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Matthew J. Cannon

*Department of Animal Sciences, The Ohio State University/Ohio Agricultural Research and Development Center, Wooster, OH*

John S. Davis

*Olson Center for Women's Health, Department of Obstetrics and Gynecology, University of Nebraska Medical Center, Omaha, NE*

Joy L. Pate

*Veterans Affairs Medical Center, Omaha, NE*

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# Presence and regulation of messenger ribonucleic acids encoding components of the class II major histocompatibility complex-associated antigen processing pathway in the bovine corpus luteum

Matthew J Cannon, John S Davis<sup>1</sup> and Joy L Pate

Department of Animal Sciences, The Ohio State University/Ohio Agricultural Research and Development Center, 1680 Madison Avenue, Wooster, Ohio 44691, USA and <sup>1</sup>Olson Center for Women's Health, Department of Obstetrics and Gynecology, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA and Veterans Affairs Medical Center, Omaha, Nebraska, 68105, USA

Correspondence should be addressed to J L Pate; Email: pate.1@osu.edu

## Abstract

Luteal cells express class II major histocompatibility complex (MHC) molecules and can stimulate T lymphocyte proliferation *in vitro*. However, it is unknown whether luteal cells express the intracellular components necessary to process the peptides presented by class II MHC molecules. The objective of the present study was to examine the expression and regulation of three major class II-associated antigen processing components – class II MHC-associated invariant chain (Ii), DM $\alpha$  and DM $\beta$  – in luteal tissue. Corpora lutea were collected early in the estrous cycle, during midcycle and late in the estrous cycle, and at various times following administration of a luteolytic dose of prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ) to the cow. Northern analysis revealed the presence of mRNA encoding each of the class II MHC-associated antigen processing proteins in luteal tissue. Ii mRNA concentrations did not change during the estrous cycle, whereas DM $\alpha$  and DM $\beta$  mRNA concentrations were highest in midcycle luteal tissue compared with either early or late luteal tissue. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) reduced DM $\alpha$  mRNA concentrations in cultured luteal cells in the presence of LH or PGF<sub>2 $\alpha$</sub> . DM $\alpha$  and DM $\beta$  mRNA were also present in highly enriched cultures of luteal endothelial (CLENDO) cells, and DM $\alpha$  mRNA concentrations were greater in CLENDO cultures compared with mixed luteal cell cultures. Expression of invariant chain, DM $\alpha$  and DM $\beta$  genes indicates that cells within the corpus luteum express the minimal requirements to act as functional antigen-presenting cells, and the observation that CLENDO cells are a source of DM $\alpha$  and DM $\beta$  mRNA indicates that non-immune cells within the corpus luteum may function as antigen-presenting cells.

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## Introduction

Luteal regression is a complex cascade of events, initiated by prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ), that causes cessation of luteal function and subsequent structural demise of the corpus luteum (CL). Several lines of evidence suggest participation of the immune system in this process. Macrophages and T lymphocytes are present in the CL of several species, and infiltrate the CL near the time of luteal regression and in response to PGF<sub>2 $\alpha$</sub>  (Bagavandoss *et al.* 1990, Brännström *et al.* 1994, Lawler *et al.* 1999, Penny *et al.* 1999, Krusche *et al.* 2002, Townson *et al.* 2002, Komatsu *et al.* 2003, Neuvians *et al.* 2004). Cytokines produced by these cells – including tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$ , interleukin (IL)-1 $\beta$  and

IL-6 – decrease progesterone production, increase prostaglandin synthesis and stimulate apoptotic death in cultured luteal cells (Nothnick & Pate 1990, Fairchild & Pate 1991, Benyo & Pate 1992, Gorospe *et al.* 1992, Townson & Pate 1994, 1996, Jo *et al.* 1995, Del Vecchio & Sutherland 1997, Young *et al.* 1997, Petroff *et al.* 2001). The presence of these cytokines has been detected in luteal tissue (Bagavandoss *et al.* 1990, Ji *et al.* 1991, Penny *et al.* 1999, Petroff *et al.* 1999, Krusche *et al.* 2002, Townson *et al.* 2002, Komatsu *et al.* 2003, Neuvians *et al.* 2004), indicating that macrophages and T cells present within the CL are activated. Class II major histocompatibility complex (MHC) molecules are also expressed by luteal cells of several species (Khoury & Marshall 1990, Benyo *et al.* 1991, Kenny *et al.* 1991, Penny *et al.* 1999),

providing a means of activation of T lymphocytes present in luteal tissue.

Class II MHC molecules, which are typically expressed on antigen-presenting cells of the immune system (i.e. macrophages, dendritic cells and B lymphocytes), present small peptides processed intracellularly to T lymphocytes (Sant 1994). When cells expressing class II MHC molecules (antigen-presenting cells) interact with T cells, the peptides bound to the MHC molecules determine whether the antigen-presenting cell will activate T cells. The immune system is tolerant of normally expressed self-peptides, but T lymphocytes can be activated by cells expressing peptides derived from a foreign source (i.e. viral or bacterial peptides) as well as self-peptides that are presented by MHC molecules under abnormal conditions (i.e. tumor antigens). As class II MHC molecules are synthesized, they immediately bind to a chaperone protein called invariant chain (Ii), a portion of which occupies the peptide-binding groove of the MHC molecule (Roche & Cresswell 1990, Avva & Cresswell 1994). Invariant chain serves a dual function, directing intracellular transport of class II MHC molecules (Bakke & Dobberstein 1990, Lotteau *et al.* 1990) and ensuring that binding of 'inappropriate' peptides does not occur prematurely (Roche & Cresswell 1990). Class II MHC-Ii complexes are transported into specialized intracellular compartments in which proteases degrade the Ii into a peptide called class II-associated invariant chain peptide (CLIP) (Maric *et al.* 1994, Riese *et al.* 1996). Prior to binding of antigenic peptides by class II MHC molecules, the CLIP fragment bound to the peptide binding groove must be removed. Removal of CLIP is carried out by a class II MHC-like heterodimeric protein referred to as DM (Fling *et al.* 1994, Morris *et al.* 1994). Following CLIP removal, DM stabilizes class II MHC molecules to allow efficient binding of antigenic peptides (Denzin *et al.* 1996).

In addition to the primary roles for Ii and DM described above, these intracellular proteins have been implicated in a secondary role as 'editors' of the repertoire of peptides presented to T cells in the context of class II MHC, since they control in part the type of peptides that bind to class II MHC molecules and that are presented to T lymphocytes (Castellino *et al.* 1997, Vogt *et al.* 1999). Antigen-presenting cells taken from Ii or DM knockout animals stimulate activation and proliferation of otherwise non-reactive T cell clones and reveal the presence of 'self-reactive' T cells in normal animals (Bodmer *et al.* 1994, Katz *et al.* 1996, Marieke van Ham *et al.* 1996, Lightstone *et al.* 1997), indicating that alterations in Ii or DM expression may alter the ability of antigen-presenting cells to stimulate T cell activation.

Expression of class II MHC molecules is not limited to cells of the immune system, and in many cases expression of class II MHC molecules by other cell types is associated with inflammation and/or autoimmune disorders. Cells of the bovine CL express class II MHC molecules, and class II MHC expression increases near the end of the estrous

cycle and in response to a luteolytic dose of PGF<sub>2α</sub> (Benyo *et al.* 1991). Bovine luteal cells also stimulate class II MHC-dependent *in vitro* proliferation of T cells isolated from the same animal (autologous T cells) (Petroff *et al.* 1997). Following administration of a luteolytic dose of PGF<sub>2α</sub> to the cow, the ability of luteal cells to stimulate T lymphocyte proliferation is enhanced (Petroff *et al.* 1997). The enhancement of luteal cell-stimulated T lymphocyte proliferation may be due at least in part to the effect of PGF<sub>2α</sub> on progesterone synthesis *in vitro* (Cannon *et al.* 2003), which decreases following *in vivo* exposure to PGF<sub>2α</sub> (Pate & Nephew 1988). However, we speculate that changes in expression of class II MHC genes and associated antigen-processing genes may also be involved in the enhancement of luteal cell-stimulated T cell proliferation, and that processing of antigens by luteal cells may be altered near the time of luteal regression. Alteration of antigen processing within luteal cells could result in binding of a different repertoire of peptides to class II MHC molecules, allowing luteal cells to stimulate T cell activation during luteal regression. The hypothesis tested in the present study is that expression of either Ii or DM changes near the end of the estrous cycle. Due to observations in the initial portion of the present study, we wished to determine whether steady-state concentrations of DMα and DMβ mRNA are regulated by luteinizing hormone (LH) and PGF<sub>2α</sub>, the classic hormonal regulators of luteal function, or by TNF-α, which is suspected to play a significant role in regulation of luteal function. We have also examined highly enriched cultures of luteal endothelial (CLENDO) cells for the presence of DMα and DMβ mRNA, to determine whether endothelial cells of the CL express the components of the class II MHC antigen-processing pathway.

## Materials and Methods

### Reagents

Powdered Ham's F-12 culture medium, gentamicin, fetal bovine serum, *Escherichia coli* DH5α chemically competent cells, and TRIzol Reagent were all purchased from Life Technologies (Grand Island, NY, USA). Recombinant murine TNF-α was purchased from Life Technologies and R&D Systems (Minneapolis, MN, USA). The National Hormone and Pituitary Program (Baltimore, MD, USA) provided bovine LH (NIAMMD-bLH-4). Insulin-transferrin-selenium (ITS) premix was obtained from BD Biosciences (Bedford, MA, USA). Bovine serum albumin (fraction V), PGF<sub>2α</sub>, HEPES buffer and Bouin's fixative were purchased from Sigma. Prefer fixative was purchased from Anatech Ltd (Battle Creek, MI, USA). Type I collagenase was acquired from Worthington Biochemical Corp. (Freehold, NJ, USA). SDS and MOPS buffer were acquired from Amresco (Solon, OH, USA). Digoxigenin-labeled rNTP mix, alkaline-phosphatase-conjugated anti-digoxigenin Fab fragments, CDP-Star chemiluminescent substrate, blocking

reagent, LightCycler-FastStart DNA SYBR Green I kits and ampicillin were all purchased from Roche. Random hexamer primers were purchased from Amersham. SuperScript II reverse transcriptase was acquired from Invitrogen. DyNAmo HS SYBR Green qPCR kits were purchased from MJ Research (Boston, MA, USA). Restriction enzymes, RNA polymerases, dNTPs, recombinant RNase inhibitor, Moloney murine leukemia virus (M-MLV) reverse transcriptase, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) were purchased from Promega. Hybond N+ membranes were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Tissue culture flasks were from Corning (Acton, MA, USA). All other chemicals were purchased from Sigma or Fisher Scientific (Fairlawn, NJ, USA).

### Animals and tissue collection

Multiparous, lactating dairy cows between 3 and 6 years of age were used in the present studies. Animals were housed indoors and had complete freedom of movement; they were given regular access to a total mixed ration and were allowed to feed *ad libitum*. Corpora lutea were removed transvaginally from regularly cycling dairy cows. Blood samples were collected from all animals prior to CL removal to determine circulating concentrations of progesterone. Corpora lutea were cut into four equal pieces, three of which were immediately snap frozen in liquid nitrogen, transported to the laboratory and stored at  $-80^{\circ}\text{C}$  until RNA extraction was performed. In the first experiment, CL were removed early in the estrous cycle (day 5,  $n = 6$ ), during midcycle (days 9–12,  $n = 6$ ) or late in the estrous cycle (day 18,  $n = 6$ ), or at 0.5 ( $n = 5$ ), 1 ( $n = 5$ ), 4 ( $n = 5$ ), 12 ( $n = 4$ ) or 24 h ( $n = 5$ ) following i.m. administration of 25 mg  $\text{PGF}_{2\alpha}$  (Lutalyse; Upjohn Co., Kalamazoo, MI, USA). In the second experiment, CL were removed during midcycle, immediately placed in ice-cold Ham's F-12 culture medium on ice and transported to the laboratory for dissociation. Handling of animals and surgical procedures were carried out in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

### RNA isolation and Northern analysis

Frozen tissue collected for the first experiment was homogenized in TRIzol reagent using a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY, USA). In the second experiment, RNA from cultured cells was isolated by adding TRIzol reagent directly to culture flasks. Following tissue homogenization or cell lysis, total cellular RNA was isolated according to procedures outlined by the manufacturer. The final RNA precipitate was resuspended in double-distilled water containing 0.1% diethyl pyrocarbonate (DEPC) and RNA concentration was determined spectrophotometrically.

Fifteen micrograms of total cellular RNA were electrophoretically separated on 1.5% agarose denaturing gels containing 20 mM MOPS buffer, 5 mM sodium acetate, 1 mM EDTA and 0.66 M formaldehyde. Following electrophoresis, gels were stained for 15 min with 1  $\mu\text{g}/\text{ml}$  ethidium bromide in DEPC-treated water, destained for 1 h in DEPC-treated water and photographed. RNA was transferred to Hybond-N+ membranes, and membranes were baked at  $80^{\circ}\text{C}$  for 2 h to crosslink RNA to the membranes. Gels were restained with ethidium bromide following transfer and examined under u.v. light to confirm complete transfer of RNA.

Digoxigenin-labeled riboprobes were synthesized using plasmids containing full-length Bovine Lymphocyte Antigen (BoLA)-DM $\alpha$ , BoLA-DM $\beta$  or bovine class II MHC-associated Ii cDNA sequences. Plasmids containing these sequences have been described previously (Niimi *et al.* 1995, 1996) and were provided by Dr Yoko Aida (The Institute of Physical and Chemical Research, Tsukuba, Japan). Digoxigenin-labeled riboprobes were generated using linearized plasmids according to the instructions in Roche Molecular Biochemicals' *DIG Nonradioactive Nucleic Acid Labeling and Detection System* manual. Membranes were prehybridized at  $68^{\circ}\text{C}$  for 1 h with hybridization core buffer (250 mM sodium phosphate, 1 mM EDTA, 5% (w/v) SDS containing 0.5% (w/v) Roche DIG Nucleic Acid Detection System Blocking Reagent), and hybridization was carried out for 16 h at  $68^{\circ}\text{C}$ . Following hybridization, membranes were washed and detection was performed using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments in concert with the chemiluminescent CDP-Star substrate, according to the specifications in Roche Molecular Biochemicals' *DIG Nonradioactive Nucleic Acid Labeling and Detection System* manual. Membranes were exposed to Biomax ML film (Eastman Kodak Co., Rochester, NY, USA) to detect chemiluminescence.

Quantification of steady-state concentrations of each message was performed densitometrically using a PDI 420oe scanning densitometer. The densitometric value (in arbitrary densitometric units) of the band of interest in each sample was standardized to the densitometric values of the corresponding 18S rRNA.

### Luteal cell dissociation and culture

Dissociation of luteal cells was carried out according to procedures described previously (Pate & Condon 1982). Cell culture was performed in a humidified atmosphere of 5%  $\text{CO}_2$  in air at  $37^{\circ}\text{C}$ . Dispersed luteal cells ( $4 \times 10^6$  cells/flask) were cultured in Ham's F-12 medium supplemented with insulin (5  $\mu\text{g}/\text{ml}$ ), transferrin (5  $\mu\text{g}/\text{ml}$ ), selenium (5 ng/ml), gentamicin (20  $\mu\text{g}/\text{ml}$ ) and LH (1 ng/ml) in serum-coated 25  $\text{cm}^2$  flasks, in a total of 4 ml of culture medium. Cells were allowed to adhere overnight, medium was replaced and cultures were treated with LH (10 ng/ml), TNF- $\alpha$  (50 ng/ml) or  $\text{PGF}_{2\alpha}$  (10 ng/ml) in a



3 × 3 factorial arrangement. Treatment concentrations used in this study have been shown previously to affect function, viability and gene expression in cultured bovine luteal cells (Pate & Condon 1984, Townson & Pate 1994, Petroff *et al.* 2001, Cannon & Pate 2003). Medium and treatments were replaced after 24 h. Total RNA was extracted from cultured cells after 48 h of culture. The experiment was replicated a total of four times using CL from different animals.

### **Isolation and culture of purified luteal endothelial cells**

Purified endothelial cells from bovine CL (CLENDO cells) collected during early pregnancy were purchased from Cambrex BioScience (BioWittaker, Walkersville, MD, USA) as described previously (Cavicchio *et al.* 2002, Pru *et al.* 2003, Liptak *et al.* 2005). In the present study, endothelial cells from frozen aliquots (passages 3–5; 5000 cells/cm<sup>2</sup>) were cultured in EGM-2MV media, as recommended by the supplier with 3% fetal bovine serum (Cambrex Bioscience) in 60 mm dishes. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Culture medium was replaced every 48 h until 80–90% confluent. On the day of harvest, cells were equilibrated for 2–3 h in serum-free EBM-2 medium. CLENDO cells were collected in RLT lysis buffer and RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's specifications.

### **Quantitative reverse transcription-PCR (RT-qPCR)**

Primers used to amplify DM $\alpha$  and DM $\beta$  cDNAs were designed based on the published sequences for DM $\alpha$  and DM $\beta$  (Niimi *et al.* 1995; GenBank accession numbers D76416 and D76417 respectively). Primer sequences were 5'-GCTCTGAGTCCTACGACGA-3' and 5'-GCACAGCACATTCTCCAGAAG-3' for DM $\alpha$  forward and reverse primers respectively, and 5'-CACCTGTGTGTTGGACGATGA-3' and 5'-AGCGTTACCAGAGCCACAGAA-3' for DM $\beta$  forward and reverse primers respectively. Amplification using DM $\alpha$  primers resulted in a single 526 bp DM $\alpha$  cDNA fragment, whereas amplification using DM $\beta$  primers resulted in a single 597 bp DM $\beta$  cDNA fragment. Primer sequences used for amplification of steroidogenic acute regulatory protein (StAR) and CD31 (an endothelial cell adhesion molecule) mRNA in cultures of mixed luteal cells or luteal endothelial cells were based on the published sequences (Hartung *et al.* 1995, GenBank accession number NM\_174189; Stewart *et al.* 1996, GenBank accession number U35433) and were as follows: 5'-CCTCTCTACAGCGACCAA-3' and 5'-TCGTGATGATGACCGTG-3' for StAR forward and reverse primers respectively; 5'-GTTACGCGAAGTTCTGCGAG-3' and 5'-CTTGCTGGCTGTGGTCTTGT-3' for CD31 forward and reverse primers respectively. Amplification using the CD31 primers resulted in a 229 bp CD31 cDNA product and amplification using StAR primers yielded a 311 bp StAR cDNA product. The identity of all PCR products was

confirmed by DNA sequence analysis. An 854 bp glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA fragment was amplified in parallel reactions, using ovine specific primers described previously (Tsai *et al.* 1996), and concentrations of DM $\alpha$ , DM $\beta$ , CD31 and StAR in each sample were standardized to the corresponding G3PDH concentrations.

Steady-state concentrations of mRNA encoding StAR, CD31, DM $\alpha$  and DM $\beta$  were determined in total RNA extracted from cultures of mixed luteal cells and luteal endothelial cells, using an MJ Research Opticon 2 real-time PCR thermal cycler. Prior to PCR, reverse transcription using random hexamer primers was performed on 2  $\mu$ g of total RNA extracted from cultures. PCR was then performed, using 200 ng of reverse transcribed cDNA, using the DyNAmo HS SYBR Green qPCR kit according to the manufacturer's instructions. Thermal cycling was carried out using the following conditions: denaturation, 94 °C for 30 s; annealing, 56 °C for 30 s; extension, 72 °C for 60 s, for a total of 32 cycles. Melting curve analysis was performed after the end of the last cycle and, in conjunction with gel electrophoresis of amplified products, was used to verify the amplification of a single product in each sample. Fluorescence values in each tube were measured at the end of each cycle using single acquisition mode. Fluorescence values of the product of interest in each sample were standardized to the corresponding G3PDH fluorescence values, and these standardized values were then used to calculate the mean steady-state amounts of each message. Since no standard curve was run for the messages of interest, values were expressed in arbitrary units of fluorescence for the purposes of analysis.

Steady-state concentrations of DM $\alpha$  and DM $\beta$  mRNA were determined in total RNA extracted from luteal cells treated with LH, PGF<sub>2 $\alpha$</sub>  and TNF- $\alpha$  using a Roche LightCycler real-time PCR thermal cycler. Prior to quantitative PCR, DM $\alpha$ , DM $\beta$  or G3PDH reverse primer was used to reverse transcribe 200 ng of total cellular RNA extracted from cultured cells. Quantitative PCR was then performed on 40 ng of reverse transcribed RNA, using the Roche LightCycler-FastStart DNA Master SYBR Green I kit according to the manufacturer's instructions. As a standard curve, DM $\alpha$ , DM $\beta$  and G3PDH cDNAs were amplified from lymph node total RNA, electrophoretically separated on a 1.2% agarose gel and eluted. The concentration of purified cDNA products was determined spectrophotometrically, and serial dilutions were prepared and used as an external standard curve in the quantitative PCR assays. Thermal cycling was carried out using the following conditions: denaturation, 94 °C for 30 s; annealing, 58 °C for 30 s; extension, 72 °C for 60 s, 40 cycles total. The ramping speed for transition from denaturation to annealing steps and annealing to extension steps was set manually at 1°/s, while the ramping speed from extension to denaturation steps was set at 20°/s to minimize total running time. Melting curve analysis was performed after the end of the last cycle in order to verify the amplification of a single

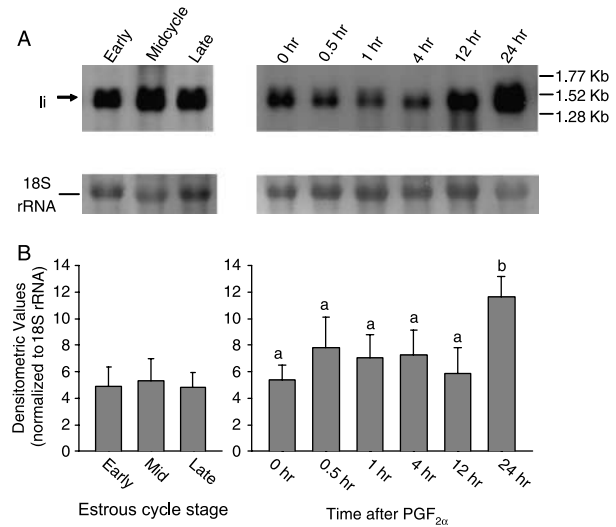
product in each sample. There was no evidence of primer dimers or bands other than the desired amplicons in the reactions as determined by melting curve analysis, and this was confirmed by gel electrophoresis of amplified products. The lack of undesired products allowed fluorescence measurements to be taken at the same temperature (72 °C) at which extension was performed. Fluorescence values in each tube were measured at the end of each cycle using single acquisition mode. Samples were run in duplicate and fluorescence values for duplicate samples were averaged. The baseline fluorescence reading and noise band cut-off were set manually for samples and standards, in order to eliminate background fluorescence values from calculation of the slope and  $y$ -intercept of the standard curve. A standard curve was generated using average fluorescence values of duplicate standards. Average fluorescence values of samples were then used to calculate the concentration of target cDNA in each amplification reaction, using the Fit Points methods of the accompanying LightCycler data analysis software.

### Statistical analysis

Normalized densitometric values collected from Northern analyses in the first experiment were subjected to two-way analysis of variance to determine whether differences in mean densitometric values of DM $\alpha$ , DM $\beta$ , or Ii RNA existed between stages of the estrous cycle. In the instance of a significant  $F$  value, the Student–Newman–Kuels (S–N–K) test was used to determine differences between specific means. Relative differences in steady-state concentrations of CD31, StAR, DM $\alpha$  and DM $\beta$  mRNA between cultures of mixed luteal cells or luteal endothelial cells were determined using Student's  $t$  test. In the third experiment, steady-state concentrations of DM $\alpha$  or DM $\beta$  mRNA in treated luteal cell cultures were subjected to one-way ANOVA to determine if differences existed between treatment means. In the incidence of a significant  $F$  value, the S–N–K test was used to determine differences between specific treatment means. Differences were considered to be significant at  $P < 0.05$ . A  $P$  value between 0.05 and 0.08 was considered to show a tendency towards a significant difference. All statistical procedures were performed using the SigmaStat statistical analysis software package (Jandel Corporation, San Rafael, CA, USA).

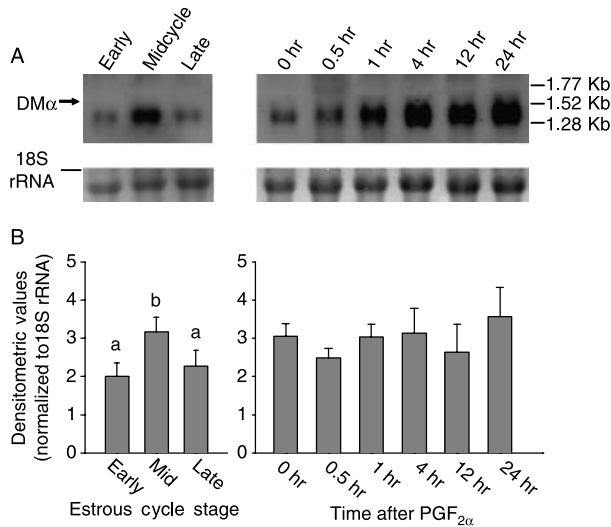
### Results

The results of the Northern analyses to detect and quantify Ii, DM $\alpha$  and DM $\beta$  mRNA in luteal tissue are displayed in Figs 1 to 3. Figure 1A demonstrates the presence of an Ii transcript of approximately 1.3 Kb in all luteal tissue samples. Muscle tissue, which served as a source of negative control RNA, was devoid of any corresponding band (not shown). There was a large amount of variability



**Figure 1** Northern analysis of Ii mRNA in bovine luteal tissue. (A) Northern blot showing Ii mRNA in a single CL collected from each of the following times of the estrous cycle: early (day 5,  $n = 6$ ); during midcycle (days 10–12,  $n = 6$ ); or late (day 18,  $n = 6$ ); and at 0 ( $n = 5$ ), 0.5 ( $n = 5$ ), 1 ( $n = 5$ ), 4 ( $n = 5$ ), 12 ( $n = 4$ ) or 24 h ( $n = 5$ ) following administration of 25 mg PGF $_{2\alpha}$  to the cow. 18S rRNA corresponding to each sample is also shown. (B) Steady-state concentration of Ii mRNA in luteal tissues collected at each time point (mean  $\pm$  S.E.M.,  $n$  values are as stated above). Bars represent densitometric values of Ii mRNA standardized to 18S rRNA. Values with different letters are significantly different ( $P < 0.05$ ).

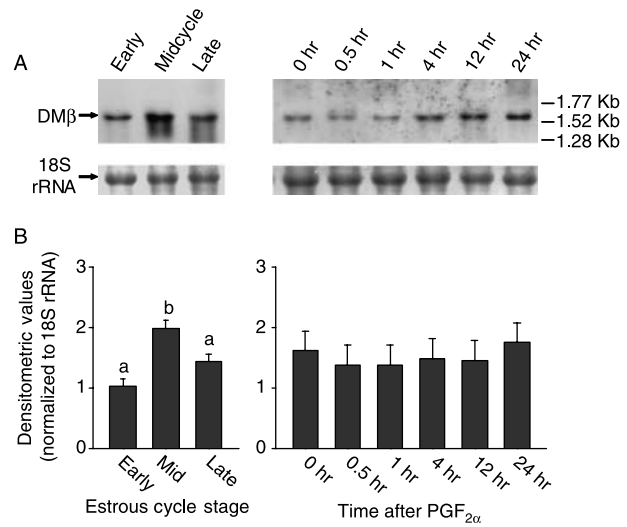
among animals in the steady-state concentration of Ii mRNA following *in vivo* administration of PGF $_{2\alpha}$ . This is responsible for the apparent discrepancy between the density of bands in the Northern blot shown in Fig. 1A and the graph shown in Fig. 1B, which represents the mean of data from four or five animals at each time after PGF $_{2\alpha}$  administration. There were no differences in concentrations of Ii mRNA between CL collected at any stage of the estrous cycle; however there was a greater than two-fold increase in steady-state concentrations of Ii mRNA in CL collected 24 h after PGF $_{2\alpha}$  administration ( $P < 0.05$ , Fig. 1B). Figure 2A shows a Northern blot of DM $\alpha$  mRNA demonstrating the presence of a single DM $\alpha$  transcript in luteal tissue. Muscle tissue RNA was devoid of any corresponding band (not shown). Similar to the results of Ii Northern analysis, a large amount of variability among animals was also found in DM $\alpha$  mRNA concentrations following PGF $_{2\alpha}$  administration, which is responsible for the apparent difference between the density of bands in the Northern blot shown in Fig. 2A and the graph shown in Fig. 2B, which represents the mean of data from four or five animals at each time after PGF $_{2\alpha}$  administration. Such variability was not observed among animals in CL collected during the estrous cycle. Concentrations of DM $\alpha$  mRNA tended ( $P > 0.08$ ) to be greater in CL collected during midcycle compared with CL collected early (day 5) or late (day 18) in the estrous cycle (Fig. 2B). There were no differences ( $P > 0.10$ ) in concentrations of



**Figure 2** Northern analysis of DM $\alpha$  mRNA in bovine luteal tissue. (A) Northern blot showing DM $\alpha$  mRNA in a single CL collected from each of the following times of the estrous cycle: early (day 5,  $n = 6$ ); during midcycle (days 10–12,  $n = 6$ ); or late (day 18,  $n = 6$ ); and at 0 ( $n = 5$ ), 0.5 ( $n = 5$ ), 1 ( $n = 5$ ), 4 ( $n = 5$ ), 12 ( $n = 4$ ) or 24 h ( $n = 5$ ) following administration of 25 mg PGF<sub>2 $\alpha$</sub>  to the cow. 18S rRNA corresponding to each sample is also shown. (B) Steady-state concentrations of DM $\alpha$  mRNA in luteal tissues collected at each time point (mean  $\pm$  S.E.M.,  $n$  values are as stated above). Bars represent densitometric values of DM $\alpha$  mRNA standardized to 18S rRNA. Values with different letters tended to be significantly different ( $P < 0.08$ ).

DM $\alpha$  mRNA in CL collected following administration of a luteolytic dose of PGF<sub>2 $\alpha$</sub> , possibly due to the large degree of animal-to-animal variation mentioned earlier. Messenger RNA encoding DM $\beta$  was also detected in luteal tissue by Northern analysis, as demonstrated by the presence of a single DM $\beta$  transcript in all luteal tissue samples (Fig. 3A). Muscle tissue RNA was also devoid of any band corresponding to DM $\beta$  (not shown). Concentrations of DM $\beta$  mRNA were greater ( $P < 0.05$ ) in midcycle CL as compared with CL collected early or late in the estrous cycle (Fig. 3B), similar to DM $\alpha$ . There were no differences in concentrations of DM $\beta$  mRNA following PGF<sub>2 $\alpha$</sub>  administration.

Steady-state concentrations of DM $\alpha$  and DM $\beta$  mRNA in CLENDO cells and mixed luteal cells were compared in order to determine whether endothelial cells derived from the bovine corpus luteum expressed DM $\alpha$  and DM $\beta$ . Purity and composition of the CLENDO cell cultures and mixed luteal cell cultures was assessed using quantitative RT-PCR to determine steady-state concentrations of mRNA encoding CD31 and StAR protein. CD31 mRNA was present both in mixed luteal cell and CLENDO cultures, with much greater steady-state concentrations present in CLENDO cultures. STAR mRNA, while abundant in mixed luteal cell cultures, was undetectable in some CLENDO cultures, and was present in concentrations near the detection limit of the PCR assay in others (data not shown). Messenger RNA encoding DM $\alpha$  and DM $\beta$  was



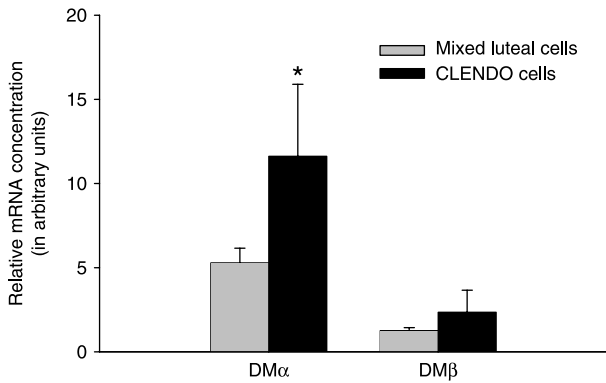
**Figure 3** Northern analysis of DM $\beta$  mRNA in bovine luteal tissue. (A) Northern blot showing DM $\beta$  mRNA in a single CL collected from each of the following times of the estrous cycle: early (day 5,  $n = 6$ ); during midcycle (days 10–12,  $n = 6$ ); or late (day 18,  $n = 6$ ); and at 0 ( $n = 5$ ), 0.5 ( $n = 5$ ), 1 ( $n = 5$ ), 4 ( $n = 5$ ), 12 ( $n = 4$ ) or 24 h ( $n = 5$ ) following administration of 25 mg PGF<sub>2 $\alpha$</sub>  to the cow. 18S rRNA corresponding to each sample is also shown. (B) Steady-state concentrations of DM $\beta$  mRNA in luteal tissues collected at each time point (mean  $\pm$  S.E.M.,  $n$  values are as stated above). Bars represent densitometric values of DM $\beta$  mRNA standardized to 18S rRNA. Values with different letters are significantly different ( $P < 0.05$ ).

detectable in cultures of mixed luteal cells as well as CLENDO cell cultures. Steady-state concentrations of DM $\alpha$ , but not DM $\beta$ , mRNA were greater in CLENDO cultures (Fig. 4).

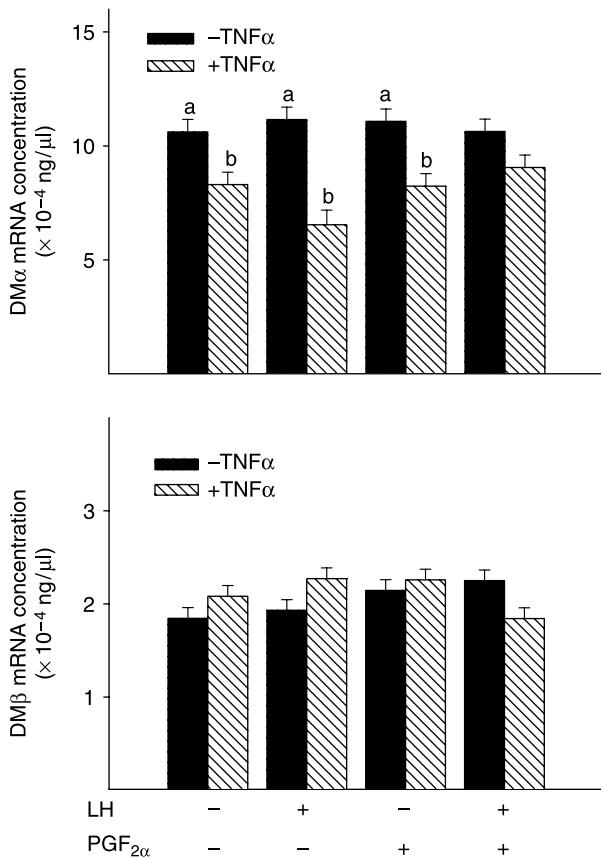
The effects of LH, PGF<sub>2 $\alpha$</sub>  and TNF- $\alpha$  on the concentrations of DM $\alpha$  mRNA in cultured bovine luteal cells are displayed in Fig. 5. Concentrations of DM $\alpha$  were less in TNF- $\alpha$ -treated cultures compared with controls ( $P < 0.05$ ). Similarly, in the presence of LH or PGF<sub>2 $\alpha$</sub> , TNF $\alpha$  caused a reduction in DM $\alpha$  mRNA concentrations. Treatment with TNF- $\alpha$  in the presence of both LH and PGF<sub>2 $\alpha$</sub>  also caused slight, but non-significant, reductions in DM $\alpha$  mRNA. LH, PGF<sub>2 $\alpha$</sub>  and TNF- $\alpha$  were all without effect on concentrations of BoLA-DM $\beta$  mRNA in cultured luteal cells (Fig. 5).

## Discussion

The present study is the first to demonstrate the presence of mRNA encoding Ii, DM $\alpha$  and DM $\beta$  in the CL of any species. These results are significant when considering the increase in the ability of luteal cells to stimulate T cell proliferation in response to PGF<sub>2 $\alpha$</sub> . Although we have previously demonstrated that reduction in progesterone synthesis is at least partly responsible for the increase in the ability of luteal cells to stimulate T lymphocyte proliferation following PGF<sub>2 $\alpha$</sub>  administration (Cannon *et al.* 2003), alteration in intracellular antigen processing could



**Figure 4** Steady-state concentrations of DM $\alpha$  and DM $\beta$  mRNA in cultures of mixed luteal cells and CLEND0 cells. Bars represent concentrations of DM $\alpha$  and DM $\beta$  mRNA, as determined by RT-qPCR, in RNA extracted from cultures of mixed luteal cells (gray bars) or luteal endothelial cells (black bars) (mean  $\pm$  S.E.M.,  $n = 3$ ). \*Significant difference between mixed luteal cell and luteal endothelial cell cultures.



**Figure 5** Steady-state concentrations of DM $\alpha$  mRNA (upper panel) and DM $\beta$  mRNA (lower panel) in cultures of mixed luteal cells. Bars represent concentrations of DM $\alpha$  and DM $\beta$  mRNA, as determined by RT-qPCR, in RNA extracted from luteal cells cultured in the presence or absence of LH and/or PGF $_{2\alpha}$ , with (hatched bars) or without (black bars) TNF- $\alpha$ . Different letters denote significant differences between cultures in the presence or absence of TNF- $\alpha$  (mean  $\pm$  S.E.M.,  $n = 4$ ,  $P < 0.05$ ).

also contribute to the enhanced ability of luteal cells from regressing CL to stimulate T cell proliferation. Alterations in DM $\alpha$  and DM $\beta$  expression near the end of the estrous cycle could catalyze a change in luteal cells that facilitates the process of luteal regression.

Steady-state concentrations of Ii mRNA were not different at any stage of the estrous cycle. The reason for this is not clear. Class II MHC expression on bovine luteal cells increases throughout the estrous cycle and following PGF $_{2\alpha}$  administration (Benyo *et al.* 1991), and since Ii is required for transport of class II MHC molecules to the cell surface, expression of Ii would be expected to parallel class II MHC expression. Given the observed lack of change in Ii mRNA during the estrous cycle or following administration of PGF $_{2\alpha}$ , it appears that this gene is constitutively expressed in luteal tissue. It is possible that constitutive expression of Ii is sufficient to allow transport of class II MHC molecules to the cell surface. It must be noted, however, that the present study measured mRNA whereas the previous study measured cell surface proteins (Benyo *et al.* 1991). In that study, expression of class II MHC molecules by luteal cells was not measured beyond 12 h after a luteolytic dose of PGF $_{2\alpha}$ . It is possible that class II MHC expression may increase dramatically by 24 h after PGF $_{2\alpha}$ , similar to the increase in Ii mRNA concentrations observed in the present study.

Concentrations of DM $\alpha$  and DM $\beta$  mRNA were greatest in the CL during midcycle, and declined near the time of luteal regression. Since previous studies have shown that Ii and DM are coordinately regulated by the class II transactivating factor CIITA (Steimle *et al.* 1994, Chang & Flavell 1995), this result was unexpected in light of the observed lack of change in Ii mRNA concentration throughout the estrous cycle. The number of bovine luteal cells expressing class II MHC molecules, as well as the degree of class II MHC expression, both increase near the time of luteal regression (Benyo *et al.* 1991). The decline in DM $\alpha$  and DM $\beta$  mRNA concentrations in luteal tissue concurrent with increases in class II MHC expression may result in presentation of a repertoire of peptides to T cells during luteal regression that is different from that presented during midcycle. Administration of a luteolytic dose of PGF $_{2\alpha}$  to the cow did not induce changes in the steady-state concentrations of DM $\alpha$  or DM $\beta$  mRNA. The reason for the lack of effect of PGF $_{2\alpha}$  on DM $\alpha$  and DM $\beta$  expression is unknown, but it is evident that the control of expression of these genes in the CL is not regulated by PGF $_{2\alpha}$ . Treatment of cultured luteal cells with TNF- $\alpha$  reduced concentrations of DM $\alpha$ , and elevation of TNF- $\alpha$  during luteal regression has been demonstrated (Ji *et al.* 1991, Shaw & Britt 1995). Elevation of TNF- $\alpha$  in the CL was not observed until after the decline in circulating and tissue concentrations of progesterone, but intra-luteal concentrations of TNF- $\alpha$  may increase prior to the decline in circulating concentrations of progesterone, which would explain the decline in DM $\alpha$  mRNA observed in late cycle CL. Steady-state concentrations of DM $\beta$  mRNA were not



affected by LH, TNF- $\alpha$  or PGF<sub>2 $\alpha$</sub> , indicating that multiple factors control the expression of DM in the CL.

The present study demonstrates the presence of Ii, DM $\alpha$  and DM $\beta$  mRNA in luteal tissue in amounts detectable using the relatively non-sensitive method of Northern analysis. That these messages are detectable by Northern analysis is suggestive of a source of mRNA other than infiltrating macrophages and B lymphocytes, which are relatively few in number compared with the total number of cells in the bovine CL. DM $\alpha$  and DM $\beta$  mRNA were also found in CLENDO cultures in the present study, which provides support for the hypothesis that a cell type in addition to macrophages in the CL expresses these components of the antigen-processing pathway. It is worth noting that the CLENDO cultures used in the present study are composed of very highly purified populations of luteal endothelial cells, since the concentration of CD31 mRNA in CLENDO cells was approximately ten-fold greater than in mixed luteal cell cultures, whereas StAR mRNA is almost undetectable. Conversely, the mixed luteal cell cultures contain largely steroidogenic cells, but are likely to also contain small numbers of endothelial cells; large amounts of StAR mRNA were present in the mixed luteal cell cultures, whereas concentrations of CD31 mRNA were much less than those in CLENDO cultures. Steady-state concentrations of DM $\alpha$  were greater in CLENDO cell cultures compared with mixed luteal cell cultures, and although it was not significant, concentrations of DM $\beta$  mRNA also appeared to be greater in CLENDO cell cultures compared with mixed luteal cell cultures. However, DM $\alpha$  and DM $\beta$  mRNAs were also present in mixed luteal cell cultures in relatively high abundance. From the analysis of CD31 concentrations in the CLENDO and mixed luteal cell cultures, we have concluded that endothelial cells make up a very small proportion of the total cells in mixed luteal cell cultures. Therefore, it seems unlikely that endothelial cells account for the relatively large amount of DM $\alpha$  and DM $\beta$  mRNA present in mixed luteal cell cultures. We have not assessed the presence of macrophages in these cultures, but while it is unlikely that macrophages are present in the CLENDO cultures, due to the purification procedures used to derive these cultures, it is possible that significant numbers of macrophages are present in mixed luteal cell cultures. This would account for the presence of the relatively large amount of DM $\alpha$  and DM $\beta$  mRNA found in mixed luteal cell cultures. It is unfortunate that it was not possible to obtain a pure population of steroidogenic luteal cells for these studies, and it is not possible to conclude from this experiment whether or not steroidogenic cells express DM $\alpha$  and DM $\beta$ . However, these results clearly demonstrate that luteal endothelial cells express DM $\alpha$  and DM $\beta$ .

In conclusion, the present study demonstrates the expression of Ii, DM $\alpha$  and DM $\beta$  genes in luteal tissue. These genes encode intracellular antigen-processing proteins required for processing and presentation of

antigenic peptides to T lymphocytes. The changes in DM $\alpha$  and DM $\beta$  expression observed near the end of the estrous cycle could result in presentation of an altered repertoire of self-peptides in the context of class II MHC molecules. This alteration of antigen presentation may have significant implications for regulation of luteal function via interaction of resident T lymphocytes with cells of the CL. However, since expression of the antigen-processing components examined in this study is greatest during mid-cycle, it is possible that presentation of antigen by class II MHC is necessary in the fully functional CL to maintain a state of immune tolerance to the tissue. Since immune tolerance would no longer be essential at the end of the estrous cycle, the decline in DM $\alpha$  and DM $\beta$  expression may serve to facilitate activation of immune cells that may participate in the process of regression. Future studies are needed to determine the role(s) played by the immune system in regulation of luteal function.

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