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Biological characters of bats in relation to natural reservoir of emerging viruses

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Abstract

Many investigators focused on bats (Chiroptera) for their specific character, i.e. echolocation system, phylogenic tree, food practice and unique reproduction. However, most of basic information about the vital functions related to anti-viral activity has been unclear. For evaluating some animals as a natural reservoir or host of infectious pathogens, it is necessary that not only their immune system but also their biology, the environment of their living, food habits and physiological features should be clarified and they should be analyzed from these multi-view points. The majority of current studies on infectious diseases have been conducted for the elucidation of viral virulence using experimental animals or viral gene function in vitro, but in a few case, researchers focused on wild animal itself. In this paper, we described basic information about bats as follows; genetic background, character of the immunological factors, histological character of immune organs, the physiological function and sensitivity of bat cells to viral infection.

Keywords: Chiroptera; Mitochondrial DNA; Immune factors; Body temperature; Spleen; Retina; ELISA; YOKV

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Résumé

De nombreux chercheurs se sont intéressés aux chauves-souris (Chiroptères) pour leurs particularités, notamment pour leur système d'écholocalisation, leur phylogénèse, leurs habitudes alimentaires ou leur mode de reproduction unique. Néanmoins nous n'avons presque pas d'informations de base quant à leurs fonctions vitales en ce qui concerne leur résistance aux virus. Il est indispensable, lorsqu'on considère une espèce animale réservoir naturel d'agents pathogènes, de ne pas se limiter à la seule étude de son système immunitaire, mais de faire également des analyses portant sur plusieurs points de vue: biologie, habitat, habitudes alimentaires et caractéristiques physiologiques. La plupart des études récentes portant sur des maladies infectieuses consistent en des analyses in vitro des fonctions génétiques virales ou en des études pathologiques sur des animaux de laboratoire. Ainsi les chercheurs ne s'intéressent que très rarement aux animaux sauvages directement. La présente étude se propose donc de donner des informations de base sur les chauves-souris, en particulier leur background génétique, les caractéristiques de leurs facteurs immunitaires, les caractéristiques histologiques de leurs organes immunitaires, leurs fonctions physiologiques, leur sensibilité cellulaire face aux infections virales.

Mots-clés: Chauves-souris (Chiroptères); ADN mitochondrial; Facteurs immunitaires; Température corporelle; Rate; Rétine; ELISA; Virus YOKV

1. Introduction

Bats (Mammalian: Chiroptera) have been studied exclusively on their specific character, i.e. echolocation system, phylogenic tree, food practice and unique reproduction [1–5]. However, it is recently reported that bats are also natural reservoirs or natural hosts of the emerging and re-emerging infectious disease, for examples Nipah virus, Hendra virus and lyssavirus infections including rabies, which lead to serious disorders in humans as well as in some animals [6–8]. Moreover, bat-SARS-CoV and Ebola virus were also isolated from bats, and the infectious disease derived from bats has been increasing in number [9–11]. Among these viruses, few investigators reported the result of experimental infection to bats and especially for Ebola virus, it was reported that bats supported the replication and circulation of high titers of this virus without any illness [12]. Many investigators focused on bats from the above-mentioned standpoints, however, most of basic information about their vital functions, such as the specific immune system and others associated with anti-viral activity, has been unclear until now. Therefore, basic researches for immunology in bats are important to advance investigation of the bat-derived infectious diseases.

The majority of current studies on infectious diseases have been aimed at the elucidation of viral virulence using experimental animals or viral gene function using cell lines in vitro, but in a few case, researchers focused on wild animal itself [13,14]. In this paper, we describe basic information about bats as follows: genetic background, character of the immunological factors and the physiological function.

The study is conducted using the genus *Rousette* (Chiroptera: Megachiroptera) which includes Egyptian and Leschenault's rousettes.

2. Biological characters of the rousette bats

In the basic studies on bats, to elucidate their biological character is the most important subject, although there is a little information about it. Therefore, we investigated the bats from the genetic, histological, molecular biological and physiological points of view.

2.1. Phylogenic analysis using the mitochondria DNA

Bats consist of the second-ranking big order in mammals, having about 1000 species, and live around the world except for the northern and southern polar areas. They is only one mammal with the capacity of powered flight and are divided into two suborders, Microchiroptera and Megachiroptera. The formers have various food habits, which are mainly insectivorous, and have the ability of the laryngeal echolocation but the latter is a fruit eater and have a comprehensive visual perception similar to the non-human primate and human being [15].

The phylogenic position or the relationship of bats among other mammalians has been studied using both morphological and molecular techniques. Some morphological studies have supported the idea that bat is included in the superorder Archonta, which also include Primate, Dermoptera and Scandentia [16,17]. However, from the molecular point of view, bat is thought to be within Laurasiatheria including Cetartiodactyla, Perissodactyla, Carnivora, Pholidota and Eulipotyphla [18]. Moreover, several investigators place bats as a sister group of Fereuungulata and latest studies support that bat is the monophyletic group within Fereuungulata, by mitochondria DNA (mtDNA) sequence data [19,20].

Although Megachiroptera is more closely related to primates than to Microchiroptera based on an anatomical pattern of neural projections from eye balls to prestrinate cortex via geniculate bodies [21,22], molecular studies reject this hypothesis. The phylogenetic position of Megachiroptera within bats is placed neighboring to Rhinolophoidea [15]. However, it is not clear which species of Megachiroptera is the most close relatives to Microchiroptera, especially for Rhinolophoidea. Thus, we determined the complete mtDNA sequence of Egyptian rousette, *Rousettus aegyptiacus*, which inhabits from South Africa to Egypt, Pakistan and Cyprus, by the direct and the shotgun sequencing methods. The phylogenic analysis was conducted to decide the position of Egyptian rousette within Chiroptera and among other animals.

The number of the nucleotide base pairs of mtDNA of Egyptian rousette was 16,706. The phylogenic tree was made using maximum-likelihood method based on the amino acid sequences of 13 proteins encoded by mtDNA. The sequence was compared with two other Megachiroteras, five Microchiropteras and other mammals. The results suggested that Chiroptera was monophylic group and more closely related to the large clade including Carnivora, Artiodactyla and Perissodactyla. Moreover,

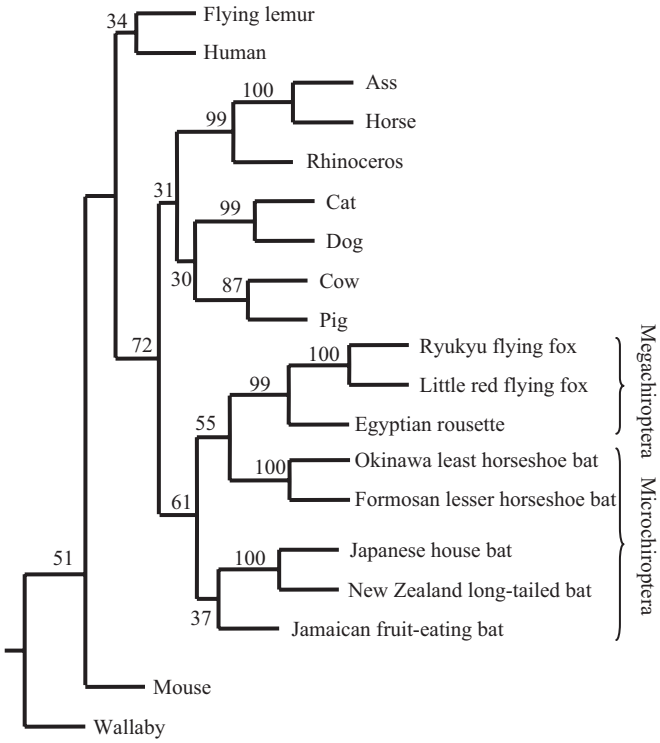

Fig. 1. Maximum likelihood tree constructed by PHYLIP using the sequence of nucleotide 13 protein genes encoded by mtDNA. Numbers at nodes indicate bootstrap value for maximum likelihood method.

within Chiroptera group, Egyptian rousette might be divided earlier from Rhinolophoidae (Chiroptera: Microchiroptera) than two other Megachiropteras, the little red flying fox and the Ryukyu flying fox (Fig. 1).

Our data supported two former hypotheses. One is that Megachiroptera makes a monophyletic group together with Microchiroptera. The second is that Chiroptera share a common ancestor with the large cluster including Carnivora, Perissodactyla and Artiodactyla, and Chiroptera diverged from a common ancestor before the large cluster diverged [23,24]. Moreover, this result created a new possibility that the genus *Rousette* may be the prototype of the suborder of Megachiroptera which was divided from Microchiroptera (one of missing rings of the small bats to mega bats). Thus, it is considered that the genus *Rousette* is the most suitable group for comparative studies on bat species.

2.2. Immunological cross-reactivity of bat IgG epitopes

The difference of antigenic determinants in serum proteins is a convenient tool for examining the molecular evolution of a certain protein among animal species [25–27]. A polyclonal antibody includes different kinds of antibodies recognizing

different epitopes, and it can specifically bind to the definite antigenic determinants of the serum protein. A high degree of cross-reactivity reflects similarity of antigenic determinants on the target protein among different species, which might have been conserved through the course of evolution. Therefore, we quantified the difference of antigen determinants using bat immunoglobulin G (IgG) within Chiroptera as well as between Chiropera and other closely related species based on the cross-reactivity of polyclonal anti-bat IgG antibody.

The rabbit anti-bat IgG was prepared using purified bat IgG obtained from the serum of *Rousettes aegyptiacus* as antigen. The purification of bat IgG was checked by SDS-PAGE. To assess the specificity of the anti-bat IgG antibody, we performed western blot analysis. Only one band with a molecular weight of about 160 kDa was detected in both bat serum and purified bat IgG fraction. These results suggested that the prepared anti-bat IgG antibody had high specificity. To compare the specificity, sera were obtained from four orders, i.e. Primate (one squirrel monkey, two lemurian monkeys and one human), Carnivora (four dogs), Insectivora (five large Japanese moles and four house shrews) and Chiroptera including two Rousettes, one Microchiroptera and three other Megachriopteras. The cross-reactivity of IgG epitopes in each serum sample using anti-bat IgG antibody was determined by the competitive enzyme-linked immunosorbent assay (ELISA). Fetal calf serum (FCS) was used as negative control. As a result, all serum samples obtained from Chiroptera showed high cross-reactivity, over 95% of inhibition, and the cross-reactivity of other animal samples was very low, mostly less than 20% of inhibition. The mean and standard deviation of percent inhibition of sera from Primates, Carnivores, Insectivores and Chiroptera were 15.2 ± 4.6, 12.2 ± 2.8, 8.4 ± 2.2 and 100.9 ± 4.5, respectively.

This result indicated that the anti-Rousette IgG polyclonal antibody had a high specificity to Chiroptera IgG, and will be useful for the screening and detection of pathogens. It supported also that two suborders of Chiroptera were closely related to each other or are monophyletic. The relationship between Chiroptera and other species, which have been considered as close relatives of bats such as Primate, Insectivora and Carnivora, were obviously distinct. However, the present result indicated that Primate and Carnivora might be more closely related to Chiroptera than Insectivora ($p < 0.05$).

It is considered that the large difference between Chiroptera species and Primate, Carnivora or Insectivora was caused by their own evolution as well as living environment; i.e. the former basically lives in the sky and the latter on or in the ground. The result also suggested that Chiroptera might be monophyly.

2.3. Phylogenic analysis using anatomical and histological methods

The echolocation system, which is the main recognition ability of Microchiroptera, has been reported frequently [28–30]. The cognitive methods of two suborders within Chiroptera are completely different, i.e. Microchiroptera uses mainly echolocation, and Megachiroptera uses visual perception. In the former all fibers of the optic nerve entered into the contralateral optic tract, however, in the

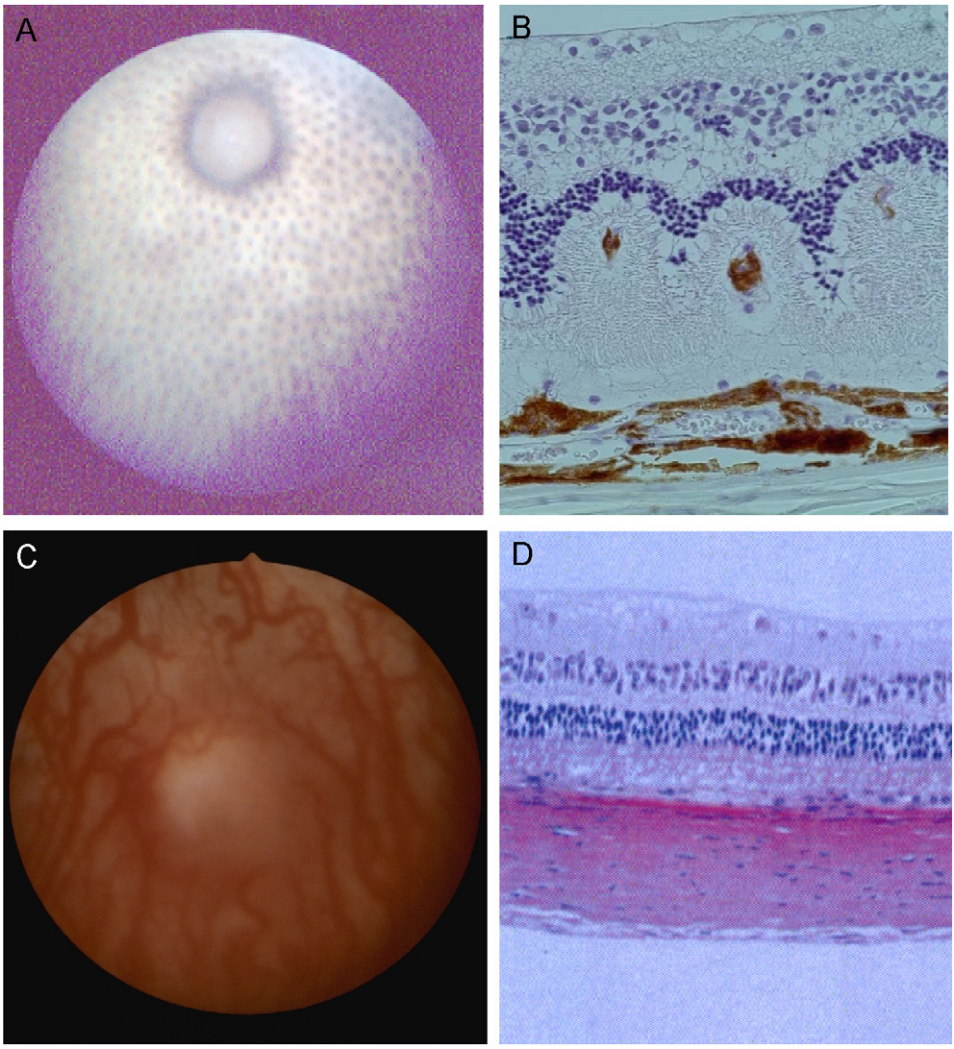

Fig. 2. Anatomical and histopathological analysis of retina of Egyptian rousette, *Rousettus aegyptiacus*: (A, C) funduscopy of eye ball, (B, D) histopathology of retina, (A) Tinny spots were observed in bats retina, (B) papillary structure was observed between outer granular layer and retinal pigment epithelium and (C, D) funduscopy and histopathology of guinea-pig, as a control.

latter the optic nerve connect to both contrarateral and ipsilateral superior colliculus, being similar to the projection pattern of primates. Thus, we investigated on the optical system of *R. aegyptiacus* and structure of their retina in comparison with those of Michrochiroptera.

In the funduscopy finding of the eye balls of Egyptian rousette, there were large numbers of tiny spots, which scatter onto the whole retina except for the optic disc. This peculiar structure could not be observed onto other animals' retina including

small bats. Although blood vessels distributed from the optic nerve papilla are observed in the retina of other mammals, in the case of rousette retina, the blood vessels were not observed at all. By the histological examination, rousette retina had a papillary structure between outer granular layer and retinal pigment epithelium, unlike other animals' smooth-layered retina including small bats. In the immunohistological examinations, rhodopsin could be detected from the outer segment of the internal photoreceptor matrix (IPM) to outer nuclear layer (ONL) but mainly in the outer segment of IPM, and recoverin-positive cells were detected from inner segment of IPM to ONL. Although both recoverin and rhodopsin proteins were expressed in the same area as those in the rat retina, the ratio of rhodopsin-positive area to that of recoverin in the Egyptian rousette retina was wider than that in rat retina (Fig. 2).

These results suggested that the tiny spots observed by the funduscopy on the retina of Egyptian rousette assumed to be due to the papillary protrusion of pigment epithelium, which might result in enlargement of the surface area receiving light wave, because they need to increase the number of photoreceptor or the light-receiving area for gathering information in the dark night regarding the environment, such as enemies or prey without tapetamu lucidum structures or only simple tongue echolocation system.

2.4. Change of body temperature

Body temperature regulates the basic metabolic rate and various body activities, but it is settled within narrow range in majority of mammalians by the homeostatic control, except for some emergencies such as viral or bacterial infections. In the virus replication process, it has been reported that the ratio of virus production is dependent on the body temperature [31]. It is noted that bats, especially for Microchirotpera, have specific character, torpor, which is similar to hibernation, when they are exposed in low temperature [32]. It was also reported that some kinds of Megachiroptera showed torpor-like response under low-temperature condition [33]. In torpor and hibernation, the lowering of the internal body temperature is observed and animals get large tolerance to nuclear radiation, cancerogenic substance and infectious diseases. Whereas the investigation about torpor in bats had already reported, there was no report on circadian rhythm of the internal body temperature of bats using a telemeter. We examined the internal body temperature of *Rousettus leschenaulti* using telemetry system. In order to know environmental temperature effects, the rousette bat was kept in an incubator, which was settled light and dark cycle consisted of 12 h of light per day (light on from 08:00 to 20:00) and the thermal conditions were settled following three conditions; 24 °C constant, from 10:00 to 16:00 at 30 °C and another time at 24 °C, from 10:00 to 16:00 at 33 °C and another time at 27 °C.

Under the first condition, 24 °C constant, rousette bat body temperature was around 36 °C in light period when bats took a rest, but in dark period when bats became active, the body temperature was up to and sometime over 39 °C. Under the second and third conditions, in the dark phase the average of their body temperature

were over 38 °C, but in the light phase was depended on the environmental temperature (from 36 to 38 °C). In the case of the second condition, the gap of body temperature between light and dark phase was more tight than others. These results indicated that the thermal gap of body temperature between the rest and active phase was wider than other mammals, and that the infected virus might be difficult to replicate constantly in rousette bats. It was thought that higher body temperature in dark phase over 38 °C was due to the movement.

3. Evaluation of the immune systems of bats

Bats are thought to be a natural reservoir or vector of rabies virus, Nipah virus, Hendra virus, European bat lyssavirus types 1 and 2 and Australian bat lyssavirus [34–38]. In addition, other viruses related to the emerging and re-emerging infectious diseases were also isolated from bats, for examples Ebola virus and bat-SARS-CoV [9–11]. In the case of experimental infection of Ebola virus, bats supported the replication and the circulation of high titers of virus without any clinical signs [39].

It has already been reported that bats have three types of immunocompetent cells, i.e. plastic adherent cells with pesudopodia, nylon wool adherent cells with small microvilli and nylon nonadherent cells with comparatively smooth surfaces. These cell types resemble to macrophage, B cells and T cells of other mammals, respectively [40]. However, there is a little basic information about the immune system of bats and the comprehensive studies in vitro are not conducted. So, we examined the molecular and biological characters of their immune factors and performed histopathological and immunohistochemical analyses of their immune organs.

3.1. Molecular and biological characters of bat immune factors

The nucleotide sequences of almost all immune factors of bats are not identified until now. We determined the nucleotide sequences of several immune factors including full open reading frame (ORF) of CD4 on the helper T cells, which induces the acquire immunity, and IFN-α and β, which relate to the innate immunity. The nucleotide and deduced amino acid sequences of these molecules were analyzed by phylogenic and molecular biological point of view.

In the acquired immunity, the antigen recognition between T cells and antigen-presenting cells (APC) requires interaction of T cell receptor (TCR) and major histocompatibility complex (MHC) molecules. The CD4 (MHC class II) and CD8 (MHC class I) molecules are the surface key proteins engaged in immune reactions. Thus, the surfaces of mature T cells expressed either CD4 or CD8 [41,42]. CD4-expressing T cells react to the antigen presented by the MHC classII molecule of APC [43]. CD4 molecule is a glycoprotein belonging to an adherent molecule group classified to the immunoglobulin superfamily. It has four Ig-like domains in the extracellular region and intracellular region connects to tyrosine kinase, Lck, belonging to the Src family specific for lymphoid cells [44]. We sequenced the full

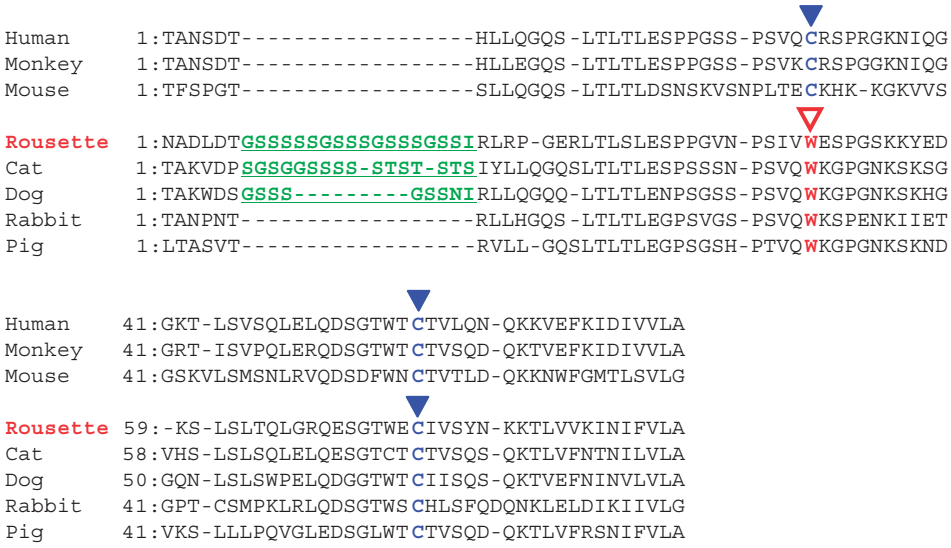

Fig. 3. Comparison of the CD4 Ig-like C region between the bat and human, monkey mouse, cat, pig, dog and whale. Identical amino acid residues are indicated by dots (•) and gaps are indicated by bars (–). The cystein residues consisting of the disulfide bond are indicated by closed triangles. The residues in which tryptophan is substituted for cystein are indicated by open triangles. Underlines show amino acids insertions provided CD4 of human and mouse being as a standard.

ORF of CD4, and analyzed the bat CD4 from phylogenic and comparative zoological viewpoints.

The nucleotide sequence of bat CD4 consisted of a total of 1905 bases, in which 1419 nucleotides encoded 472 amino acids. When the homology of the nucleotide and the deduced amino acid sequences were compared between the bat CD4 and those of other animals, such as human, mouse, cat, dog and chicken, bat CD4 had a higher homology to both cat and dog CD4 than those of other animals. The phylogenic tree using neighbor-joining method with the amino acid sequence of CD4 showed that bat CD4 was closely related to cat and dog CD4 and was distant from duck and chicken, which agreed with the homology of the nucleotide and the amino acid sequence. This result supported that Chiroptera was included in the same clade including cat, dog, pig and whale [45,46]. Moreover, the amino acid sequence of the CD4 Ig-like C-type 1 domain was compared between bat and other animals. In bat CD4, there was an insertion of 18 extra amino acids in the beginning of this domain, where dog and cat CD4 have 9 and 16 amino acids insertions, respectively, provided CD4 of human and mouse being as a standard. The insertion might influence the relationship between V-type domain and C-type 1 domain. In the N-terminus side, cystein pair might be lost because one cystein was replaced by tryptophan in the bat CD4, whereas in the case of human, monkey and mouse CD4 it was still cystein. The latter animals might have a disulfide bond formed by the two cysteins in this region. However, bat CD4 lacked the disulfide bond, as in cat, dog and whale. As a result

the conformation of bat CD4 Ig-like C-type 1 region might be different from that of the human and mouse (Fig. 3). The conformational change of the bat CD4 might influence the antigen presentation process between APC and helper T cells by means of CD4–MHC classII connection [47,48].

In the innate immunity, virus-infected cells provoke many responses to viral infection. One of these responses is to secrete type-I (α/β) interferons (IFNs) [49]. Type-I INFs are antiviral cytokines, and composed of the multiple subtypes of α and the single type of β. When some microbes infect to cells or are detected by anti-microbe intercellular or extracellular sensors, type-I IFNs are secreted from most of the cells, especially from dendritic cells. Secreted type-I IFNs bind to the interferon receptor (IFNAR) and activate the expression of numerous interferon-stimulated genes (ISGs), such as the protein kinase R (PKR), the 2′–5′ oligoadenylate synthetases (OAS), the myxovirus resistance gene (Mx), which have anti-viral activities [50]. However, the nucleotide sequences of type-I IFNs of bats were not determined until now.

The ORFs of IFN-α and β had 562 base pairs that encoded 187 amino acids, 558 base pairs that encoded 186 amino acids, respectively. Since IFN-α genes have many subtypes, it is difficult to compare its nucleotide sequence among several animal species. Therefore, we checked homology of the bat IFN-β to those of human, pig, cat, horse and mouse. The homologies were 77.5%, 82.0%, 78.3%, 77.5% and 66.5% at nucleotide level, and 64.2%, 72.0%, 61.8%, 61.3% and 49.5% at amino acid level, respectively. The phylogenic tree was constructed based on the amino acid sequences obtained from the bat INF ORFs with several representative eutherian type-I IFNs as well as chicken type-I IFN using maximum likelihood method. The results showed that both bat IFN-α and bat IFN-β were included in each type of mammalian IFN group, but far from those in avian. Moreover, both bat IFN-α and β were more closely related to pig IFN-α and β than the other mammal ones.

There have been few studies on the immune systems of the bat that has been thought to be an important vector or a natural host of various pathogenic microbes. We believe that these basic information will help to elucidate the ecology of infectious agents derived from bats as wells as our understanding of bat immunological factors.

3.2. Histopathological and immunohistochemical analyses of the immune organs

It has been reported that many microbes were isolated from bats in field sampling and the antibody to several pathogens was detected in the epidemiological studies using viral neutralizing test. In these epidemiological studies and experimental infections with microbes in vivo, the histopathological finding is one of the most important information for investigating whether and how they react to the invading microbes. However, there are limited numbers of reports about their normal histology, especially for the immune organs. We examined histopathologically on thymus, spleen and mesenteric lymph nodes, which are the main immune organs, of six normal *R. aegyptiacus*, which were kept at conventional containment grade in our laboratory. Their thymus, spleen and lymph nudes were sampled, fixed with 4%

paraformaldehyde, embedded in paraffin and sliced. We examined their normal histology using hematoxylin–eosin staining, Masson–trichrome staining and immunostaining with anti-bat IgG rabbit polyclonal antibody.

In the hematoxylin–eosin staining, thymus structure was not different from other animals, but most of all germinal centers in splenic white pulp and lymph node of all samples were hypertrophic. By Masson–trichrome staining, these hypertrophic germinal centers were not for the deposition of fibrin but for cells with abundant cytoplasm. In the immunostaing using the anti-bat IgG antibody, these cells correspond to B cells which were positive to anti-bat IgG antibody. This result indicated that the immunological cells, especially for B cells, in Rousette bats kept in our laboratory under the conventional condition were naturally activated, or they might be coexisted with some microbes, which were apparently no pathogenic to Rousette bat. It is needed further study on natural state of T and B cells in different species of bats at various ages for basic information.

4. The susceptibility of bat to virus infection

There are also a few studies on the experimental infection of virus, which was isolated from bats or antibody positive to the virus in the field survey and/or virus was thought to be concerned in the infectious cycles [51–53]. Thus, we established primary cell cultures from rousette bat kidneys and evaluated their susceptibility to the virus infections compared with the commercially available bat lung cell line, Tb-1 Lu.

4.1. Sensitivity of bats cells to viruses in vitro

Only a few cases of experimental infection of virus to bats in vivo or cell lines derived from bats have been documented, and the established and available cell line from bat is only Tb-1 Lu, which is the alveolar epithelial cell lines [54]. Therefore, first we tried to prepare bat primary kidney cells (BPKC) and examined the sensitivity of them to some viruses.

Viruses used in the experiment were following; Yokose virus (YOKV; family Flaviviridae), which is only one virus isolated from Japanese Microhiroptera, Akabane virus (AKAV; family Bunyaviridae), Fukuoka virus (FUKV; family Rhabdovirus), Kawanabe virus (KAWV; family Reoviridae), canine parainfluenza virus (CPIV; family Paramyxoviridae), pseudorabies virus (PRV; family Herpesvidirae) and Japanese encephalitis virus (JEV; family Flaviviridae). All of these viruses had the ability to infect and replicate in both BPKC and Tb-1 Lu, and in BPKC these viruses, except for JEV, developed a cytopathic effect (CPE) and the infected cells were dead until 3 days after infection (d.a.i.). Meanwhile, Tb-1 Lu cell lines released the infectious virus, although it showed lower levels of all viruses replication than BPKC without CPE. JEV could infect and produce the infectious virus in BPKC showing a peak at 2 d.a.i., but the virus did not induce any CPE until 7 d.a.i.

To examine whether the presence or absence of CPE depend on the cell type with IFN, we treated polyinosinic–polycytidylic acid [poly(I:C)] to two types of cell and examined the difference of type-I IFN expression. Poly(I:C) is synthetic mimetic viral

double-stranded RNA (dsRNA) and a strong inducer of type-I IFNs in vivo and in vitro [55].

Type-I INFs are antiviral cytokines, and composed of the multiple subtypes of α and the single type of β. Cells make use of two signal transduction pathways to express type-I IFN genes, the classical pathway and the new IFN induction pathway. In the first case, the intracellular sensors detect viral components in the cytoplasm and activate interferon regulatory factor 3 (IRF-3) and NF-κB which transactivate IFN-β gene [56]. Synthesized IFN-β secretes and binds to type-I IFNAR. Viral dsRNA activates this signal through Toll-like receptors 3 (TLR3), RIG-I and MDA5. In the case of latter, cells recognize viral materials with TLR7, TLR8 and TLR9 expressed on the cell surface or in endosomes. TLR7 and 8 recognize viral single-stranded RNA, and TLR9 recognizes double-stranded CpG-rich DNA [57]. TLR signaling activates IRF-7 and regulates multiple IFN-α and single β gene expression [58]. Synthesized type-I IFNs bind to IFNAR and activate the expression of numerous ISGs, such as the PKR, the OAS and the Mx, through JAK-STAT signaling pathway [50]. These products control viral infection, for examples PKR inhibits the viral protein translation, OAS degrades cellular and viral RNA and Mx sequesters viral ribonucleoproteins to specific subcellular compartments.

Several investigators reported that fruit and insectivorous bats supported the replication and circulation of high titers in experimental inoculation of Ebola virus without any clinical signs [12]. In this virus, it was already reported that VP35 protein blocks activation of IRF-3 and PR24 protein inhibit IFN signaling [59,60]. Therefore, as the origin of some viruses, it is important to investigate the IFN system of bats. However, there are a few basic studies subject to bat immune systems in the world, including the IFN system.

In BPKC, the expression of only IFN-β mRNA was increased 3 h after poly(I:C) treatment. In the case of Tb-1 Lu, however, both IFN-α and β mRNA expression were not detected at any time. This results suggested that BPKC had a capacity of the responsiveness to poly(I:C) through TLR3, RIG-1 and MAD5 and expressed IFN-β mRNA. But new IFN induction pathway through IFNAR did not reach to the enough stimulation of IFN-α gene at 3 h after treatment. While, it was thought that Tb-1 Lu did not respond to poly(I:C) through TLR3, RIG-1 and MAD5.

To examine whether these two types of cells react to bat type-I IFNs and express type-I IFNs mRNA, we treated bat type-I IFNs to BPKC and Tb-1 Lu, and examined mRNA expression of bat type-I IFNs at 0, 4 and 8 h after treatment. We used the supernatant of BPKC treated with poly(I:C) as bat type-I IFN-including medium. Briefly, poly(I:C) exposure was conducted in BPKC with DEAE-dextran for 3 h. After that, culture medium was removed and the cells were washed by PBS and then cultured for 24 h with new 5% FCS medium. The whole supernatant was collected and used as bat type-I IFN-including medium (conditioned medium). In the case of BPKC, IFN-α mRNA expression was detected at every time and increased gradually, while IFN-β mRNA expression was detected at 4 and 8 h and peak at 4 h. However, Tb-1 Lu did express neither IFN-α nor β mRNA at any time. It indicated that the reaction of IFN-β was sooner for a reaction to virus or microbes as soon as possible and that of IFN-α was longer for the expression of antiviral

activity proteins, including the PKR, OAS and Mx protein, for long time. Meanwhile, when Tb-1 Lu were treated with poly(I:C) and the conditioned medium, these cells did not express type-I IFNs mRNA at any time. From these results, we suggested that the mechanism from the recognition of poly(I:C) through TLR3 or bat IFNs through IFNAR to the expression of type-I IFNs was not working right in Tb-1 Lu cell line, whereas BPKC did work. Therefore, it might be better to use primary cell culture than using an established cell line to evaluate host response to virus or microbes.

4.2. Epidemiological study of bats

Bats, the only flying mammals, have a great diversity and account for 20% of the 4800 mammalian species recorded in the world. During the past decade, bats have been associated with a number of emerging zoonotic agents, including Hendra, Nipah, Lyssa, Ebola and SARS coronavirus-like viruses. Therefore, bats are thought to be an important reservoir of many mammalian viruses. Serological surveys of viruses that infected bats have been already reported. Most of the surveys were conducted by using neutralization test (NT) or fluorescent antibody tests [9,11,61–64]. However, it is not easy to obtain the sufficient amount of blood samples to perform these tests, because of the size of bats, particularly in Microchiroptera (microbats). Moreover, these assays are not so suitable for testing a large number of samples at the same time. For these points of view, ELISA is a powerful tool for the serological survey viruses that infect bats. However, there are no conventional ELISA systems, except the systems using protein G or competitive techniques with monoclonal antibodies [65–68].

In our study, the ELISA system using biotinylated anti-bat IgG rabbit sera was developed. We used polyclonal anti-bat IgG rabbit sera reported in our previous paper [69]. The antibody reacted only with bat IgG, not with IgG of other mammalian species. The ELISA system detects the specific IgG antibodies of bats. As there are few reports on viruses that isolated from bats in Japan [70,71], we decided to use YOKV as an ELISA antigen in this study. YOKV belongs to Entebbe bat virus group, the genus *Flavivirus*, family Flaviviridae, and was isolated from a bat in Oita Prefecture in Japan in 1971 [70]. Before the virus was isolated, attempts were done to isolate JEV from bats by Oya et al., to investigate the possibility that bats served as a reservoir for JEV in winter period. During this investigation, YOKV was isolated from bats, *Miniopterus fuliginosus,* which seemed to be different from JEV by serological analysis.

To examine the availability of the ELISA developed in this work, serological survey was carried out on bat serum samples collected from the Philippines and Malaysia. In this survey, 2.7% of the samples collected from the Philippines and 19% from Malaysia showed detectable levels of antibodies. Serum samples were also tested by NT, and the correlation rate between ELISA and NT was 0.79. These data suggest that YOKV is distributed not only in Japan but also in other Asian countries. However, the antibody titer against YOKV was not so high in this survey. And, it is known that antibodies against flaviviruses show cross-reactivity with other

flaviviral antigens [68]. Therefore, to examine the specificity of ELISA, ELISA substituting the antigen to JEV, which was widely distributed in South-East Asian countries, was conducted. Although ELISA with JEV antigen reacted with the positive serum against YOKV, the ELISA titer was much lower than homologous titer with YOKV antigen. These results suggest that this conventional ELISA system is useful to detect the specific YOKV antibody in bat sera. The method is so simple, and easy to establish without obtaining specific antibodies against target virus. This system has a possibility to be applied to other viruses by substituting only the coating virus antigen.

4.3. Experimental infection of bats

There are few reports on experimental viral infection of bats except lyssaviruses [53,72]. Although neurovirulence was observed in suckling mice that were intracerebrally inoculated with YOKV, the pathogenicity of this virus is still unknown. Therefore, to examine the pathogenicity of YOKV in bats, and to confirm whether bat is an amplifying host for YOKV or not, an experimental infection was conducted.

In this study, at first, surveillance was conducted on the sera collected from the orbital sinus from Leshenault's Rousette bats (frugivorous bats) which were kept in our farm. These bats were kindly obtained from the zoo. The fruit bats were kept in separate cages in the farm away from any other animal species. ELISA test was used to exclude the bats which have antibody against YOKV. The results showed that 14% of these bats had ELISA antibodies. The seronegative bats were experimentally infected with YOKV, and no clinical signs were observed. Moreover, no significant amplification of virus genome was detected by RT-PCR from the sera and organs. These results reveal that YOKV replicates poorly in bats, suggesting that bats do not seem to serve as an amplifying host for YOKV. Our results coincide with the previous reports on West Nile virus [73], conveying that insectivorous bats have antibodies against the virus, but the level of virus growth is low.

Recently, Tajima et al. [70] have determined the complete nucleotide sequence of YOKV and compared the nucleotide and deduced amino acid sequences with those of other flaviviruses. They concluded that YOKV is genetically closer to yellow fever virus than JEV, and is more closely related to the partially reported amino acid sequences of Entebbe bat virus, Sokuluk virus and Sepik virus. Previous phylogenenic analysis of the genus *Flavivirus* revealed that flaviviruses could be divided into three groups: mosquito-borne, tick-borne, and unknown vector groups [74,75]. Tajima et al. indicated that YOKV would belong to mosquito-borne group, although YOKV is classified in the Entebbe bat virus group of vector unknown group. Previous report indicated that Entebbe bat and Sokluk viruses could replicate in mosquito cells in vitro [76]. These findings may suggest that YOKV as well as Entebbe bat virus and Sokluk virus is related to mosquito-borne flaviviruses. These facts might suggest that the fruits bats, which showed ELISA antibodies, were infected by mosquitoes. YOKV might have other amplifying host except bats, and mosquitoes might be candidate for an amplifying host for YOKV.

Further studies on virus isolation from mosquitoes are needed to confirm the epidemiology of YOKV.

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