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MHC Class II+ (HLA-DP-like) Cells in the Cow Reproductive Tract: I. Immunolocalization and Distribution of MHC Class II+ Cells in Uterus at Different Phases of the Estrous Cycle

U. Eren*, M. Sandikci, S. Kum and V. Eren[1]

University of Adnan Menderes, Faculty of Veterinary Medicine, Department of Histology -Embryology
09016 Isikli-Aydın, Turkey

ABSTRACT : This study was undertaken to investigate the distribution of major histocompatibility complex class II positive (MHC II+) (HLA-DP-like) cells in the cow uterus (cervix, corpus and cornu uteri) and to compare these cells between the estrus and diestrus phases of the estrous cycle. Twenty-nine multiparous cows were used. Tissue samples from the middle of the cervix, the corpus and the right cornu were taken immediately after slaughter at the estrus or diestrus phase. Streptavidin-biotin peroxidase complex staining was used to detect MHC II+ cells. The number of MHC II+ cells per unit area of tissue was counted using image analysis software under a light microscope. Numerous MHC II+ cells were found in the endometrium (cervix, corpus and cornu uteri) in both estrus and diestrus. MHC II+ cells were found in the surface epithelium of the cervix uteri in diestrus, but in the corpus uteri in both estrus and diestrus and in the cornu uteri in estrus. MHC II+ cells were also found freely in the lumen of the glands and between the gland epithelia of the corpus and cornu uteri in both estrus and diestrus. There were also MHC II+ cells in the connective tissue of the myometrium and perimetrium (outside the endometrium) and around the blood vessels. Endothelial cells were frequently positive for MHC II staining. More MHC II+ cells were found in the endometrium than outside the endometrium in both estrus and diestrus ($p<0.001$). However, there was no difference in the numbers of positive cells between estrus and diestrus either in the endometrium or outside it. These results are the first evidence for HLA-DP-like MHC II+ cells in the bovine uterus. They indicate that antigen presentation by HLA-DP-like MHC II+ cells of the uterus is not influenced by hormonal status. (**Key Words :** MHC Class II, Cow, Genital Tract, Estrous Cycle)

INTRODUCTION

The mucosal defence mechanisms of the female genital tract of mammals and chickens have become a focus of attention for researchers.

Endometrial lymphoid tissue in humans comprises lymphocytes related to subepithelial and periglandular macrophages, interstitial lymphocytes, macrophages and lymphoid aggregates in the stratum basalis (Morris et al., 1985). The hormonal changes of the estrous cycle, especially of estrogen, which is a regulator of the process, have an effect on the defence mechanism in mammals (Wira and Sullivan, 1985; Wira and Rossoll, 1995a, b). It has also been reported that resistance against infections of the uterus in estrus is better than that in diestrus (Canning and Billington, 1983; Rachman et al., 1983; Watson, 1985; Tunon et al., 1999).

Ia+ cells, macrophages, granulocytes and dendritic cells are numerous in the endometrial stroma and glandular epithelium of the uterus in the estrus phase of rats (Kaushic et al., 1998). However, Wira and Rossoll (1995 a, b) proposed that estradiol induces antigen presentation by rat uterus epithelial cells while inhibiting this in the uterine stroma and vagina. Inhibition of stromal cell antigen presentation is mediated through the stimulatory effect of estradiol on transforming growth factor-β production by epithelial cells (Wira and Rossoll, 2003).

In mares, the epithelium and stratum compactum have more MHC class II+ cells during estrus than during diestrus (Watson and Thomson, 1996).

Macrophages (Barua et al., 1998a, b) and MHC II+ antigen-presenting cells (APCs) (Barua and Yoshimura, 1999a; Barua et al., 2001) have also been investigated in the follicular tissue and ovarian stroma of chickens. MHC II+

* Corresponding Author: U. Eren. Tel: +90-256-2470915, Fax: +90-256-2470720, E-mail: ueren@adu.edu.tr
[1] University of Adnan Menderes, Cine Vocational High School, Cine, Aydın, Turkey.
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cells were observed in all segments of the oviduct, in the subepithelial and middle part of the stroma, and in the mucosal epithelium of the infundibulum and the vagina of laying hens (Yoshimura et al., 1997).

In acquired immunity, antigens are received, processed and presented with major histocompatibility complex (MHC) class II molecules by APCs to helper T lymphocytes (Diker, 1998; Yoshimura, 2004). Human MHC class II molecules are classified in three isotypes: HLA-DR, -DQ and -DP. HLA-DR and -DQ are the best structurally and functionally characterized. The functional role of HLA-DP molecules in the immune system is still under investigation. HLA-DP may serve as a marker for susceptibility to arthritis, multiple sclerosis, heavy metal lung disease and coeliac disease, indicating a potential role for HLA-DP in antigen presentation and HLA-DP-restricted T-cell responses (Begovich et al., 1989; Bugawan et al., 1989; Richeldi et al., 1993; Potolicchio et al., 1997; Yu et al., 1998).

Expression of the classical class II heterodimers on cattle leucocytes was identified using cross-species-reactive monoclonal antibodies (mAb) (Lewin et al., 1985). These mAb were later confirmed to be specific for DR and DQ products by sequential immunoprecipitation and one-dimensional isoelectric focusing (Janzer-Pfeil and Splitter, 1989; Bissumbhar et al., 1994). Ababou and coworkers (Ababou et al., 1994; Ababou et al., 1995) reported evidence for the expression of a third class II molecule that is cross-reactive with HLA-DP.

Because MHC genes play a major role in the development of acquired immune responses, it is essential to obtain comparative information on their organization, expression and possible functional dichotomies in different species.

There have been very few studies of the location and distribution of APCs (HLA-DR alpha) (Cobb and Watson, 1995), and no studies have been undertaken on the location and distribution of HLA-DQ- or HLA-DP-like MHC II+ cells in the bovine uterus. The objectives of this study were to investigate the presence of MHC II+ (HLA-DP-like) cells in the cow uterus, and to determine whether there is any significant difference in their distribution between the estrus and diestrus phases of the estrous cycle.

MATERIAL AND METHODS

Tissue collection

A total of 63 uteri from 3 to 6-year-old multiparous Holstein cows without genital problems were used. They wait and rest 12 h in summer and 8 h in winter before slaughtering. The stage of the estrous cycle was determined from the structure of the ovaries and blood progesterone levels. Jugular blood samples were collected before slaughter. Immediately after slaughter, the genital organs were collected and examined. Animals that were pregnant or had organs with a pathological appearance were excluded from the study. Tissue samples were taken from the uteri of animals that were judged to be in estrus or diestrus by inspection of the ovarium (Arthur et al., 1996; Palta et al., 1998). For immmunocytochemical examination, tissue samples with an approximate volume of 5-7 mm^3 were taken from the middle part of the cervix, the corpus and the right cornu. The specimens were placed in Optimal Cutting Temperature compound (Tissue-Tek; Sakura Ltd, USA) and immediately frozen by plunging into liquid nitrogen. The frozen samples were stored in a freezer at -80°C until used for immunocytochemistry. Samples from the same areas were also fixed in 10% neutral buffered formalin for subsequent analysis. Blood and tissue samples from 63 animals were brought to the laboratory in this manner.

Progesterone determination in blood samples

Sera were obtained from blood samples and stored at -20°C until analysis. Progesterone levels were determined by radioimmunoassay (RIA Progesterone, IM 118; Immunotech Corp.) (Becker et al., 1975). The levels obtained confirmed the findings of macroscopic observation of the ovaries; that is, 16 uteri were in estrus (0.00-0.38 ng/ml progesterone) and 16 uteri were in diestrus (2.11-11.51 ng/ml progesteron).

Microscopy of healthy tissues

Tissue samples fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned (5 μm) and stained with haematoxylin and eosin. Examination of the sections showed that two samples in estrus and one in diestrus phase had inflammation. These samples were excluded from the study.

Preparation of tissue sections and immunocytochemistry

A total of 29 healthy uteri, 14 in estrus and 15 in diestrus, were used. Cryostat sections were cut at intervals of 50 μm and a thickness of 6 μm. Tissue sections taken serially were placed on organosilane (3-aminopropyltriethoxy-silane, A3648; Sigma) coated slides. The sections were kept at -20°C until use. Streptavidin-biotin peroxidase complex (StreptABC) staining was applied to three sections from each region of the uterus (cervix, corpus and cornu uteri) (Hsu et al., 1981; www.vmrd.com/docs/ab/ihc.htm, 2001). The sections were fixed in acetone for 10 min at room temperature and incubated in a methanol mixture including 0.6% H_2O_2 for 5 min after drying in air. After washing with distilled water, they were then soaked in PBS for 5 min. Non-specific protein binding was reduced by incubation with 10% normal goat serum for 30 min. The

Table 1. Mean blood progesterone levels of the cows at estrus and diestrus phases

Phase of cycles	Progesteron levels (ng/ml) (mean±SEM)
Estrus (n = 14)	0.13±0.03
Diestrus (n = 15)	5.19±0.7

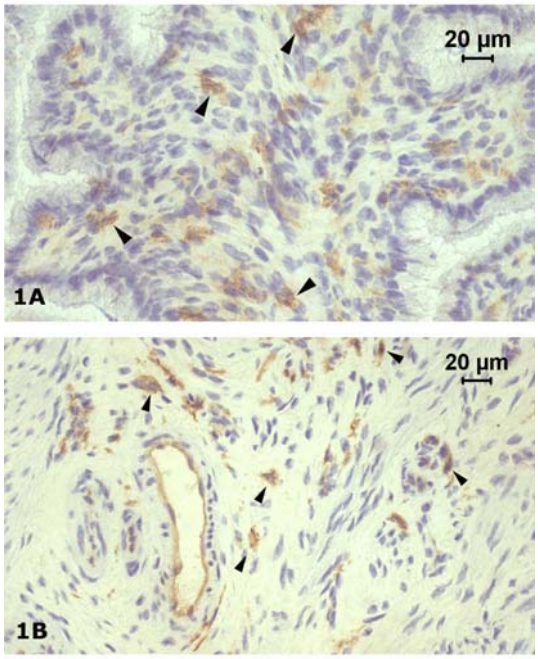

Figure 1. (A): MHC II+ cells in the cervical mucosa at estrus (arrowheads). B: MHC II+ cells in the cervical mucosa at diestrus (arrowheads). Streptavidin-biotin immunoperoxidase technique.

sections were then overlaid with 40 µl of primary antibody (anti-bovine monoclonal MHC II antibody, H 42 A; Veterinary Medical Research and Development, VMRD) (Martinez et al., 2005), and incubated in a humidified chamber for 4 h at room temperature. The primary antibody was diluted with PBS in a ratio of 1:250. The sections were placed in TBS for 5 min after washing with TBS. The secondary biotinylated antibody (goat anti-mouse Ig-0492; Dako) was applied for 30 min. The sections were held in TBS for 5 min after shaking in TBS. They were then incubated with avidin peroxidase complex (Strept ABC/HRP working solution/0492; Dako) for 30 min. Finally, they were placed in TBS for 5 min, and the immune reaction products were visualized by incubation with substrate-chromogen solution (3,3′-diaminobenzidine, D-5905; Sıgma). The sections were counter-stained with Harris hematoxylin and mounted in mounting medium (S3025-; Dako) after washing in distilled water. Some sections were not counter-stained, for photography. Positive control sections from bovine spleen were also stained. For negative controls, the primary antibody was not applied to the sections.

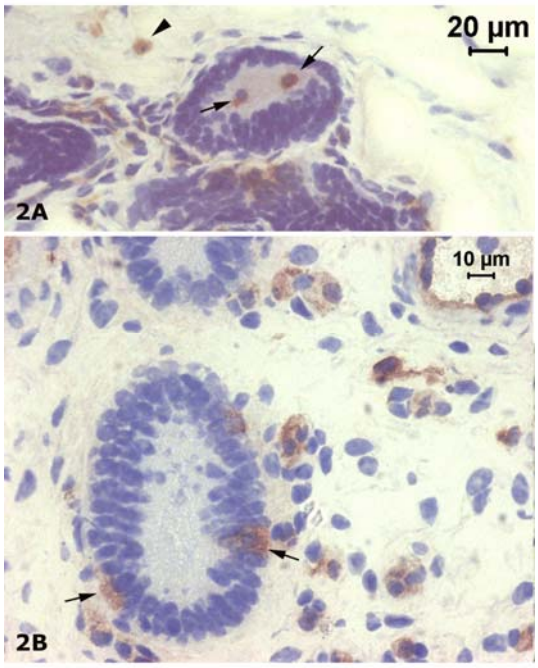

Figure 2. A MHC II+ cells among the glands (arrowheads) and in the gland lumen (arrows) in the corpus uteri at estrus. (B) MHC II+ cells between gland epithelia in the cornu uteri at diestrus (arrows). Streptavidin-biotin immunoperoxidase technique.

Examination of tissue sections and cell counting

Prepared sections were examined under a light microscope (Leica DMLB) and by computer with images transferred through a video camera (Leica DC200) compatible with the microscope. Cell counting was performed using computer-assisted image analysis software (Leica Q Win Standart Version 2.8) (Barua and Yoshimura, 1999a; German et al., 1999). To count the MHC II+ cells, two regions were chosen in each tissue section. The endometrium was the first region examined (surface epithelia to lamina propria), while the region outside of the endometrium was the second (connective tissue areas of myometrium and perimetrium). For each animal, three sections from the cervix, corpus and cornu uteri were scored. Counting was done in five areas of 0.518 mm^2 in both chosen regions. The mean count and the count per mm^2 were calculated.

Statistical analysis

Data were analyzed by using t-tests for dependent and independent groups. The SPSS 10.0 for Windows® statistics package was used.

RESULTS

The mean blood progesterone levels of the cows are shown in Table 1.

There was no evidence of any labelling in the negative

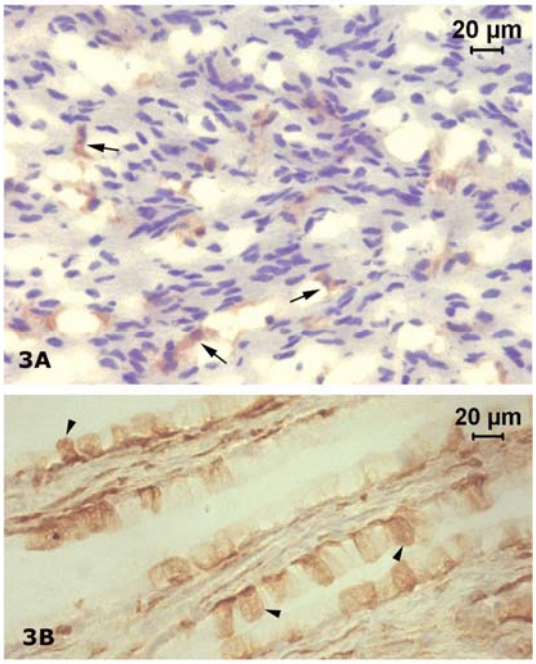

Figure 3. (A) MHC II+ cells in the connective tissue of the myometrium in the cornu uteri at diestrus (arrows). Streptavidin-biotin immunoperoxidase technique. (B) MHC II+ secretory cells in the surface epithelia of the cervix uteri at diestrus (arrowheads). Streptavidin-biotin immunoperoxidase technique (no counter-staining).

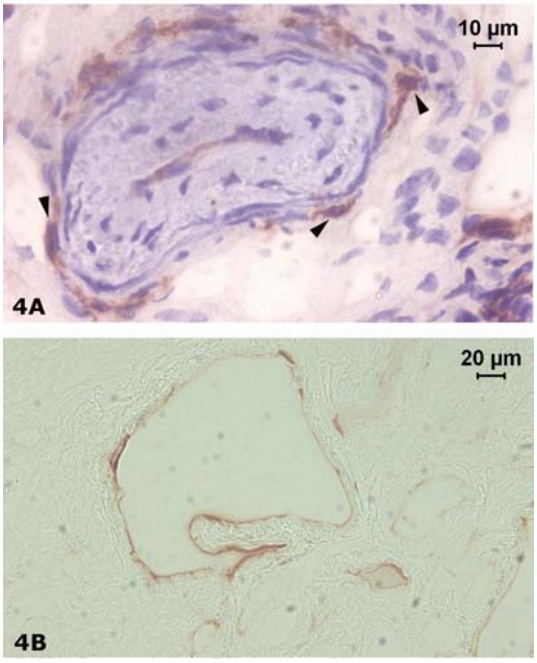

Figure 4. (A) MHC II+ cells in the surrounding vessels of the cornu uteri (arrowheads). Streptavidin-biotin immunoperoxidase technique. (B) MHC II+ reaction in the endothelial cells of the vessels. Streptavidin-biotin immunoperoxidase technique (no counter-staining).

control sections, and sections of the spleen used as positive controls showed labelling appropriate for the antibody.

When the sections were examined microscopically, an extremely large number of MHC II+ cells of different shapes were seen in the sections of cervix (Figure 1A, B), corpus (Figure 2A) and cornu (Figure 2B, 3A) uteri in the first region (surface epithelia+lamina propria/endometrium). Although no MHC II+ reaction was observed in the surface epithelia of the cervix uteri in estrus, MHC II+ reactive secretory cells (Figure 3B, arrowheads) were seen in diestrus. MHC II+ cells were also found in the surface epithelia of the corpus uteri in estrus and diestrus. Furthermore, free MHC II+ cells were seen in the lumens of the glands (Figure 2A, arrows) of the corpus uteri. In the surface epithelia of the cornu uteri, an MHC II+ reaction was seen in estrus but not in diestrus. MHC II+ cells were found in the lumens of the glands and between the gland epithelia in estrus and diestrus (Figure 2B, arrows).

Subepithelial connective tissue among the glands showed intense MHC II+ cells, and there were MHC II+ cells in the connective tissue of the second region (myometrium+perimetrium) (Figure 3A) and around the vessels (Figure 4A, arrowheads). The MHC II+ cells morphologically resembled macrophages, dendritic cells, fibroblasts and mast cells, especially in the connective tissue surrounding blood vessels. Endothelial cells also gave a positive reaction in the cervix, corpus and cornu uteri (Figure 4B).

Mean cell counts are shown in Table 2. Although the largest numbers of MHC II+ cells were found in the first region of the cornu uteri in estrus and the first region of the corpus uteri in diestrus, there were no statistically significant differences in cell numbers per mm^2 between estrus and diestrus in the cervix, corpus and cornu uteri (Table 2). The difference in cell number between the first and second regions of the cervix, corpus and cornu uteri was found to be statistically significant ($p<0.001$) (Table 2).

DISCUSSION

MHC II+ cells in different regions of genital tract

MHC II+ cells have been determined in different regions of the female genital tract in various animal species. Kaushic et al. (1998) suggested that, in rats, MHC II+ cells, macrophages, granulocytes and dendritic cells are numerous in the uterus, in the surrounding glandular epithelia and in the endometrial stroma. In another study on rats (Head and Gaede, 1986), Ia+ cells were observed in myometrial and endometrial connective tissue, especially near the glandular and luminal epithelia. Intense MHC II+ cells were seen in the lamina propria of the cervix and vaginal mucosa, especially in the subepithelial connective tissue, in women (Tabibzadeh et al., 1987; Johansson et al., 1999).

Table 2. Number of MHC II+ cells per mm^2 area in cervix, corpus and cornu uteri at estrus and diestrus

Phase of cycles	MHC II+ cell/mm (mean±SEM)								
	Cervix uteri			Corpus uteri			Cornu uteri		
	First region	Second region	P	First region	Second region	P	First region	Second region	P
Estrus (n:14)	152.7±14.5	58.7±9.8	***	157.1±12.2	40.0±8.0	***	191.4±17.8	49.5±7.9	***
Diestrus (n:15)	154.9±18.1	58.7±11.9	***	199.0±19.1	49.0±9.5	***	153.5±18.7	48.2±6.6	***
P	NS	NS		NS	NS		NS	NS	

NS: Non significant, *** $p<0.001$.

Macrophages (Barua et al., 1998a, b) and MHC II+ APCs (Barua and Yoshimura, 1999a; Barua et al., 2001) were observed in the ovarian stroma and follicular tissue of hens. In another study, MHC II+ cells were seen in all segments of the oviduct, the middle parts of the stroma and the subepithelial region, and the mucosal epithelia of the infundibulum and vagina of hens (Yoshimura et al., 1997). Some researchers have propounded that thecal fibroblasts express MHC II molecules in hens (Barua and Yoshimura, 1999a; Barua et al., 2001). Tunon et al. (1999) found that the numbers of MHC II+, CD4+ and CD8+ cells were higher in the uterine body than in the cornu uteri of mares during estrous, but the difference in MHC II cells was not significant.

In this study, tissue samples from the cervix, corpus and cornu uteri were examined, and MHC II+ cells were found in surface and glandular epithelia, in connective tissue surrounding the glands and in connective tissue areas outside the endometrium, surrounding the blood vessels. Many more MHC II+ cells were found in the endometrium of the cervix, corpus and cornu uteri than in the other parts ($p<0.001$). The results of this study are similar to those gathered from cervical tissue of women (Johansson et al., 1999), but are not in agreement with the findings from rats (Head and Gaede, 1986). Greater numbers of MHC II+ cells were seen in the cornu in estrus than in the corpus. This finding differs from that described by Tunon et al. (1999) in mares. As in the mares, there was no statistically significant difference between the two regions.

Different MHC II+ cell types observed in genital tract

Studies suggest that different cell types in the female genital tract express the MHC II molecule. An MHC II+ reaction was determined in surface epithelia (Donaldson et al., 1990; Watson and Dixon, 1993; Wallace et al., 2001), in glandular epithelia (Donaldson et al., 1990), and with lymphocytes (Tabibzadeh et al., 1987; Low et al., 1990; Watson and Dixon, 1993), monocytes (Tabibzadeh et al., 1987; Watson and Dixon, 1993), macrophages (Tabibzadeh et al., 1987; Watson and Dixon, 1993), dendritic cells (Watson and Dixon, 1993), fibroblasts (Zheng et al., 1998; Barua and Yoshimura, 1999a; Barua et al., 2001; Zheng et al., 2001) and endothelia (Tabibzadeh et al., 1987; Watson and Dixon, 1993; Kaeoket et al., 2001).

In the present study, MHC II+ cells were found in the surface epithelium of the cervix, corpus and cornu uteri. Wallace et al. (2001) suggested that MHC II-expressing endometrial epithelia can process antigens and present them to T lymphocytes or can transmit them to APCs in the subepithelial area in humans, and this may also occur in the cow uterus. While free MHC II+ cells were observed in the lumens of the glands, they were also found between gland epithelia in the cornu uteri. These APCs could have passed from connective tissue to the gland lumens. MHC II+ cells of different shapes were observed in the endometrium and under the endometrial layer. The morphology of some of these cells was similar to that of macrophages or dendritic cells. These findings support those described by others (Tabibzadeh et al., 1987; Watson and Dixon, 1993). The observation that the cells that resembled fibroblasts gave a positive reaction also supports previous studies (Barua and Yoshimura, 1999a; Barua et al., 2001; Zheng et al., 1998, 2001).

Henz et al. (2001) reported that mast cells could phagocytize diverse particles, take up antigens, and express a number of receptors, particularly MHC class I and II antigens, ICAM-1 and -3, CD43, CD80, CD86 and CD40L, and allow them to interact with T and B lymphocytes. In previous research, Eren et al. (1999) found many more mast cells in the subepithelial layer, surrounding the glands, and surrounding the vessels in cow endometrium. They also observed that cells resembling mast cells gave an MHC II-positive reaction. This finding is similar to that of Henz et al. (2001). However, these cells could not be definitely identified immunocytochemically in this study. An MHC II+ reaction was observed with the most of the endothelial cells of the cervix, corpus and cornu uteri. Similar observations have been reported from humans (Johansson et al., 1999), sows (Kaeoket et al., 2001) and mares (Watson and Dixon, 1993).

MHC II+ cell distribution at estrus and diestrus

Estrus increases MHC II+ cell numbers in epithelia and subepithelial connective tissue (Tabibzadeh et al., 1987; Watson and Dixon, 1993; Frayne and Stokes, 1994; Kaushic et al., 1998; Kaoeket et al., 2001). Injection of estrogen into

immature hens increased MHC II+ cells, T lymphocyte subtypes, and Ig-synthesizing cells in the ovarium and oviduct (Yoshimura, 2004). De and Wood (1990) reported that quantitative differences in macrophages were not important in the uteri of mice at proestrus, estrus and diestrus, but the distribution of these cells could change in the tissue during the estrous cycle, with the greatest numbers in the subepithelial stroma in proestrus and estrus. Also in cows, MHC II+ cells were abundant in the epithelia and subepithelial connective tissue of the uterus, and their numbers increased in the follicular phase (Cobb and Watson, 1995). In contrast, though the cervix, corpus and cornu uteri were examined in this study, no positive reaction was observed in the epithelium of the cervix in estrus or the cornu in diestrus. The absence of a positive reaction by the epithelium of the cervix in estrus could enable free passage of spermatozoa. Although we found more MHC II+ cells in the first region of the cornu uteri in estrus and in the first region of the corpus uteri in diestrus, no statistically significant difference in the number of positive cells was found between estrus and diestrus in the endometrium or outside the endometrium (Table 2). These results are not in agreement with a report that MHC II+ cell numbers increase in the follicular phase (Cobb and Watson, 1995). The difference could be a result of the use of a different mAbs (HLA- DR alpha).

In summary, we identified HLA-DP-like MHC class II-bearing cells in the cow uterus in both estrus and diestrus. Qualitative and quantitative information on the distribution of these cells was obtained using mAb (H42A/VMRD). It was concluded that different cell types have APC functions in the uterus, especially in the endometrium, and there was no difference in cell counts between estrus and diestrus. Future work could involve the investigation of HLA-DP-like MHC II+ cells in the cow oviduct and vagina, and identifying MHC II+ cells throughout the genital tract.

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