FULL PAPER *Pathology*

Distribution of CD163-positive cell and MHC class II-positive cell in the normal equine uveal tract

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ABSTRACT. Antigen-presenting cells (APCs) in the uveal tract participate in ocular immunity including immune homeostasis and the pathogenesis of uveitis. In horses, although uveitis is the most common ocular disorder, little is known about ocular immunity, such as the distribution of APCs. In this study, we investigated the distribution of CD163-positive and MHC II-positive cells in the normal equine uveal tract using an immunofluorescence technique. Eleven eyes from 10 Thoroughbred horses aged 1 to 24 years old were used. Indirect immunofluorescence was performed using the primary antibodies CD163, MHC class II (MHC II) and CD20. To demonstrate the site of their greatest distribution, positive cells were manually counted in 3 different parts of the uveal tract (ciliary body, iris and choroid), and their average number was assessed by statistical analysis. The distribution of pleomorphic CD163- and MHC II-expressed cells was detected throughout the equine uveal tract, but no CD20-expressed cells were detected. The statistical analysis demonstrated the distribution of CD163- and MHC II-positive cells focusing on the ciliary body. These results demonstrated that the ciliary body is the largest site of their distribution in the normal equine uveal tract, and the ciliary body is considered to play important roles in uveal and/or ocular immune homeostasis. The data provided in this study will help further understanding of equine ocular immunity in the normal state and might be beneficial for understanding of mechanisms of ocular disorders, such as equine uveitis.
KEY WORDS: CD163, horse, immunohistochemistry, MHC II, uveal tract

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Resident antigen-presenting cells (APCs) in the normal uveal tract participate in local ocular immunological homeostasis and systemic immunological regulation. The uveal tract has previously been demonstrated to be a site of rich APC distribution in the human [12], murine [10, 12] and rat [12, 13] eyes. APCs in the uveal tract were classified into different immunohistochemical phenotypes, such as macrophage and dendritic cells. They not only play important roles in the normal state but also were considered to be involved in initiation and propagation of uveitis and/or uveal immune-mediated disease [11].

MHC class II (MHC II) expression is a specific characteristic of cells with the ability to present antigens. Normally, the expression is limited to three types of cells that are classified as APCs, i.e., macrophages, dendritic cells and B cells [18]. MHC II-positive cells are distributed throughout the ocular tissue and are involved in the formation of immune privilege, and they are considered to play an important role in ocular immunity [14, 17]. They are also considered to play an important role in progression of ocular inflammation and immunoresponse.

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In horses, uveitis is the most important of all ophthalmic disorders, and as in other animals, it has been reported to have a variety of causes [2, 3, 7]. However, resident cells, such as tissue macrophages and MHC II-positive cells, have not been sufficiently elucidated. For better understanding of the etiology and mechanisms of immune-inflammatory responses affecting the equine uveal tract, we must develop our knowledge of their distribution in the normal state.

The present study aimed to elucidate the distribution of tissue macrophages and MHC II-positive cells in the equine uveal tract so as to clarify their role in uveitis. The basic knowledge provided by the present study will be valuable in understanding the mechanisms of equine ocular immune homeostasis. Moreover, it might provide beneficial data to develop our understanding of equine uveitis.

MATERIALS AND METHODS

Animals and tissue preparation: Eleven normal eyes from 10 horses were studied. The horses used in the present study were submitted for necropsy to the Laboratory of Veterinary Pathology of Rakuno Gakuen University. All the horses were Thoroughbreds. The ages of the horses ranged from 1-year-old to 24-year-old, and there was 1 male and 10 females. There were various clinical and pathological diagnoses, but ocular abnormalities were not observed in any horses. The characteristics of the horses used in this study are summarized in Table 1. The eyes were fixed in 10% buffered formalin. For adequate fixation, appendages of the eyes were trimmed, and an incision about 1 cm long was made in the

Table 1. The horses used in the present study

No.	Eye	Age	Sex	Diagnosis
1	L	1 Y	F	Pelvic fracture
2	R			
3	L	1 Y	F	Osteochondritis dissecans
4	R	1 Y	M	Wobbler syndrome
5	L	2 Y	F	Fracture of the left third metacarpal bone
6	R	8 Y	F	Rupture of the adductor muscle
7	R	8 Y	F	Colonic torsion
8	L	9 Y	F	Spinal cord injury
9	R	15 Y	F	Uterine artery rupture
10	R	17 Y	F	Colonic torsion
11	L	24 Y	F	Mammary gland adenocarcinoma

L: left, R: right, Y: year(s), M: male, F: female.

dorsal and sagittal ora serrata. Formalin was injected via the incision, and then the eyes were immersed for 24 to 48 hr in formalin. The fixed eyes were cut into two or three 0.5–1.0 cm strips of tissue containing anterior and posterior ocular segments. The divided tissues were embedded in paraffin wax following immersion in graded ethyl alcohols and xylene. The tissues were sectioned at a thickness of 5 μm and used for immunohistochemical examinations.

Immunohistochemical examinations: Indirect immunofluorescence studies were performed using primary mouse monoclonal antibodies against human CD163 (AM-3K; Trans Genic Inc., Tokyo, Japan; diluted 1:50) and MHC II (HLA-DR; TAL.1B5; Dako, Glostrup, Denmark; diluted 1:50) and a rabbit polyclonal antibody against human CD20 (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.; diluted 1:200). Dewaxed sections were subjected to antigen retrieved by incubation with Proteinase K (Dako) for CD163 and by heating in an autoclave in the presence of 0.01-M sodium citrate buffer (pH 6.0) for MHC II. For CD20, no pretreatment was performed. After pretreatment, the sections were incubated with 10% normal goat serum (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37°C for 30 min as a blocking step. Subsequently, the sections were incubated with the diluted primary antibodies at 4°C overnight in moist chambers. Following primary incubation, the sections were reacted with Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.; diluted 1:200) for CD163 and MHC II, or with Alexa Fluor 546-labeled goat anti-rabbit IgG (Molecular Probes; diluted 1:200) for CD20, at room temperature for 30 min in shaded moist chambers. The sections were covered with a water-soluble mounting medium and then were examined using a confocal microscope (C2; Nikon Instech Co., Ltd., Tokyo, Japan). With each immunofluorescence run, equine lymph node was used as a positive control, and for negative controls, sections were incubated without the primary antibodies.

Statistical analysis: The numbers of CD163+ and MHC II+ cells in different uveal tissues, the ciliary body, iris and choroid, were manually counted, respectively. Counting of these cells was performed with an immunofluorescence staining microscope image using 400× high-power magnifications, and the tissue area was measured in each image. The measurements were carried out until the total of the measured areas in each tissue exceeded 1 mm^2. The counted numbers of positive cells are presented as the average per 1 mm^2, and statistical analyses were performed using specific software (Excel and Ekuseru-Toukei 2012; Social Survey Research Information Co., Ltd., Tokyo, Japan). To determine the greatest site of CD163+ and MHC II+ cells, the average numbers of CD163+ and MHC II+ cells in each tissue were analyzed by Kruskal-Wallis test and Scheffe's test for a priori and post hoc comparison, respectively. For each analysis, $P<0.01$ was considered to be significant.

RESULTS

Immunohistochemical examinations: *CD163+ cells*: *Ciliary body*. Main location of CD163+ cells was found in the neighborhood of the ciliary epithelium, and most of the cells were lying closely beneath and along the basal side of the pigmented ciliary epithelium (Fig. 1). The majority of the cells appeared to be fusiform and/or elongating in shape, and the minority of the cells were round to oval in shape (Fig. 2). Fusiform CD163+ cells were also scattered throughout connective tissue of the ciliary stroma including the ciliary base.

Iris. CD163+ cells were scattered throughout the iris and were detected in the stroma including the anterior surface of the iris and lying directly beneath and along the basal side of the posterior pigment epithelium (Fig. 3). Most of the cells were morphologically round to oval in shape, but elongated CD163+ cells were occasionally observed. Lower densities of immunopositive cells were observed at the pupil margin and mid-iris compared with the iridal base.

Choroid. CD163+ cells were scattered throughout the choroidal stroma and were morphologically round to oval in shape, but elongated positive cells were also observed (Fig. 4).

MHC II+ cells: *Ciliary body*. Abundant distributions of MHC II+ cells were observed in the ciliary body, and their main location in the ciliary body was in the neighborhood of the ciliary epithelium. The MHC II+ cells, which had similar immunomorphological appearances to CD163+ cells, lay directly beneath and along the basal side of the pigmented ciliary epithelium (Figs. 5 and 6). In addition to these findings, some MHC II+ cells displayed a dendritic appearance. The cells were occasionally interposed between the layers of the ciliary epithelium (Fig. 7), and the cells were also detected at the basal side of the pigmented ciliary epithelium. MHC II+ processes sometimes extended from the MHC II+ cells were observed between the lateral and apical surface of ciliary epithelial cells (Fig. 6). MHC II+ cells were randomly scattered throughout the connective tissue stroma, and the majority of the cells displayed a fusiform shape, whereas the minority of the cells displayed a round to oval shape. Also, dendriform MHC II+ cells were scattered throughout the base of the stroma in the ciliary body.

Iris. MHC II+ cells were randomly scattered throughout the connective tissue stroma and were also found at the anterior surface of the iris and lying directly beneath and along the basal side of the posterior pigment epithelium. Morphologically, the cells were round to oval in shape and were occasionally elongated. Lower densities of MHC II+ cells

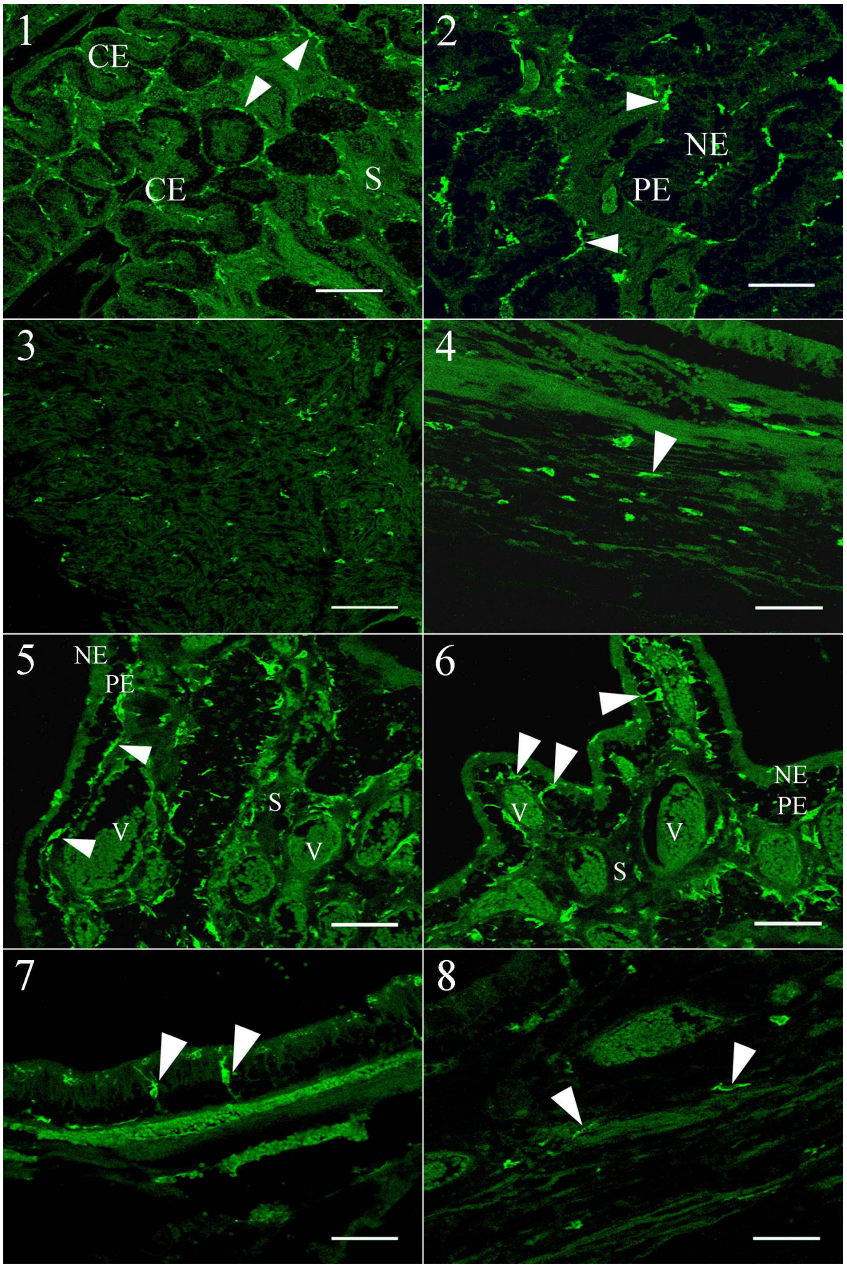

Fig. 1. CD163, ciliary body in horse No. 3. CD163-positive cells are located in the neighborhood of the ciliary epithelium (arrowheads). CE: ciliary epithelium. S: stroma. Bar=100 μm.

Fig. 2. CD163, ciliary body in horse No. 3. Fusiform and elongate CD163-positive cells lie closely beneath and along the basal side of the pigmented ciliary epithelium (arrowheads). NE: nonpigmented epithelium. PE: pigmented epithelium. S: stroma. Bar=50 μm.

Fig. 3. CD163, iris in horse No.1. CD163-positive cells are scattered throughout the iridal stroma. Bar=50 μm.

Fig. 4. CD163, choroid in horse No. 1. CD163-positive cells are morphologically round to oval in shape, but elongated positive cells (arrowhead) are occasionally found. Bar=50 μm.

Fig. 5. MHC II, ciliary body in horse No. 9. MHC II-positive cells lie directly beneath and along the basal side of pigmented ciliary epithelium (arrowheads). The positive cells are also scattered throughout the stroma. NE: nonpigmented epithelium. PE: pigmented epithelium. S: stroma. V: vessel. Bar=50 μm.

Fig. 6. MHC II, ciliary body in horse No. 11. MHC II-positive cells and MHC II-positive processes are occasionally interposed between the layers of the ciliary epithelium (arrowheads). NE: nonpigmented epithelium. PE: pigmented epithelium. S: stroma. V: vessel. Bar=50 μm.

Fig. 7. MHC II, ciliary body in horse No. 11. Interposed dendriform MHC II-positive cells between the layers of the ciliary epithelium (arrowheads). Note that some processes are extended from the cytoplasm. Bar=50 μm.

Fig. 8. MHC II, choroid in horse No. 1. MHC II-positive cells are morphologically round to oval in shape, but elongated positive cells (arrowheads) are occasionally found. Bar=50 μm.

were observed at the pupil margin and mid-iris compared with the iridal base.

Choroid. Scattered MHC II+ cells, which were morphologically round to oval in shape and elongated, were observed in the choroidal stroma (Fig. 8).

CD20+ cells: No cells exhibiting immunoreactivity for the CD20 antibody were observed in the ciliary body, iris and choroid.

CD163+, CD20+ and MHC II+ cells in equine lymph nodes: Cells exhibiting immunoreactivity for CD163 were detected in the lymphatic sinus, and cells exhibiting immunoreactivity for CD20 were detected in the lymphatic cortex. MHC II+ cells were sporadically found in structures of the lymph nodes including the lymphatic sinus and cortex. Dendriform MHC II+ cells were also detected in the T and B zone. The detection of CD163+, CD20+ and MHC II+ cells in equine lymph nodes indicated that the primary antibodies were appropriately used for identification of the immunoreactive cells of each antibody in the equine uveal tract.

Statistical analysis: By statistical analysis, the largest numbers of CD163+ cells and MHC II+ cells were found in the ciliary body compared with the iris and choroid. The analyzed data are summarized in Figs. 9 and 10.

DISCUSSION

This is the first study on the distribution of resident tissue macrophages in the normal equine uveal tract. CD163 expression has been specifically identified in subpopulations of resident tissue macrophages in normal tissues of various animal species including the horse [20]. Thus, the CD163-expressed cells in this study corresponded to the resident uveal tissue macrophages. Macrophages are professional phagocytes and play a pivotal role as effector cells in cell-mediated inflammation and immunity [18]. In normal tissues, they form a first line of defense in which they recognize and eliminate potential pathogens, but they also secrete various physiologically active substances and play important roles in processing of immune regulation, tissue reorganization and angiogenesis [4]. CD163-expressed macrophages, which were distributed in the equine uveal tract, are classified as alternatively activated macrophages, which have been suggested to play a major role in immune suppression and resolution of inflammation [5]. CD163 is a type B crystalline-rich scavenger receptor, and the best-characterized function of the receptor is related to clearance via endocytosis of hemoglobin-haptoglobin complexes, which possibly protect tissues from the oxidative effects of free hemoglobins. Additionally, it has also been reported to function as an immune sensor for bacteria [6]. The present study revealed CD163-positive resident tissue macrophages, which potentially participate in immunological homeostasis and inflammatory disorders in the equine uveal tract.

Eyes are known as an immune-privileged site, and MHC II-positive cells distributed throughout the ocular tissue contribute to the ocular immune homeostasis [14, 17]. Therefore, it is important to recognize their distribution, localization and number to understand equine ocular immune homeostasis, and the present study evaluated the MHC II-positive cells in the equine uveal tract. A previous study investigated MHC II-positive cells in the equine uveal tract, and a few scattered positive cells were detected in the stroma, but the study did not clearly demonstrate a specific number of MHC II-positive cells [15]. On the other hand, the present study detected a novel distribution of cells at a contiguous ciliary epithelium and demonstrated that the equine uveal tract contained further MHC II-positive cells. For further understanding of equine ocular immune homeostasis, the findings and characterizations described in the present study should be considered. The concept of ocular immune privilege in the past was dependent on a lack or paucity of distribution of MHC II antigen-bearing cells in ocular tissues including the uveal tract [1, 8, 19], and it was believed that the minimum distribution of the MHC II antigen-bearing cells minimized ocular inflammatory and immune response [17]. Subsequently, reevaluation of MHC II-positive cells in the human, mouse and rat proved that there is a rich cell network in the uveal tract [10, 12, 13]. At present, ocular immune privilege is considered to be formed by a number of MHC II-positive cells distributed throughout the uveal tract.

In addition to macrophages, dendritic cells and B cells are classified as APCs [18]. In the present study, the distribution of B cells was not detected in the normal equine uveal tract. However, comparing the distribution and morphology of CD163-positive and MHC II-positive cells suggested the presence of dendritic cells, which were identified as dendriform MHC II-positive cells in the ciliary body. Dendritic cells have weak phagocytic activity, but they are potent professional antigen-presenting cells. In peripheral tissues, these cells have functions involved in disposal of endogenous antigens, sensing of endogenous antigens and surveillance of the immune system [16]. The present study exhibited the presence of dendritic cells as dendriform MHC II-positive cells in the equine uveal tract immunomorphologically; however, further investigation is needed to identify their functional role as dendritic cells.

The statistical analyses in the present study demonstrated the greatest site of the distribution of CD163-positive and MHC II-positive cells. The results confirmed that the ciliary body is the largest site of their distribution in the equine uveal tract. The localizations of the CD163- and MHC II-positive cells in the ciliary body were considered to make a cytological contribution to the strategic formation of the blood-aqueous barrier. Moreover, the greatest localization of MHC II antigen-bearing cells at the ciliary body suggested that this might be the most competent site to recognize endogenous and/or exogenous antigens. The implication is that antigen presentation by the cells to circulating T cells is more likely to occur locally in the ciliary body than in the iris and choroid and that the ciliary body might play a central role in initiation and propagation of equine uveal and/or ocular inflammatory disorders. In the mouse, rat and human, the distribution of resident APCs in the uveal tract has been described as a rich cell network composed of MHC II-positive and/or negative macrophages and MHC II-positive dendritic cells [10–13]. High-density networks were especially found in the connective tissue stroma. The findings regarding CD163- and MHC II-positive cell distribu-

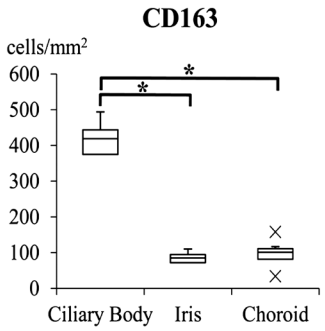

Fig. 9. Quantitative analysis of CD163+ cells located on the ciliary body, iris and choroid. There were significant differences between the ciliary body and iris (P<0.001) and between the ciliary body and choroid (P=0.0021), respectively. ˣStandard error of the mean. *Statistically significant difference (P<0.01).

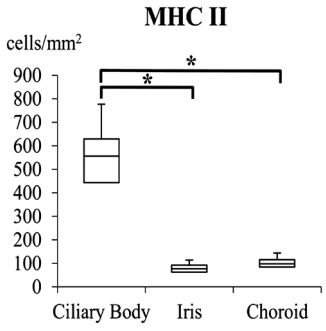

Fig. 10. Quantitative analysis of MHC II+ cells located on the ciliary body, iris and choroid. There were significant differences between the ciliary body and iris (P<0.001) and between the ciliary body and choroid (P=0.0029), respectively. *Statistically significant difference (P<0.01).

tion in the equine uveal tract might indicate that the difference of the predominant site plays a key role in the difference in ocular immunity between the horse and other animals.

The present study demonstrated and characterized the distribution of resident tissue macrophages and MHC II-positive cells in the normal equine uveal tract, which is considered to be potentially involved in immune homeostasis. The data presented in this study will help further understanding and elucidation of equine ocular immunity. In horses, uveitis is induced by various causes, which can be classified into local and systemic infection or immune-mediated disease [2, 3]. The immunological responses are important factors for promotion of its pathogenesis [7, 9]. Greater knowledge of the resident cells in the normal equine uveal tract might provide more understanding of the pathological mechanisms of uveitis.

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