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Molecular Evolution Inferred from Immunological Cross-reactivity of Immunoglobulin G among Chiroptera and Closely Related Species

Tsutomu OMATSU¹⁾, Yoshiyuki ISHII¹⁾, Shigeru KYUWA¹⁾,
Elizabeth G. MILANDA²⁾, Keiji TERAOKA³⁾, and Yasuhiro YOSHIKAWA¹⁾

¹⁾Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, ²⁾Research Institute for Tropical Medicine, Filinvest Corporate City, Alabang, Muntinlupa City, 1770, Philippines, and ³⁾Tsukuba Primate Center, National Institute of Infectious Diseases, 1-Hachimandai, Tsukuba, Ibaraki, 305-0843, Japan

Abstract: We examined the relationships between Megachiroptera and Microchiroptera, and between Chiroptera and other closely related species by the cross-reactivity of immunoglobulin epitopes. Rabbit polyclonal antibody to bat IgG was used for determining the cross-reactivity by a competitive ELISA method. Megachiroptera and Microchiroptera showed high cross-reactivity, over 95.3%, with each other. However, primates and insectivores showed very low cross-reactivity, 8.6 to 20.2% and 5.3 to 12.7%, respectively. These results suggest that suborders of Chiroptera are monophyletic and Chiroptera have a relatively closer relationship to primates than to insectivores.

Key words: bat IgG, competitive ELISA, megachiroptera

About 4,500 species of mammals are known around the world, and Chiroptera is about one quarter of all species of mammals, with about 1,000 species. It is divided into two suborders, Megachiroptera, fruit-eaters, and Microchiroptera, mainly insectivores. Chiroptera are one of the few orders of mammals that echolocate, and the only group with the capacity for powered flight. Chiroptera has traditionally been considered as a monophyletic group. However, in the flying primates hypothesis, Pettigrew objected to this traditional view by proposing that Megachiroptera are more closely related to primates than to Microchiroptera, thereby suggesting the diphyle of Chiroptera [14]. This

hypothesis is based on the analysis of neural connections between the retina and midbrain, but it is inconsistent with the conclusion of molecular [10, 15, 18] and morphological studies [1, 9, 19].

Chiroptera was once considered as the same super-order as Archonta, represented by primates, tree shrews, and flying lemurs [6]. However, in recent molecular studies a sister-relationship with Eulipotyphlans was shown, and this clade closely relates to Fereuungulates (Cetartiodactyla, Perissodactyla and Carnivora) [2, 8, 12, 13, 16]. Thus, some investigators have suggested that Chiroptera is most closely related to Eulipotyphla with Carnivora as the sister group [11]. In this way,

(Received 7 February 2003 / Accepted 28 April 2003)

Address corresponding: T. Omatsu, Department of Biomedical Science, Graduate School of Agriculture and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

the intrarelations within the order Chiroptera and the relationships with other orders of mammals have been debated for some time.

The difference in antigenic determinants of serum protein is a convenient tool for examining the molecular evolution among species [4, 5, 7, 17]. Since polyclonal antibodies consist of different kinds of molecular antibodies, which can specifically bind to the definite antigenic determinant of the serum protein, a high degree of cross-reactivity reflects similarity of antigenic determinants on the target protein among species, which might have been conserved through the course of evolution.

In this study, we quantified the difference of immunoglobulin G (IgG) among Chiroptera and other closely related species based on the cross-reactivity of polyclonal anti-bat IgG antibody, and analyzed phylogenetic relationships among them.

Sera was obtained from 20 mammals related to the bat: two Egyptian flying foxes (*Rousettus aegyptiacus*), three Greater musky fruit bats (*Ptenochirus jagoraii*), one Philippine insectivorous bat (unidentified species), one cynomolgus monkey (*Macaca fascicularis*), one squirrel monkey (*Saimiri sciureus*), two lemurian monkey (*Microcebes murians*), one human being (*Homo sapiens*), five large Japanese moles (*Mogera koubeae*), and four house shrews (*Suncus murinus*). The sera were separated by centrifugation and stored at -80°C until use. This experiment was performed in accordance with the Animal Experimentation Guideline of the University of Tokyo, and approved by the Institutional Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

In order to prepare purified IgG, whole blood was collected by cardiac puncture from ketamine chloride anesthetized bats, Egyptian flying foxes. Sera were obtained by centrifugation at 10,000 rpm for 10 min. IgG was precipitated by 50% ammonium sulfate and then purified by a MabTrapG II Kit (Amersham Bioscience, Uppsala, Sweden). The isolated bat IgG was desalted by Column PD-10 (Amersham Bioscience, Uppsala, Sweden). The purified bat IgG was adjusted to 10 $\mu\text{g}/\text{well}$ and bat serum was diluted at 1: 20; then purity of the bat IgG was checked using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% acrylamide gel). The proteins in the gel were stained by Coomassie brilliant blue (Fig. 1A).

Fig. 1. Purification of bat IgG and preparation of rabbit anti-bat IgG antibody. To confirm the purity of bat IgG and the specificity of anti-bat IgG, SDS-PAGE analysis (A) and western blot analysis (B) were conducted using bat serum (lane 1) and purified bat IgG (lane 2). The anti-bat IgG was diluted at 1: 100,000, serum at 1: 20, and 1 μg of bat IgG was used. The position of molecular weight markers are indicated with arrows on the left side.

Although several dominant and many faint bands were observed in the bat serum, bat IgG was detected as only one band, about 160 kDa, almost the same molecular weight of human IgG as described previously [3]. The results suggest that the immunogen for anti-bat IgG antibody production described below was properly prepared with high purity.

To obtain anti-bat IgG antibody, two rabbits were immunized with the purified bat IgG every two weeks, six times. Blood was collected from the rabbits and serum was separated. The IgG fraction was obtained by protein G column chromatography and stored at 4°C until use. To assess the specificity of the anti-bat IgG antibody, we performed western blot analysis. The purified bat IgG was adjusted to 1 $\mu\text{g}/\text{well}$, serum was diluted at 1: 20, and then these samples were separated by electrophoresis using SDS-PAGE with 12.5% acrylamide gel. The proteins in the gel were transferred and blotted onto polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA).

After blocking with 5% nonfat dried milk in 20 mM PBS (pH 7.0) and 0.1% Tween-20 for one hour at room temperature, the blotted membrane was incubated with primary antibody diluted at 1: 100,000 overnight at 4°C. Then, it was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1: 5,000, Jackson Lab, West Grove, PA) for 1 h at room temperature. The immunoreaction was visualized by enhanced chemiluminescence (ECL, Amersham, UK).

Only one band with a molecular weight of about 160 kDa was detected, which was supposed to be the bat IgG of both bat serum and purified bat IgG (Fig. 1B). These results suggest that the prepared anti-bat IgG antibody had high specificity.

The cross-reactivity of immunoglobulins in each serum sample with anti-bat IgG antibody was determined by competitive enzyme-linked immunosorbent assay (ELISA). A 96-well flat bottom Immuno Plate (Greiner, Kremsmuenster, Germany) was coated with 80 µl/well of bat IgG diluted with phosphate buffered saline (PBS; pH 7.6) and stood at 4°C overnight. The content of bat IgG was 5 µg/ml. The plate was washed three times with 200 µl/well of PBS containing 0.05% of Tween-20 (PBS-Tween) and blocked with 160 µl/well of Block Ace (Dainippon Pharmaceutical, Osaka) at 4°C overnight. The Block Ace was removed before use.

The anti-bat IgG rabbit IgG was diluted with Block Ace at 10 µg/ml. In a 96-well flat bottom culture plate, 60 µl/well of bat, monkey, and rat sera diluted with Block Ace was reacted with 60 µl/well of diluted anti-bat IgG at 4°C overnight. Fetal calf serum (FCS) was used as a negative control.

The second reaction was done by moving 80 µl/well of the first reaction mixture into each well of a bat IgG-coated ELISA plate. The plate was allowed to stand at 4°C overnight. After washing the ELISA plate five times with 200 µl/well of PBS-Tween, 80 µl/well of horseradish peroxidase-labeled mouse anti-rabbit IgG antibody (Jackson Lab) diluted at 1: 5,000 was added to each well and allowed to stand at room temperature for 4 h in a dark box. After washing the ELISA plate five times with 200 µl/well of PBS-Tween, 80 µl/well of substrate solution was added to each well. The plate was incubated at room temperature for 30 min and additionally 80 µl/well of 2N H₂SO₄ solution was added to terminate the enzyme reaction. The optical density (OD) at 490 nm of each well was measured by a

Fig. 2. (A) The reactivity between anti-bat IgG antibody and diluted bat, rat, cynomolgus monkey sera and FCS in competitive ELISA. The cross-reactivity was measured by the reaction between anti-bat IgG and diluted bat, rat, monkey sera and FCS. FCS was used as a negative control. (B) The reactivity between anti-monkey IgG antibody and diluted bat, rat, cynomolgus monkey sera and FCS in competitive ELISA. 1E+05 = 1×10^5 dilution.

microplate reader (Bio Rad).

Figure 2 shows the reactivity of anti-bat IgG antibody with plate-fixed bat IgG after the first reaction between anti-bat IgG antibody and diluted bat, rat, cynomolgus monkey sera, and FCS. The contents of the free anti-bat IgG antibody reflecting the OD values were measured. The OD value increased in relation to decrease of bat serum concentration in the first reaction. On the other hand, there was no difference of the OD value between high and low concentrations of other sera and that of FCS as negative control.

Similar results were obtained in the same assay using combinations of monkey IgG and anti-monkey IgG.

From these results, we decided that sample serum should be diluted at 1:100 with Block Ace. The cross-reactivity of anti-bat IgG with each serum evaluated by

Fig. 3. The cross-reactivity of anti-bat IgG with sera of other animal species. Percent inhibition indicates the cross-reactivity of anti-bat IgG with serum from: Egyptian flying fox (lanes 1, 2), Philippine insectivorous bat (lane 3), Greater musky fruit bat (lanes 4–6), large Japanese mole (lanes 7–11), house shrew (lanes 12–15), lemurian monkey (lanes 16, 17), cynomolgus monkey (lane 18), squirrel monkey (lane 19) and human being (lane 20).

competitive ELISA was indicated by % inhibition, which was calculated by the following formula.

$$\% \text{ inhibition} = (\text{OD in sample serum} - \text{OD in fetal calf serum}) / (\text{OD in bat serum} - \text{OD in fetal calf serum}) \times 100$$

Each sample obtained from Chiroptera showed high cross-reactivity, over 95.3 of % inhibition (Fig. 3). There was little difference among the Egyptian flying fox and other Megachiroptera and a Microchiroptera. However, cross-reactivity of other animal samples was low, mostly less than 20. The mean and standard deviation of % inhibition (range) of sera from primates, insectivores and Chiroptera were 13.1 ± 4.4 (8.6–20.2), 8.4 ± 2.2 (5.3–12.7) and 101.2 ± 4.1 (95.3–104.7), respectively.

The reaction between Chiroptera and non-chiropteran species and between primates and rodents showed a significant difference. It indicates that the bat polyclonal antibody, which we had prepared a high specificity to Chiroptera, and will be useful for the screening and detection of pathogens.

The present result indicates that the two suborders of Chiroptera are 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 primates, insectivores and rodents, were obviously distinct. However, the present result indi-

cates that primates might be more closely related to Chiroptera than insectivores ($p < 0.05$). We consider that the large difference between Chiroptera and primates, or insectivores is caused by the differences in their evolution as well as living environment; i.e. chiroptera basically have lived in the sky, and the others on the ground. The result also suggests that Chiroptera might have diverged firstly from an ancestor of rodents and then from an ancestor of primates.

In conclusion, this is the first report to describe the relationships among Chiroptera and other species from the point of view of molecular evolution of IgG. In this study, we suggest that Chiroptera is monophyletic. To confirm this hypothesis, it will be necessary to test sera of other Chiroptera.

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