suborder Yinpteropterae are important pollinators and seed dispersers, but sometimes can be pests when they damage fruit crops.

Bats comprise the second largest order of mammals (next to rodents) in total number of species and may exceed all other groups in overall abundance (Kunz, 1982; Wimsatt, 1970). Of the >5400 recognized species of mammals, bats comprise 20% (Teeling et al., 2002; Simmons, 2005). Bats are believed to have evolved early and morphologically have changed very little over the past 52 million years (Hill and Smith, 1984; Simmons et al., 2008). Bats are divided into 18 families that comprise two major suborders: Yinpteropterae and Yangchiroptera. The former suborder includes the family Pteropodidae which comprises the so-called “megabats” or “flying foxes” and the families Rhinolophidae, Hipposideridae, Megadermatidae, Rhinopomidae and Crasoz cysticercidae while the Yangchiroptera includes all other families including the insectivorous “microbats” (Teeling et al., 2002). Except for the genus Rousettes, all of the families Pteropodidae are non-echolators compared to all other species of both suborders. The appearance of certain megabats (flying foxes) and neurological features of the eye and prestrinate cortex lead Pettigrew to propose that the order was...
diphyletic, i.e. the Yinpteropodidae arose from a primate/lemurs stock while all other bat families evolved from the Insectivora (Pettigrew, 1986). This was an intriguing hypothesis because it implied that mammalian flight evolved twice. However, advances in molecular analyses, including what we report here, have largely laid to rest the diphyletic hypothesis for the Chiroptera (Murphy et al., 2007; Teeling et al., 2005; Adkins and Honeycutt, 1991; Mindell et al., 1991). Bats were originally considered members of the superorder Archonta which also included primates, tree shrews and flying lemurs (Gregory, 1910; Simpson, 1945). Recent analyses place bats closer to ungulates, whales, horses and carnivores (Teeling et al., 2002, 2005) [a clade referred to as Laurasiatheria].

All bat species can be vectors for human viral diseases (Messenger et al., 2003; Calisher et al., 2006; Wibbelt et al., 2009). There are >85 viruses known from bats (Calisher et al., 2006) and several are associated with animal and human epidemics (Wong et al., 1991). Since the work of Teeling et al. (2002, 2005) leaves little doubt that bats are indeed part of the mammalian lineage, we did find immunological evidence for the diversification of the two suborders. Our analysis revealed a surprising and unexplained diversity among expressed VH3 genes of the little brown bat, M. lucifugus, which raises the question of whether bats might use SGC similar to that of birds, extensive somatic hypermutation (SHM) or combinatorial diversity to develop their specific antibody repertoire. The latter question has been addressed in a separate study that shows extensive germline diversity but a surprising lack of SHM (Bratsch et al., in press).

2. Materials and methods

2.1. Source of materials

Spleen and other lymphoid tissues were removed from euthanized little brown bats (M. lucifugus) that had been collected at a maternity roost in eastern Massachusetts. The same tissues were also collected from the big brown bat (Eptius fuscus) from western Indiana. Both species are members of the family Vespertilionidae, suborder Yangochiroptera. The same tissues were collected from the Seba’s short-nosed fruit bat, Carollia perspicillata (family Phyllostomidae suborder Yangochiroptera) as by-products of viral research at Northern Colorado University. CDNA from the short-nosed fruit bat Cynopterus sphinx (family Pteropodidae), was prepared at the East China Normal University and at the China Agricultural University. Apart from the latter, all samples were collected and frozen in liquid nitrogen and shipped on dry ice to the University of Iowa.

2.2. Preparation of RNA and cDNA

RNA was extracted from pulverized frozen tissue or from TriZol-stored material following the manufacturer’s protocol (Invitrogen, Carlsbad, CA) and as previously described (Sun et al., 1998). RNA concentration was determined spectrophotometrically using a NanoDrop 1000. CDNA was prepared using Superscript II as previously described (Butler et al., 2006).

2.3. Recovery of bat Ig transcripts by PCR

Transcripts encoding bat IgM, IgD, IgG, IgA and IgE were recovered by PCR using degenerate primer sets. Table 1 indicates the

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Primer forward</th>
<th>Reverse</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>PCR1 FR1 5'</td>
<td>actctccttgcaagcccttg</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>PCR1 FR1 3'</td>
<td>aatctccttgcaagcccttg</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>PCR1 FR1 5'</td>
<td>actctccttgcaagcccttg</td>
<td>1400</td>
</tr>
<tr>
<td></td>
<td>PCR1 FR1 3'</td>
<td>aatctccttgcaagcccttg</td>
<td></td>
</tr>
<tr>
<td>IgD</td>
<td>PCR1 FR1 5'</td>
<td>actctccttgcaagcccttg</td>
<td>1450</td>
</tr>
<tr>
<td></td>
<td>PCR1 FR1 3'</td>
<td>actctccttgcaagcccttg</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>PCR1 FR1 5'</td>
<td>actctccttgcaagcccttg</td>
<td>1300</td>
</tr>
<tr>
<td></td>
<td>PCR1 FR1 3'</td>
<td>actctccttgcaagcccttg</td>
<td></td>
</tr>
<tr>
<td>IgE</td>
<td>PCR1 FR1 5'</td>
<td>actctccttgcaagcccttg</td>
<td>1500</td>
</tr>
</tbody>
</table>

a FR1 5' = framework 1 of VH3 genes. FR1 3' is a nested primer that targets a sequence downstream from FR1 5'.
b Products of expected length were obtained, cloned and sequenced.
c All genes encoding Cy genes share a common UTR sequence.
primers used and the domain sequences targeted by the primers employed in first (PCR1) and second (PCR2) round PCR amplification and the expected length of the final PCR products. Since the VH3 family is considered most ancestral (Schroeder et al., 1990) we used the FR1-5′ primer based on the VH3 genes of swine and other species that have VH3 genes in their genome (Butler, 2006; Butler et al., 2006). A hemi-nested PCR system was used in which the second round used the internal forward FR1-3′ primer (Table 1), but the same reverse primers that annealed to sequences encoding the CH3, CH4 or UTR regions of the various isotypes. PCR product length was evaluated on agarose gels and those of expected length were excised from the gels and cloned into pCR2TOPO according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The ligation mixture was plated on L-B plates. Blue/white selection was used to identify transformants.

**Fig. 1.** The deduced amino acid sequences of the heavy chains of IgM, IgD, IgE and IgA from four bats fit the canonical pattern for the corresponding human Ig isotypes. Amino acid positions of interest that are referred to in the text, are in boldface and are numbered according to the IMGT system (Lefranc and LeFranc, 2001). Cysteines involved in the intradomain S–S loop are in bold with arrows indicating the loop. The cysteines involved in the covalent bond to the light chain, are also in bold and indicated as L–H. Potential N-linked glycosylation sites are in boldface. Position in each domain start with 1 as is done in the IMGT system. The functional hinge of IgA is part of the 5′ portion of the CH2 domain. Dots indicate that the residue is the same as for the reference human sequence. Human IgA2 was used for comparison since the extended hinge of human IgA1 is unique among mammals. Gaps are inserted into the alignment to obtain the best possible alignment with the human sequence that was used as a reference. Bat sequences have been deposited in GenBank and the accession numbers and those for the human sequences that were used for comparison are as follows: M. lucifugus = IgA, HM134924; IgD, HM134925; IgM, HM134926; E. fuscus = IgA, HM134927; IgM, HM134928; C. perspicillata = IgA, HM134931; IgD, HM134932; IgM, HM134933; C. sphinx = IgA, HM134944; IgM, HM134945; C. sphinx = IgA, HM134948; C. sphinx = IgA, HM134950; IgM, HM134951; IgM, HM134953.
to recover colonies that were subsequently prepared for sequence analysis. The samples were then processed using a Qiagen Quick lyses kit (Valencia, CA) and digested with EcoR1 for 2 h to release the inserted DNA after which the product was again analyzed on an agarose electrophoretic gel to confirm that products of the expected length were recovered.

2.4. Sequence analysis

Clones processed as described above were sent to the core sequencing facility of the University of Iowa where they were analyzed using an Applied Biosystems Model 3730 (96 capillary) DNA sequencer. Our data are presented using the IMGT system in which the start codon for each domain is number 1 (Fig. 1). We have no evidence for variants at each position that are described for human variants on the IMGT website http://www.imgt.org.

2.5. Recovery of genomic IgD

Sequences from expressed IgD from M. lucifugus were used to recover genomic IgD sequences (scaffold_15451, 11.5 kb) by applying BLAST to the partially sequenced genome of M. lucifugus (http://www.ensembl.org/Myotis_lucifugus/Info/Index). A new primer was made by aligning the sequences from the transcribed IgD from M. lucifugus with the sequence recovered from BLAST to identify heavily conserved areas of bat IgD. This new primer recovered IgD from E. fuscus spleen cDNA.

2.6. Analysis of VDJ transcripts

VDJ sequences, generated as by-products of efforts to identify the various Ig heavy-chain genes, were analyzed with regard to the recovery of identical VH genes (Table 2) and for the characteristics of their CDR3 regions (Table 3). Sixty-six IgG VDJ transcripts and seven VDJ transcripts for IgM, IgD, IgE and IgA were recovered from the spleen of M. lucifugus. In addition, 51 VDJ transcripts were also recovered from E. fuscus, C. perspicillata and C. sphinx during the procedure to recover their genes transcribing IgM, IgG, IgA and IgE. These transcripts allowed for a partial characterization of transcribed VDJs.

2.7. IgG subclass analysis

Putative IgG subclass/allotype sequences recovered from all four bat species were compared in PileUp. The translated sequences were then grouped and aligned based on the PileUp so that major differences within and among species could be identified. Major differences were identified in the hinge region, intraspecific comparisons of hinge sequences for different bat IgGs and for human, mouse, cattle and swine IgG subclasses were compared.

2.8. Phylogenetic comparisons of immunoglobulin heavy-chain constant regions genes

Phylogenetic trees were constructed with PHYLIP 3.67 software combined with the TreeView package (Felsenstein, 1989; Page, 1993).
Table 2

Recovery of identical FR4 sequences was common while recovery of identical transcribed VH genes was not.

<table>
<thead>
<tr>
<th>VH</th>
<th>CDR1</th>
<th>CDR2</th>
<th>JH-FR4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Identical</td>
<td>Sequence</td>
</tr>
<tr>
<td>M. lucifugus</td>
<td>73</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>SYWMK</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>SSYMK</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>SYSMK</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>DSSMN</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>TSNMN</td>
<td>2</td>
</tr>
<tr>
<td>E. fuscus</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>SHYMS</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Unique</td>
<td></td>
</tr>
<tr>
<td>C. perspicillata</td>
<td>16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NTMEN</td>
<td>2</td>
</tr>
<tr>
<td>C. sphinx</td>
<td>15</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>SSMN</td>
<td>15</td>
</tr>
</tbody>
</table>

a Same CDR1 (underlined) found in two different species.
b The same JH was found in all four species of bats (also underlined).
c JH found in two of the bat species examined.

1996). Multiple sequence alignments for tree construction were generated using CLUSTAL. Prodist and Neighbor programs in the PHYLIP package were also used for tree construction. A consensus tree was taken from 1000 bootstrapped phylogenetic trees.

3. Results

3.1. Bats possess the canonical isotype repertoire of eutherian mammals

Fig. 1 provides an alignment of the deduced amino acid sequences of IgM, IgD, IgE and IgA from four bat species with that of the corresponding isotypes from human. Given that IgG isotypes from bats appear to have diverged prior to the appearance of the major mammalian orders (Fig. 2), their sequences are separately compared (see below). The major motifs for the five human isotypes (and other eutherian mammals; data not shown) are present in all four bat species investigated in the present study. For those shown in Fig. 1, the intrachain disulfide loop in each domain is also present in the corresponding domains of bat Igs and the sequences adjacent to the cysteines involved in these bridges are generally conserved. With some exceptions, the major potential N-glycosylation sites are conserved for each isotype in bats. In the case of IgM and IgA, these are identical. This also appears to be the situation for IgD although the CH2 domain of bat IgD is absent (Figs. 1 and 5). IgE is most divergent by this criterion since the N-glycosylation site in CH1 (position

Table 3

CDR3 sequences motifs and CDR3 lengths in VDJ recovered with various heavy-chain transcripts.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isotype</th>
<th>Sequence of CDR3</th>
<th>Frequency</th>
<th>CDR3 length (range)</th>
<th>Mean CDR3 length</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. lucifugus</td>
<td>IgM</td>
<td>R^<em>DELGSPFDV W^</em></td>
<td>4</td>
<td>8–10</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>YQAGAALAY W</td>
<td>5</td>
<td>6–12</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>IgE</td>
<td>DGStVVTMEN W</td>
<td>3</td>
<td>6–12</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>IgD</td>
<td>NHUGDFDV W</td>
<td>2</td>
<td>9–10</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>DNHRDFDV W</td>
<td>66</td>
<td>4–12</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>VVCAASGICYWFDW</td>
<td>3</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>DGQVTQYLDW W</td>
<td>5</td>
<td>6–11</td>
<td>8.8</td>
</tr>
<tr>
<td>E. fuscus</td>
<td>IgE</td>
<td>DVPGLYFDW</td>
<td>3</td>
<td>7–8</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>IgD</td>
<td>PPVLVAAQFDW W</td>
<td>4</td>
<td>11–13</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>RMDAAGDFDV W</td>
<td>9</td>
<td>4–13</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>EYQFGSTY W</td>
<td>1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>GCGAYNEDW Y</td>
<td>4</td>
<td>6–14</td>
<td>9.5</td>
</tr>
<tr>
<td>C. sphinx</td>
<td>IgE</td>
<td>DLNTIDLY W</td>
<td>1</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>IgD^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>DOTQTVHPSYD W</td>
<td>10</td>
<td>8–12</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>DLNSOY W</td>
<td>5</td>
<td>7–9</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>IgA</td>
<td>CKNSDFYFY W</td>
<td>4</td>
<td>4–14</td>
<td>8.8</td>
</tr>
<tr>
<td>C. perspicillata</td>
<td>IgE</td>
<td>DVQGQTYMID W</td>
<td>2</td>
<td>10–12</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>IgD^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>SRYGHYGYSEY W</td>
<td>7</td>
<td>4–12</td>
<td>9.6</td>
</tr>
</tbody>
</table>

a The boundaries of CDR3 between the terminal arginine (R) of the FR3 of VH3 family genes and the invariant tryptophan of FR4 (W) are indicated.
b IgD has not been recovered from the cDNA of C. sphinx and C. perspicillata.
Fig. 2. Genes encoding the heavy-chain genes of IgM, IgD, IgE and IgA of bats comprise a separate group with highest similarity with carnivores. The phylogenetic tree was constructed by using amino acid sequences of the first and last CH domains (platypus δ CH7 was used as the last domain for comparison) of these heavy-chain isotype. The heavy-chain constant regions are indicated with the appropriate Greek letters. M = M. lucifugus, E = E. fuscus, Ca = C. perspicillata; Cy = C. sphinz. For the convenience of readers, the regions dealing with these four isotypes are indicated in boxes. The values supporting each node are derived from 1000 bootstrapped phylogenetic trees. The accession numbers for the sequences used are for C/H9251: cattle, AF109167; dog, L36871; dolphin, AY62310; echidina, AF416951; horse, AY247966; human, J00220; mouse, J00475; opossum, AF012110; panda, AY818387; swine, U2594; platypus, AY055778; rabbit, X51647; rat, AABR03049801; sheep, AF24645; C: cattle, AF515672; dog, DQ297185; horse, AY631942; human, BC02172; mouse, V00786 and V00788; panda, AY818394; swine, AF411239; platypus, EU503149; rat, AA01964; sheep, AF411238. C: cattle U63640; dog, L36872; echidina, AY09258; horse, AJ05046; human, J00222; mouse, X01857; opossum, AF03519; panda, AY818389; swine, U96100; platypus, AY055780; rat, K22901; sheep, M84356. Ca: cattle, U63637; camel, ABO1672; dog, EF207721 and EF207724; dolphin, AAG40853; echidina, AF416952; horse, L49414; human, X14940; mouse, V00818; opossum, AF01210; panda, AY818392; swine, U50149; platypus, AY168639; rabbit, J00666; rat, AABR0304980; sheep, X59994. Accession numbers for bat species are the same as those given in Figs. 1 and 3.
Fig. 3. The IgG subclasses of bats diversified after speciation as in other eutherian mammals. The phylogenetic tree was constructed based on the amino acid sequences of the first CH domain of all IgG subclasses which is highly species-specific. The designation for bat species is the same as for Fig. 2, i.e. M, E, C and Cy. These designations are shown in boldface. The accession numbers for the sequences compared are: cattle (X16701, M36946, U63638), camel (AJ421266, AJ131945), dog (AF354264, AF354265, AF354266, AF354267), horse (AJ302055, AJ302056, AJ312379, AJ302858, AJ12380, AJ12381), human (J00228, J00230, X03604, K01316), mouse (J00453, V00825, V00763, J00479, X00915), opossum (AF051195), swine (U03778, U03779, U03782, EU372658, EU372657, EU372655), possum (AF157619), rabbit (J29172), rat (AABB03049895, AABB03049905, AABB03049912), sheep (X09797, X09813). GenBank numbers for bats Cy sequences are: M. lucifugus (HM134929, HM134930, HM134931, HM134932, HM134933, HM134934, HM134935), E. fuscus (HM134942), C. perspicillata (HM134946), C. sphinx (HM134951, HM134952).
Fig. 4. The IgG subclasses of bats, like other eutherian mammals, differ most in their hinge regions and hinge motifs are not shared among species. The amino acid sequences of the hinge regions of IgG from different bat species and for subclasses of human, mice, cattle and swine are presented. Sequence motifs shared by the hinge regions of different subclasses within a species are underlined. The $-1$, $-2$, etc. associated with subclasses in cattle and humans indicate the use of alternative hinge exons. Superscripts for swine C$^\alpha$/H9253 genes denote allotypic variants. The accession numbers for the cg hinge sequences are the same as those listed in the legend of Fig. 3.

21) is not present in any bat species we examined. Moreover, the potential N-glycosylation motif in the CH2 (position 43) is missing in all bats and only one of the three potential N-glycosylation sites in the CH3 domain of human IgE (position 65) is found in bat IgE. Because of this observation we examined the IgE sequences of several other mammalian IgEs including the duckbill platypus and the Virginia opossum. We found that the N-glycosylation motif at position 21 in CH1 was common to human, mouse, cattle and swine but was missing in all bats, in the opossum and the platypus. Cattle have an additional site at position 22.

Of particular interest is the absence of a cysteine in the CH1 domain of bat IgA that is part of the covalent S–S bond to the light chains in human IgA1. Thus we used human IgA2 as our reference because it also lacks this cysteine. Furthermore human IgA2 lacks the unique 13 amino acid hinge of human IgA1 that is not found in any other mammal. This means that bat IgA resembles human IgA2 and that of cattle and swine but was missing in all bats, in the opossum and the platypus. Cattle have an additional site at position 22.

3.2. IGHC gene sequences of bats form a separate group most similar to carnivores

Phylogenetic analysis of IgM, IgD, IgE and IgA gene sequences from bats indicates that especially IgM and IgD share their greatest similarity to that of carnivores (Fig. 2). While IgE from bats also shares sequence homology with carnivores, it is also related to IgE from the horse, swine and the human; rodents and ruminant artiodactyls are more distantly related. A somewhat similar pattern of sequence similarity was found for bat IgA. Noteworthy is that the sequence similarity of IgE, IgM and IgA, which corresponds to the accepted taxonomic relationship for the bats included in this study (Fig. 2). Given that IgM and IgD are the two most primordial Ig isotypes among vertebrates (Ohta and Flajnik, 2006) the comparison of the Cµ and Cß from bats to those in other mammals should be most reliable. Using this criterion, a common ancestry of bats with carnivores is suggested.

3.3. Subclass diversification occurred after speciation

The branch points for the various IgG subclasses appear to have occurred after species diversification (Fig. 3). Our data also show that the IgG from the Neotropical, Seba’s short-tailed fruit bat, C. perspicillata and those from the Paleotropical, short-tailed fruit bat C. sphinx form separate branches from the one leading to the insectivorous members of the Yangochiroptera, M. lucifugus and E. fuscus, of the family Vespertilionidae. On the basis of transcripts recovered, IgG in M. lucifugus has diversified to the greatest extent; seven different transcripts were recovered, whereas the transcript of only a single IgG was recovered from C. perspicillata. While some variants may be alleles, this cannot explain all the variants in M. lucifugus or C. sphinx because all sequences were recovered from tissues of the same individuals.

3.4. IgG subclass genes in bats differ in the hinge region and differences appear order-specific

Alignment of the various Cγ transcripts recovered from different bat species revealed that the putative bat IgG subclass genes show their greatest difference in their hinge region. This follows the pattern observed in other species (Butler et al., 2009). Thus, we focused our comparison on variations in the hinge of the various Cγ genes from the four species of bats available for the present study. Since the nomenclature of subclasses in all species is based on order of discovery, same-name homology is not implied. This should also be apparent from Fig. 3, which shows that bat IgG subclasses diversified after speciation, as observed for other mammalian orders (Butler, 2006) making sequence comparisons of subclasses between different species of little phylogenetic relevance. Therefore, sequence similarities between the four bat
species in the present study are postulated to reflect either convergent or parallel evolution.

The hinge sequences of IgG from different mammalian taxa, including bats, illustrate several features of evolutionary and functional importance. First, nearly all hinges are characterized by the presence of a combination of prolines (often paired) in association with cysteines. Second, the actual motif for this feature appears to be species- (or order-) specific; these are illustrated by the underlined motifs in Fig. 4. In some cases different IgG subclasses share nearly the same hinge. This is demonstrated in swine (Butler et al., 2009; Fig. 4) but has been observed in M. lucifugus, C. perspicillata and C. sphinx. The exception is in cattle. Alternative and duplicated hinges also occur. This is best illustrated by IgG3 in humans and cattle; this is also a common feature of the domain structure of IgG in some species. Since the genomic structure of the bat Cy genes has not yet been determined, this may also be true for some bat IgG subclasses.

3.5. The domain structure of bat IgD is unique but is otherwise most similar to rodents

A partial genomic δ sequence (scaffold_15451, 11.5 kb) of M. lucifugus was retrieved from its genome database using a BLAST-based approach. Although the typical transmembrane (TM) encoding exons are missing in the retrieved sequence, two constant region (C) domain encoding exons, could be identified with one corresponding to CH1, and the other to the CH3 of other known placental mammalian IgD heavy chains. The genomic structure of IgD from a variety of vertebrates was compared with the partial genomic structure of IgD from M. lucifugus (Fig. 5). These data show that the domain structure of IgD from M. lucifugus is most similar to that of rodents in having only one hinge exon and in lacking a CH2 domain; H2 and CH3 are fused exons in M. lucifugus. Despite this rodent-like feature, sequence similarities place bat IgD closer to that of carnivores than rodents (Fig. 2). In no eutherian mammals is there evidence for the multi-domain structure of IgD seen in the duckbill platypus, the African clawed frog (Xenopus sp.) and the catfish (Ohata and Flajnik, 2006; Zhao et al., 2006, 2009).

3.6. The transcribed VH3 repertoire is diverse

The primers used to recover the various C-region genes in bats involved the use of forward primers for the ancestral VH3 variable gene family. Thus, the 140 transcripts recovered all contain VDJs encoded by VH3 genes. Because VH3 family genes in all species differ primarily in CDR1 and CDR2, our analysis focused on these gene segments. Sixty-one percent of the transcripts had CDR1 sequences that were only recovered once, 13% had CDR1s that were recovered twice and 4% had CDR1s that were recovered six times (Table 2). Being a longer sequence, a higher proportion of CDR2 sequences were unique (75%). By contrast only 14% of JH-FR4 region sequences were unique suggesting that there are a limited number of JH genes in the genome but many different VH3 genes.

Of the 73 VDJ sequences recovered form M. lucifugus, none contained identical VH genes when both CDR1 and CDR2 were considered, although some of these VH genes share CDR regions with other VH genes. Sharing was most common among the shorter (15 nucleotides) CDR1 segments. In one case an identical CDR1 from M. lucifugus was also found twice in C. sphinx. By contrast, identical FR4 regions (JH encoded) were quite frequently recovered. The number and sequences of the various JH segments is unknown since the genome has not been mapped. In any case, the frequency of FR4 recovery suggests that there are fewer JH than VH genes and that M. lucifugus probably has at least five JH gene segments in its genome. The limited diversity of JH is also supported by the observation that all four bat species in the present study share one identical FR4. Since the species studied represent different families and suborders; (E. fuscus and M. lucifugus belong to the same Vespertilionidae, suborder Yangochiroptera, C. perspicillata to the family Phyllostomidae, suborder Yangochiroptera, and C. sphinx to the family Pteropodidae, suborder Yinpterochiroptera) this JH gene is evolutionarily conserved.

All VDJ sequences were from transcripts so that the diversity among these VH3 genes may be the result of somatic hypermutation (SHM) of CDR1 and CDR2 regions. This issue has been recently addressed in a separate study (Bratsch et al., in press).

3.7. The CDR3 regions of VDJ transcripts resembles that of most mammals

The CDR3 portion of transcribed VDJs mostly results from contributions by DH segments, differences in the 5' end of JH gene segments, nucleotide additions and SHM. Table 3 summarizes data from the most frequently encountered CDR3 regions transcribed with different isotypes of the four bat species. Our data indicate that the mean length of CDR3 was 9.1 ± 1.1 amino acids; the same length found in mice, humans and swine. We found no significant differences in CDR3 length among the isotype or the various species. Identification of putative DH segments in CDR3 was not possible because: (a) the same motif was never recovered twice among the 140 transcripts and (b) there is currently no information available on the genomic DH repertoire of bats.

4. Discussion

Biologists have long recognized the importance that 20% of the world’s mammals play in the ecosystem. They suppress aerial insect populations, pollinate flowers and disperse seeds (Cleveland et al., 2006; Kalka et al., 2008; Williams-Guilén et al., 2008; Shilton et al., 1999; Fleming et al., 2009). Despite their ecological importance, interest in their immune system has been almost entirely limited to the Old World family Pteropodidae (suborder Yinpterochiroptera).
Chakraborty and Chakravarty (1984) studied the immune system of the Indian flying fox (Pteropus giganteus) using a hemolytic plaque assay and (Omatsu et al. (2003) purified IgG from the Egyptian flying fox (Rousettus aegyptiacus, family Pteropodidae) and used it in serological comparison with other mammals. In studies on histoplasmosis, McMurry et al. (1982) purified serum Igs from the New World fruit bat, Artibeus lituratus and monitored changes during infection. However, to our knowledge, no attention has been given to the Igs of insectivorous bats in the suborder Yangochiroptera, and no effort has been made to clone and characterize the heavy-chain constant region Ig genes of any bat species.

The studies herein were undertaken for three reasons. First, by addressing the growing concern for emerging diseases carried by bats, we reasoned that an understanding of their immune system might provide valuable insight that could indirectly aid in mitigating these diseases. Bats carry >85 viruses and are vectors for a variety of human pandemic disease like SARS, Hendra and Ebola (Calisher et al., 2006). WNS, a fungal disease of hibernating bats in the eastern US (Blehert et al., 2009; Gargas et al., 2009), has caused one of the most precipitous declines ever reported for North American wildlife, that is threatening regional extinction (Frick et al., 2010). Second, we wished to determine whether bats might show differences in their Ig repertoire or its formation from that seen in other mammals. Finally, we reasoned that by comparing the Ig genes of different bats species with those of other mammals, we could improve our understanding of mammalian phylogeny in general and chiropteran phylogeny in particular. Various hypotheses have been proposed linking bats with various taxa including primates, rodents, insectivores, carnivores and ungulates (Teeling et al., 2002, 2005). A relatively recent hypothesis even proposed that the two suborders (i.e. so-called mega- and microbats), evolved from two separate lineages (Pettigrew, 1986).

Our findings are unambiguous in showing that Ig genes of bats fit the canonical pattern of eutherian mammals, neither birds nor proto- or protherian mammals. All four bat species examined in the present study transcribed genes for IgM, IgG, IgA and IgE and both insectivorous species of the suborder Yangochiroptera transcribed IgD (Figs. 1 and 2) with a domain structure characteristic of eutherian (e.g. placental) mammals but not of Protheria (e.g. monotremes) or lower vertebrates (e.g. amphibians and fish; Fig. 5). We were unable to recover a transcript for IgD from either C. perspicillata or C. sphinx. IgD in mammals shows the least interspecific homology among all Ig isotypes and has therefore been elusive to recover (Butler et al., 2006; Butler et al., 1996). It is likely that when genomic projects for bats other than M. lucifugus appear, IgD may also be found in the genome of other bat species, with an altered sequence and possibly as pseudogenes. Since IgD has been lost during evolution in some cases such as in birds, marsupials and even the eutherian rabbit (Mage et al., 2006), its loss in one suborder of bats may not be surprising.

The differences among mammals in the existence of the cysteine at position 15–18 (Fig. 1) in the CH1 domain that forms a bridge to the light chain in human IgA, suggests that either bats, human IgA2, cattle and swine: (a) have light chains that are non-covalently bonded to the Cc chain or (b) the cysteine at position 77 (Fig. 1) is used in the L–H bridge. Because there is no biochemical evidence of non-covalent L chains in swine and cattle, the latter hypothesis is most likely. Our results suggest that in the diversification of mammalian IgA, the pathways diverged in Cc1 resulting in the human being the only mammal outside of the exceptional rabbit family, which has more than one subclass and retains both motifs for covalent linkage to light chains.

Motifs for N-glycosylation are less likely to be conserved than cysteine residues but may nevertheless distinguish evolutionary pathways. In this respect, the pattern of N-glycosylation sites in all bats studied to date resembles that of the Virginia opossum and duckbill platypus, but not other mammals for which data are available.

The diversification of IgG into subclasses is primarily a feature of eutherian mammals; the Virginia opossum has only a single IgG and the duckbill platypus has two very similar IgGs (Zhao et al., 2009; Wang et al., 2009). The IgG-like Igs in other vertebrates have only distinct sequence similarities to those in eutherian mammals (Fig. 3). The IgG-like IgY of the chicken and lizard also shows no subclass diversification (Wei et al., 2009), although in the African clawed frog (Xenopus sp.) a short Ig molecule, IgF, appears to have been derived from IgY and further diversified in genus Xenopus (Zhao et al., 2006). IgG has been regarded as the “flagship Ig” of mammals and is not found in other vertebrates (Butler et al., 2009). Thus, its presence in bats and in diversified form (subclasses) further supports the conclusion that bats possess the canonical isotype repertoire of other eutherian mammals.

Also consistent with the pattern seen in eutherian mammals is that post-speciation diversification of IgG into subclasses mostly affects the sequence of the hinge exon (Butler et al., 2009). We observed the same to be true in the Chiroptera. The hinge sequences of the IgG variants from bats, together with that of other eutherian mammals, are shown in Fig. 4. These data show that specific hinge sequence motifs are often shared among the hinges of different IgGs in one species but not shared between species. Seven variants were recovered from a single specimen of M. lucifugus, so that assuming this individual was heterozygous, there must be 2–4 subclass eugenes while the remainder could be alleles of these subclasses. At this time only a single IgG has been recovered from Seba’s short-tailed fruit bat (family Phyllostomidae, suborder Yangochiroptera; Fig. 3).

IgGs with extended hinges such as human and swine IgG3 are best-suited for complement activation (Butler et al., 2009; LeFranc and LeFranc, 2001). In humans all but IgG2 are actively transported across the placenta (LeFranc and LeFranc, 2001) and IgG1 of cattle is selectively transported across the acinar epithelial cells of the mammary gland (Hammer et al., 1969; Brandon et al., 1971). Subclass IgGs also differ in their affinity for FcγR1, FcγRII and FcγRIII that are distributed among phagocytic cells and lymphocytes, resulting in a further division of labor among subclasses (Ravetch and Kinet, 1991).

Differential subclass expression can depend on whether a pro- or anti-inflammatory cytokine microenvironment is present (Mossman and Coffman, 1989). Based on the known division of function among IgG subclasses in well-studied species, the expressed subclass diversity we report here for bats predicts a similar diversification of function for bat IgGs like that seen among human IgG subclasses and in other eutherian mammals.

The organization of the heavy-chain variable region appears similar to that of other eutherian mammals. However, the low frequency of recovering a particular VH gene during a second attempt in >70 chances in M. lucifugus (Table 2) suggests an exceptionally diverse germline VH3 repertoire or a high rate of somatic hypermutation. The same applies to the recovery of putative DH segments (Table 3). A separate study indicates that diversity is germline encoded and not the result of extensive SHM (Bratsch et al., in press).

As in laboratory mice, the rabbit family and humans, at least five different FR4 regions were recovered suggesting as many as five JH regions. Furthermore one of these is highly conserved since it was found in all bat species examined in this study and was recovered 20 and 13 times respectively, in species from two different suborders (Table 2). CDR3 length was in the same range as in humans, laboratory mice and swine with no evidence for the unusually long CDR3 regions that are seen in cattle (Berens et al., 1997) or camels (Nguyen et al., 2008). Data summarized in Tables 2 and 3 therefore suggests a VH, DH and JH repertoire at least similar to humans and...
laboratory mice. These results are in stark contrast to swine (Sun et al., 1998; Butler et al., 2006).

Regarding the issue of phylogenetic origins, our data offer no evidence to support the diphylectic origin of bats (Petitgrew, 1986) and thus are consistent with the conclusions reached by others (Mindell et al., 1991; Nishihara et al., 2006; Butler et al., 2009). Regarding their relationship to other mammalian orders, the sequence similarity of the genes encoding IgM and IgD may be most valuable because these are regarded as phylogenetically the two oldest Ig genes. Using this criterion, bats are closely related to carnivores (Fig. 2). While sequence similarity of carnivores and bat IgD is strong, the domain structure is more rodent-like due to the lack of a CH2 domain (Fig. 5). Analogous with IgA, the hinge exon of bat IgD is fused to the CH3 domain. In the case of IgD, this has not been reported in other mammals. When genes for IgA and IgE are considered, a relationship with carnivores is still present but there is also a relationship with hoofed mammals (e.g. ungulates), humans and the dolphin (Fig. 2). Our data linking Chiroptera, Carnivora and Perissodactyla (horses) with the Laurasiatheria is consistent with studies using retroposon insertion (Nishihara et al., 2006) and non-coding intron sequences (Matthee et al., 2007). The relationship to the Perissodactyla was also reported by Murphy et al. (2007). Thus, from our analysis there is no evidence to support the hypothesis that bats evolved from flying primates or rodents. Since IgC diversified following the appearance of bats (Fig. 3) sequence similarity among eutherian mammals provides no insight into phylogeny but suggest that IgG diversification is convergent.

Results from our study unambiguously support the hypothesis that the Ig genes of both the Yinpteropodidae and the Yangochiroptera have the same canonical pattern of organization as in other eutherian mammals, and thus are not in support of a diphylectic origin for the Chiroptera. Their surprisingly diverse variable heavy-chain diversity appears to be germline encoded (Bratsch et al., in press). All other factors predict that the humoral immune system of bats will function in much the same manner as in well-studied species such as laboratory rodents. This means that the humoral immune responses of bats to their pathogens, are likely to follow the same pattern as in well-studied, non-volant eutherian mammals.

Acknowledgement

Research supported in part by NIH Contract AI25489.

References

Nikaida, M., Harada, M., Cao, Y., Hasegawa, M., Okada, N., 2000. Monophyletic origin of the order Chiroptera and its phylogenetic position among mammals, as
inferred from the composite sequences of the mitochondrial DNA of a Japanese megabat, the Ryukyu flying fox (*Pteropus dasymailus*). J. Mol. Evol. 51, 318–328.


