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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, *Perdica 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, *Perdica 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.

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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, *Perdica 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) 2007 Act 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- μ m-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- μ m-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, Burlingame, 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 μ l of Mel_{1a} ; sc-13186P and 600 ng/100 μ l 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 and 180 μ g. 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

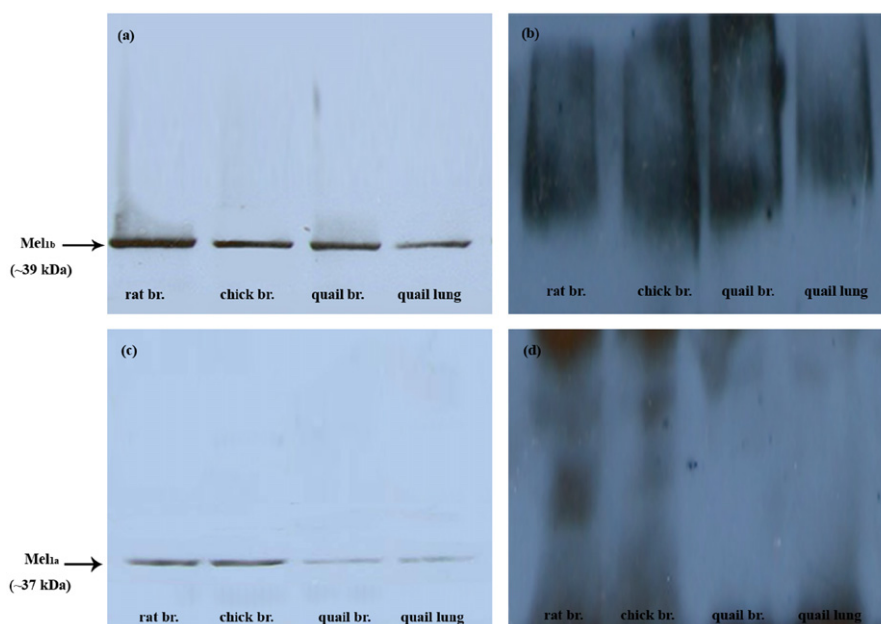


Fig. 1. (a) and (c): Western blot of isolated lung protein for Mel_{1a} and Mel_{1b} receptor of *P. asiatica* showing single specific band at 37 and ~ 39 kDa for Mel_{1a} and Mel_{1b} , respectively, using rat and chicken brain as positive control. (b) and (d): Western blot of isolated lung protein 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).

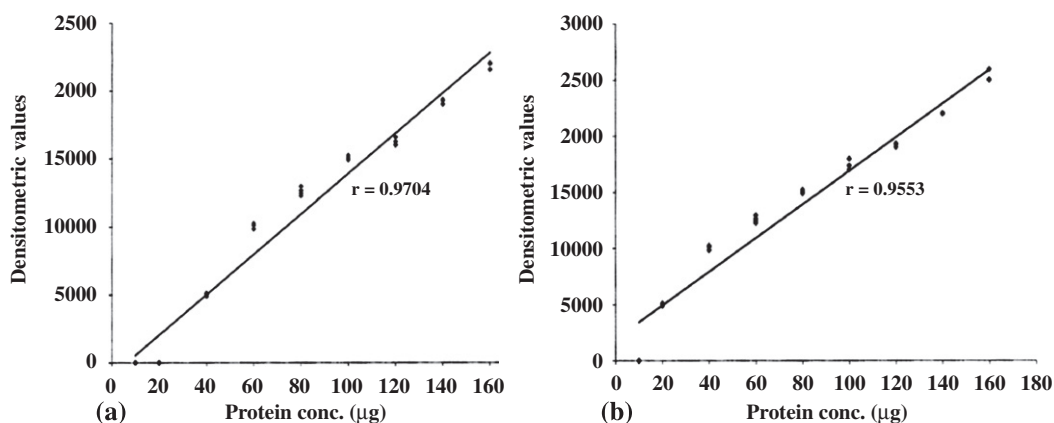


Fig. 2. Validation curve for Western blot analysis: (a) linear correlation between the amount of protein loaded and intensity of band obtained for Mel_{1a} ($r=0.9704$) and (b) linear correlation between the amount of protein loaded and intensity of band obtained for Mel_{1b} ($r=0.9553$).

the amount of protein loaded and intensity of the protein bands showed strong correlation in lung tissue protein ($r=0.9704$ for Mel_{1a} and $r=0.9553$ for Mel_{1b}) (Fig. 2a,b).

Western blot analysis

A piece of lung was removed and lysed in RIPA buffer (1% (v/v) Igel CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS) in phosphate-buffered solution (PBS) containing aprotinin and sodium orthovanadate). Aliquots containing 100 µg protein (Roy et al., 2001) were resolved by 12% (w/v) SDS-PAGE along with Spectra Multicolor Broad Range Protein Marker (SM-1841) to note the immunoreactive bands and was followed by electrotransfer to a PVDF Hybond membrane (GE Healthcare Life Sciences, Amersham, Bucks., UK). Immunodetection was carried out using melatonin receptor antibodies Mel_{1a} (R-18; sc-13186), and Mel_{1b} (T-18; sc-13177), (Santa Cruz Biotech, Santa Cruz, CA, USA, dilution 1:200) followed by horseradish peroxidase conjugated secondary antibody (anti-goat donkey IgG), using a chemiluminescence (ECL) system (GE Healthcare Life Sciences, Amersham, Bucks., UK). The antibody dilutions used in the present study were determined after testing several dilutions for the optimum results with the lung tissue of this bird. Each band was quantified with the help of Scion Image analysis software (www.scioncorp.com). The ratio of density was calculated after normalization with β actin and was expressed as % control value (Treeck et al., 2006). To test the specificity of the antibody primary antiserum in the same dilution (Mel_{1a} and Mel_{1b}; 1:200) were preabsorbed with their respective pure antigenic peptide at a concentration of 600 ng/100 µl (Mel_{1a}; sc-13186P and Mel_{1b}; sc-13177P peptides; Santa Cruz, USA) and incubated overnight at 4 °C. With this preabsorbed antiserum Western blot was performed in target tissue.

Statistical analysis

Statistical analysis of the data for Western blot analysis was performed using Student *t*-test. The differences were considered significant when $p < 0.05$ (Bruning and Knitz, 1977). Microsoft Excel program was used for statistical calculations and data presentation.

Results

Histological profiles of BALT nodules

The major immune structures of LAIS are the BALT nodules. Most of the lymphatic nodules were noted near the secondary and tertiary bronchi as a small nodule or sometimes as aggregated structures located in the lung tissue in between air capillaries. The BALT nodules were differentiated from non-BALT nodules owing to their characteristic features of aggregation of lymphocytes located at the junction of the primary bronchus and secondary bronchi (Fig. 3b), whereas non-BALT nodules had lymphocyte aggregations in other regions in the lungs (Figs. 3c, 4b). Nodules were also noted adjacent to bronchus, blood vessels and sometimes also at a distance from both the areas (Figs. 3, 4). Lymphocytes and macrophages were observed either in groups or in free form throughout the lung tissue.

Immunohistochemistry

There was differential distribution of melatonin receptor types in the lung tissue. Mel_{1b} receptor was distributed with intense immunostaining in the bronchial region of the lungs, especially in the apical margins of bronchial mucosal cells and finger-like projections of mucosal folding, with less intense immunostaining in other regions of the lung tissue. Some lymphocytes in BALT nodules, as well as in free form, also showed immunostaining (Figs. 5d, 6b). There was very low intensity of Mel_{1a} receptor immunostaining in bronchial mucosal foldings, rather than other areas of the lung tissue (Figs. 5c, 6a). When the degree of immunostaining and immunolocalization for Mel_{1a} was compared with Mel_{1b}, we found that BALT nodules always had less distribution of Mel_{1a}.

Western blot analysis

As per validation (Figs. 1, 2), Western blot of melatonin receptors using goat polyclonal antibody in lung isolated proteins showed a single immunoreactive band of approximately 37 kDa for Mel_{1a} and 39 kDa for Mel_{1b}. Statistical analysis of percent band intensity using Scion image analysis software suggest that the expression of Mel_{1b} receptor type was significantly ($p < 0.01$) higher than that of Mel_{1a} in the lung tissue (Fig. 7).

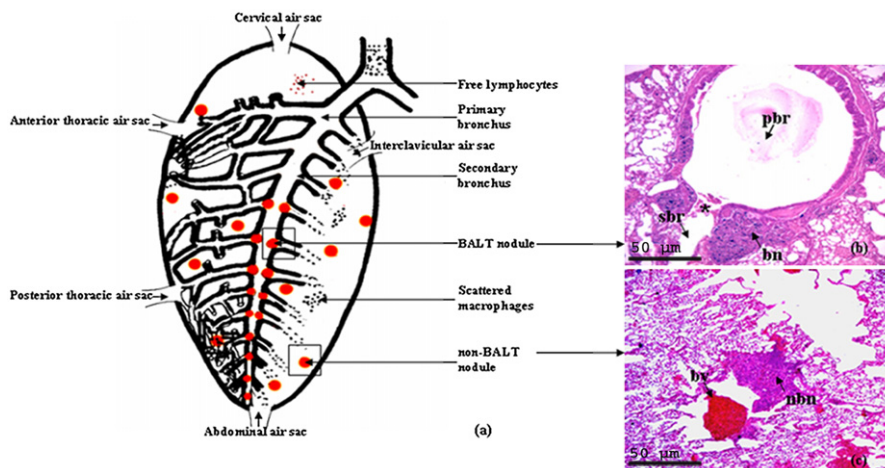


Fig. 3. (a) Diagrammatic representation of right lung of *P. asiatica* showing gross morphological distribution of BALT, non-BALT nodules, free lymphocytes, macrophages and openings to the air sacs; (b) transverse section (hematoxylin and eosin stain) of lung tissue of *P. asiatica* showing BALT nodule at the junction between primary bronchus and secondary bronchus and (c) non-BALT nodule adjacent to blood vessel. (bn, BALT nodule; *junction; pbr, primary bronchus; sbr, secondary bronchus; nbn, non-BALT nodule; bv, blood vessel).

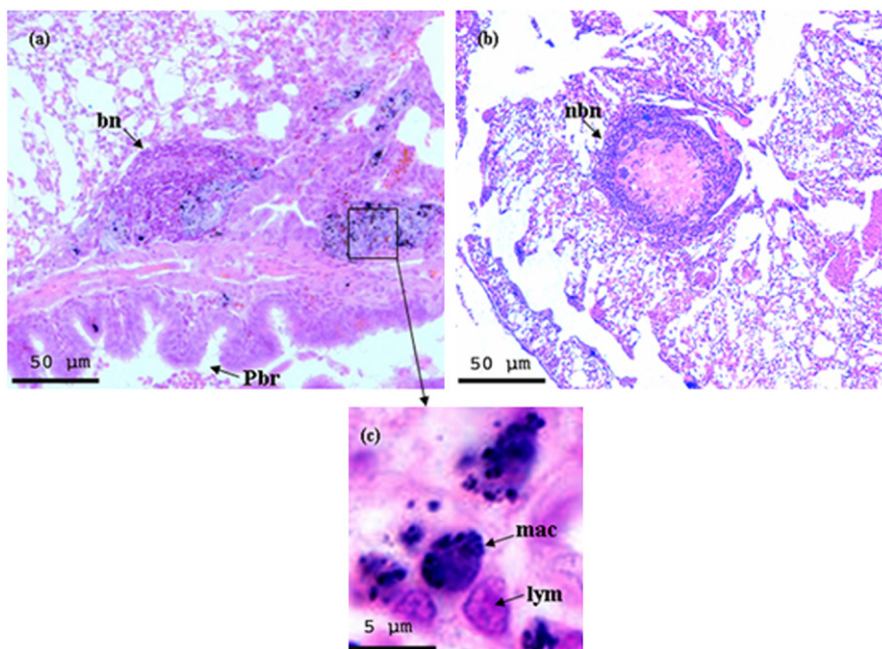


Fig. 4. Transverse section (hematoxylin and eosin stain) of lung tissue of *P. asiatica* showing: (a) BALT nodule; (b) non-BALT nodule and (c) macrophage (under oil immersion). (pbr, primary bronchus; bn, BALT nodule; nbn, non-BALT nodule; mac, macrophage; lym, lymphocyte).

Discussion

The nomenclature of avian lung-associated lymphoid structures in the bronchial mucosa of chicken was described for the first time by Beinenstock et al. in (1973a, 1973b), and the tissue was shown to be similar to Peyer's patches. The lymphoid structures designated as BALT resembled other gut-associated lymphoid tissues (GALT). Mature BALT structures with germinal centers consist of lymphocyte aggregates covered by a distinct layer of epithelial cells harbouring lymphocytes (Fagerland and Arp, 1993b), lymphoepithelium or follicle-associated epithelium (FAE) (Bienenstock and Befus, 1984; Fagerland and Arp, 1990; Fagerland and Arp, 1993a). When germinal centers are not present in BALT structure, B-cells are distributed in the margins of the lymphoid tissue, whereas lymphoid nodules are composed of aggregates of T-cells in the center and B-cells at the periphery

(Fagerland and Arp, 1993b; Jeurissen et al., 1994). BALT has been frequently studied and reported to play a crucial role in the development of local immune responses to inhaled antigens in chickens (Bienenstock et al. (1973b), Jeurissen et al., 1994; Reese et al., 2006) and turkeys (Fagerland and Arp, 1990); and our knowledge has been restricted to those species. However, the presence of well organized LAIS in other wild birds of different zones cannot be ruled out. In the present study, we demonstrated the morphology of BALT and localization of MeI_{1a} and MeI_{1b} receptors in lungs of the Indian Jungle bush quail, *P. asiatica*, a wild bird that faces more drastic changes in environmental conditions than those of poultry birds that are restricted to well defined conditions.

The histological appearance of the lymphoid tissues examined in our avian model was quite similar to that of chicken and turkey except for the location and distribution of the BALT structure

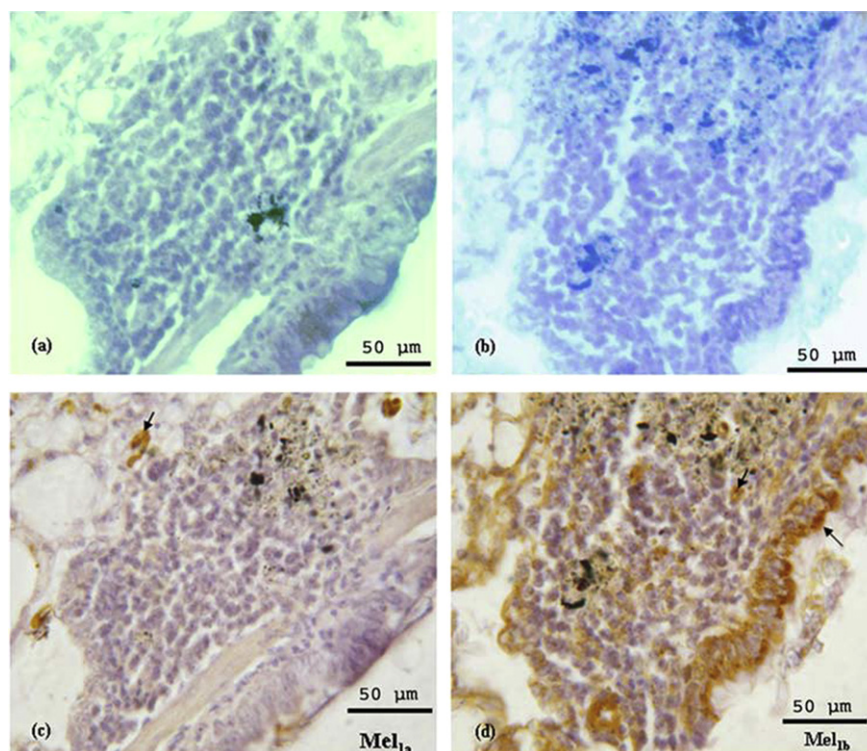


Fig. 5. Transverse section of lung tissue of *P. asiatica* showing: (a) and (b) control (preabsorbed) sections for Mel_{1a} and Mel_{1b} receptors, respectively; (c) serial section of same region (a) showing Mel_{1a} receptor immunoreactive positive cells (arrow) counterstained with hematoxylin and (d) serial section of same region (b) showing high immunoreactivity in bronchial folding and cells in nodular region for Mel_{1b} receptor (arrow) counterstained with hematoxylin.

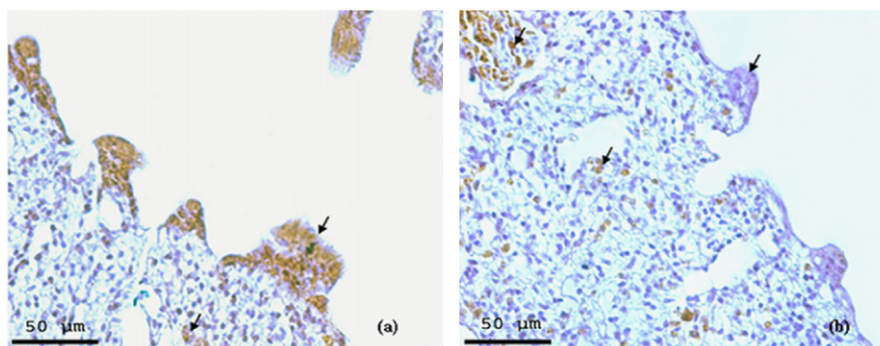


Fig. 6. Transverse section of lung tissue of *P. asiatica* showing: (a) Mel_{1a} receptor immunoreactions (arrow) and (b) Mel_{1b} receptor immunoreactive cells (arrow) in interatrial septum of the lung.

(Fig. 3). This could be due to a significant difference between the different avian species. In general, the avian lung exhibits both highly organized BALT nodules and diffusely distributed non-BALT nodules, which might be due to increased susceptibility of the lungs to various respiratory diseases. In *P. asiatica*, BALT structures were mostly noted at the junctions of the primary bronchus and caudal secondary bronchi (Fig. 3b) as noted by Fagerland and Arp (1993a) for chickens, while non-BALT nodules were diffusely distributed throughout the lung (Figs. 3c, 4b) when compared with chickens. Non-BALT nodules were also noted adjacent to the blood vessels (Fig. 3c) in *P. asiatica* suggesting an opportunity for lymphocytes and humoral products to enter directly in the vascular system, which might conceivably provide extra protection for seasonally occurring stress. Acknowledging the limitations of structure as a predictor of function, it is apparent that in the jungle bush quail, lymphoid tissue has all the

essential cell types, namely lymphocytes and macrophages (Fig. 4c) to be able to mount an effective immune response against foreign antigens.

There is some evidence to support the immunomodulatory role of melatonin in birds (Skwarlo-Sonta, 1999; Singh and Haldar, 2007). Membrane and nuclear binding sites for melatonin have been described in many different immune tissues from many species of birds and mammals, including humans and rodents (Liu and Pang, 1993; Raffi-el-Idrissi et al., 1995; Gonzalez-Haba et al., 1995). An essential fact that supports a relationship between melatonin and the immune system is the presence of melatonin receptors in immune organs and cells (Calvo et al., 1995; Carrillo-Vico et al., 2003). There is considerable evidence that melatonin, acting through its receptors, is involved in a wide range of physiological processes (Dubocovich et al., 1998; Drazen and Nelson, 2001; Pandi-Perumal et al., 2006). As an

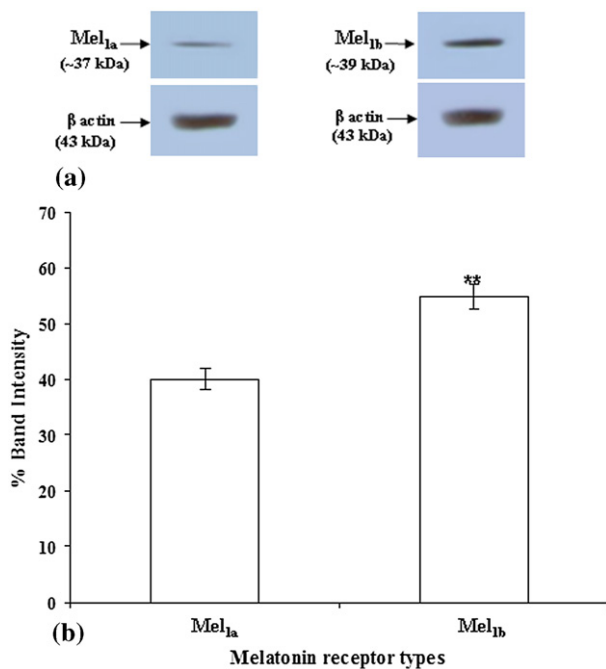


Fig. 7. (a) Western blot showing Mel_{1a} and Mel_{1b} receptor expression in lung tissue of *P. asiatica* and (b) vertical bar (mean ± SEM) showing percent band intensity of Mel_{1a} and Mel_{1b} receptor types following Scion image analysis. ****p* < 0.01, Mel_{1a} vs Mel_{1b}.

immunomodulator, melatonin has been reported to enhance cell-mediated and humoral immunity via its Mel_{1b} receptor (Drazen and Nelson, 2001).

Detailed immunohistochemical studies of BALT and non-BALT tissues are lacking for avian species, especially for wild birds. Furthermore, the role of melatonin in avian reproduction and immunity, especially in our avian model (Haldar and Singh, 2002; Singh and Haldar, 2005, 2007) has already been reported and led us to propose for the first time a functional integrity of BALT and non-BALT nodules based on the histology and receptors for melatonin. We performed immunohistochemistry and Western blot analysis for the presence of melatonin receptor types Mel_{1a} and Mel_{1b} in the BALT and non-BALT to suggest a direct action of melatonin on LAIS.

Immunohistochemical examination showed the cellular distribution of melatonin receptors (Figs. 5, 6). Both of the receptor types, Mel_{1a} and Mel_{1b}, were present in LAIS (Fig. 7), though their distribution and location was quite different (Figs. 5, 6). High immunoreactions of Mel_{1b} were noted on the smooth muscles and mucosal folds (Fig. 5d). On the other hand, Mel_{1a} immunoreactions were scanty in the bronchus region (Fig. 5c), but were more prominent in the interatrial septum of the lungs. Immunoreactions of the BALT nodule near the bronchus were more intense for Mel_{1b} than for Mel_{1a}. There were small unidentified cells stained positive for Mel_{1a} and Mel_{1b} scattered in the entire interatrial region (Fig. 6). Those cells were considered morphologically different from the cells noted in LAIS.

Sufficient distribution of melatonin receptor types in lung tissue suggests that the neuroendocrine system (pineal gland and melatonin) is certainly involved in maintenance of lung-associated immunity of wild birds. It could be that melatonin utilizes both kinds of receptors Mel_{1a} and Mel_{1b} for regulation of lung-associated immune system in this tropical bird, *P. asiatica*. In non-mammalian species a further membrane receptor, Mel_{1c}, is frequently also demonstrated within the immune system

(Markowska et al., 2004). However, specific antibodies against this receptor are not commercially available and consequently it was not technically feasible to describe its presence. It also needs to be considered here that the expression of Mel_{1a} and Mel_{1b} depends on the internal melatonin levels, as well as the season and time of day when the birds were sacrificed. We performed our study during a winter month (December) in a tropical zone when due to short day length (–10 h) basal melatonin levels are high and near an annual peak (Singh and Haldar, 2007) and hence, we expected a high receptor expression for basic study of Mel_{1a} and Mel_{1b} expression in LAIS. While our study has contributed to morphological and immunological studies on LAIS of a wild bird, it is imperative that additional functional studies are undertaken to support our conclusions. In the future we intend to study melatonin and its receptor antagonist during different reproductive phases in order to further clarify the role of melatonin in modulation of LAIS.

Acknowledgements

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