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Anatomical and histological profile of bronchus-associated lymphoid tissue and localization of melatonin receptor types (Mel_{1a} and Mel_{1b}) in the lung-associated immune system of a tropical bird, *Perdicula asiatica*

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ABSTRACT

The histological distribution of the lung-associated immune system (LAIS) and the expressional pattern of melatonin receptors are still unknown in birds. The aim of the present study was to determine the localization of the bronchus-associated lymphoid tissue (BALT nodule) in a tropical bird, the Indian jungle bush quail, *Perdicula asiatica*. We also demonstrate the expression of melatonin receptor types (Mel_{1a} and Mel_{1b}) in order to propose an immunomodulatory role of melatonin in LAIS. Localization of melatonin receptors in the lung of the Indian jungle bush quail, *P. asiatica* was supported immunohistochemically and by Western blot analysis using specific antibodies for those receptors. Immunolocalization for Mel_{1b} receptor was noted in the bronchial region of the lungs, in finger-like projections of mucosal foldings, in lymphocytes in the BALT nodule as well as in free form. In contrast, immunolocalization for Mel_{1a} receptor was noted in various areas of the lung instead of in the bronchial region. Western blot analysis showed a single band at 37 and 39 kDa for Mel_{1a} and Mel_{1b} receptors, respectively, with the latter showing higher expression. The results demonstrate a well-developed LAIS and region-specific distribution of melatonin receptors in the lung and provide evidence for a possible functional role for melatonin in the LAIS of birds.

Introduction

The avian lung differs anatomically and morphologically from that of mammals. In mammals, the lung-associated immune system (LAIS) plays a major role in fighting pathogens invading the lungs. Bronchus-associated lymphoid tissue (BALT) nodules, as described by Bienenstock et al. (1973a, 1973b), are far more frequently found in chickens than in any other species examined (Reese et al., 2006). BALT structures consisting of aggregates of lymphocytes are located at the junctions of the primary and secondary bronchi, while the non-BALT nodules with similar cell types (T-cells and B-cells) (Fig. 3a) are sparsely distributed throughout the lung (Reese et al., 2006). The Indian jungle bush quail, *P. asiatica* (order – Galliformes), is a seasonal breeder and faces more drastic environmental conditions than poultry birds and hence, might have developed a strong LAIS. It has been suggested that recent epidemics of lung-associated diseases in birds can lead to recovery from pulmonary disease by treatment with melatonin or melatonin-rich food supplements. Lymphocytes of BALT nodules are known to possess melatonin receptors. Whereas melatonin is known to play an immunomodulatory role in vertebrates including humans, so far there are no reports that LAIS could also be regulated by melatonin. The effect of melatonin on the immune system is also supported by the existence of specific binding sites for melatonin on lymphoid cells (Calvo et al., 1995). There is evidence for the presence of high-affinity binding sites for melatonin in human blood lymphocytes (Lopez-Gonzalez et al., 1992a, 1992b) and low-affinity melatonin binding sites in human granulocytes (Lopez-Gonzalez et al., 1993). In this report, we provide evidence for the localization of melatonin receptors in BALT and propose that BALT may be a target site for melatonin in the avian lung-associated immune system.

Materials and methods

Studies were conducted on healthy adult male Indian jungle bush quail, *Perdicula asiatica*, which is a sexually dimorphic species. Male birds were collected from the vicinity of Varanasi, India (Lat. 25°,18′N, Long. 83°,01′E) during the winter month of December and acclimatized to laboratory conditions for 2 weeks in an aviary exposed to ambient environmental conditions (photoperiod approx. 10 h light: 14 h darkness; maximum and minimum temperatures 15 ± 5 and 6 ± 3 °C; humidity approx. 90%). They were fed with millet seeds

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(*Pennisetum typhoideum*) along with other seasonal grains and water *ad libitum*. All the experiments were performed in accordance with institutional practice and within the framework of experimental animal studies (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA) 2007Act on Animal Welfare of the Government of India.

Histology

Male birds (n=40; weight ~45 g) were sacrificed under complete anesthesia by Nembutal (sodium pentobarbital) injection during late evening approximately 3 h after sunset (19.30–20.30). The lungs were infiltrated with Bouin's fixative *in situ* and then dissected out, cleaned and again immersed in Bouin's fixative overnight for routine histology. After fixation, tissues were dehydrated and embedded in paraffin. Transverse 6-μm-thick sections of the entire lung (-1×0.75 cm) were cut and stained with Harris hematoxylin and eosin (1% alcoholic). Histological observations of BALT and non-BALT nodules in lung tissue were performed with the help of Leitz MPV3 microscope (Germany). Ten sections of the entire lung from each bird were randomly selected for analysis of BALT and non-BALT nodules.

Immunohistochemistry

For immunohistochemical localization of melatonin receptor types (Mel_{1a} and Mel_{1b}), after Nembutal anesthesia, birds were perfused with 4% paraformaldehyde (PFA) and the entire lung was dissected out and kept in 4% PFA overnight. After dehydration, paraffin blocks were prepared and 6-μm-thick transverse sections were cut and mounted on 1% gelatin-coated slides and deparaffinized. Endogenous peroxidase activity was blocked by H_2O_2 in 80% methanol for 20 min at room temperature. Sections were washed three times with phosphate-buffered saline (PBS) and preincubated with 3% blocking serum in PBS for 40 min. Sections were then incubated with primary antibody Mel_{1a} (MEL-1A-R: R-18; sc-13186) and Mel_{1b} (MEL-1B-R: T-18; sc-13177), an affinity purified goat polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, USA, dilution 1:200) overnight at 4 °C. Sections were washed three times in PBS and were incubated with biotinylated secondary antibody (Vectastain ABC Universal kit; PK-6200, Vector laboratories, Burlinghame, CA, USA; dilution 1:10,000). Sections were washed with PBS and a pre-formed ABC reagent was conjugated to the free biotin of the secondary antibody. The antigens were visualized using the peroxidase substrate 3,3-diaminobenzidine (DAB) (Savaskan et al., 2002). For preabsorption, the antigens were added and incubated overnight at 4 °C. For the neutralization, the primary antibody was combined with Mel_{1a} and Mel_{1b} receptor blocking peptide (600 ng/100 μl of Mel_{1a}; sc-13186P and 600 ng/100 μl of Mel_{1b}; sc-13177P supplied by the manufacturer) and then incubated overnight at 4 °C. The following morning the immunohistochemical protocol was followed under the same conditions.

Validation of melatonin receptor antibodies

Antibodies for Mel_{1a} and Mel_{1b} receptor (Mel_{1a} (R-18); sc-13186 and Mel_{1b} (T-18); (sc-13177, Santa Cruz Biotech, Santa Cruz, CA, USA) were used in the study. Since, these commercial antibodies (Mel_{1a} and Mel_{1b}) have been used for the first time in this avian species, they have been validated for the use in the lung of *P. asiatica* with rat and chicken brain sections serving as positive controls. We used chicken brain because our avian model (*P. asiatica*), and chicken both belong to the same order: Galliformes. Immunoblot analysis for both Mel_{1a} and Mel_{1b} receptors in lung showed a single major band of 37 and 39 kDa, respectively (Prestained marker # SM-1841; Fermentas, Glen Burnie, MD, USA) corresponding to a similar band obtained in rat brain used as positive control (Fig. 1a,c). Validation of Western blot for Mel_{1a} and Mel_{1b} receptor protein was further confirmed using serially diluted protein (20% lung tissue homogenate) samples of lung ranging between 10 and180 μg. The intensities of the protein bands of the Western blot for Mel_{1a} and Mel_{1b} protein were quantified using densitometry software (Scion Corp. Image software, Frederick, MD, USA). The graph plotted between

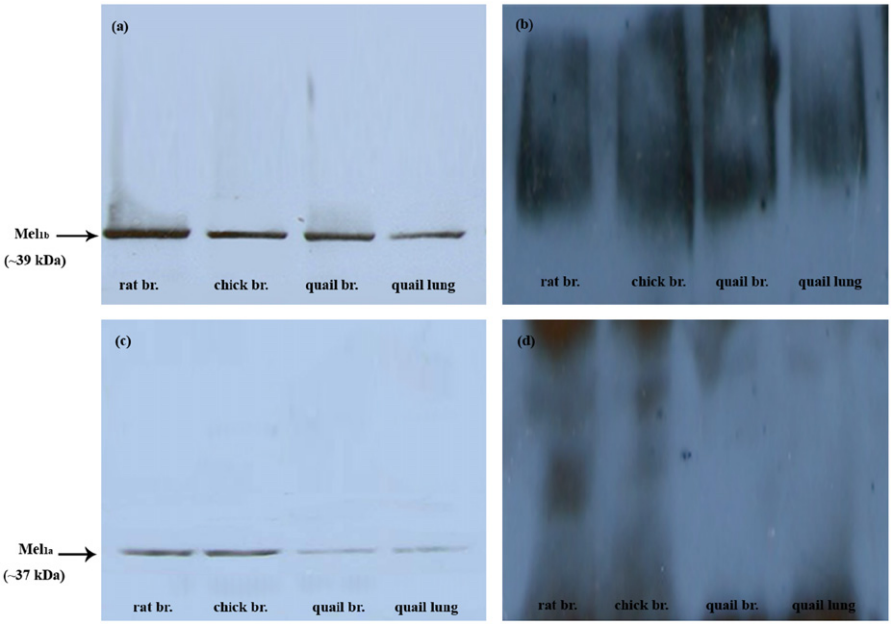

Fig. 1. (**a**) and (**c**): Western blot of isolated lung protein for Mel_{1a} and Mel_{1b} receptor of *P. asiatica* showing single specific band at 37 and ~39 kDa for Mel_{1a} and Mel_{1b}, respectively, using rat and chicken brain as positive control. (**b**) and (**d**); Western blot of isolated lung protein of *P. asiatica* preabsorbed with Mel_{1a} and Mel_{1b} receptor antibodies (600 ng/100 ml) using rat and chicken brain as positive control. (**rat br**, rat brain; **chick br**, chicken brain; **quail br**, quail brain).