

# Regulation of Lymphocyte Proliferation by Uterine Serpin: Interleukin-2 mRNA Production, CD25 Expression and Responsiveness to Interleukin-2 (44465)

MORGAN R. PELTIER, WEN-JUN LIU,<sup>2</sup> AND PETER J. HANSEN<sup>1</sup>

*Department of Dairy and Poultry Sciences, University of Florida, Gainesville, Florida 32611-0920*

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**Abstract.** During pregnancy, the endometrium of the ewe secretes large amounts of a progesterone-induced protein of the serpin superfamily of serine proteinase inhibitors called ovine uterine serpin (OvUS). This protein inhibits lymphocyte proliferation in response to concanavalin A (ConA), phytohemagglutinin (PHA), or mixed lymphocyte reaction. The purpose of these experiments was to characterize the mechanism by which OvUS inhibits lymphocyte proliferation. Ovine US caused dose-dependent inhibition of lymphocyte proliferation induced by phorbol myristol acetate (PMA), an activator of protein kinase C. The PHA-induced increase in CD25 expression was inhibited in peripheral blood mononuclear leukocytes (PBML) by OvUS. However, no effect of OvUS on Con A-induced expression of CD25 was observed. Further analysis using two-color flow cytometry revealed that OvUS inhibited ConA-induced expression of CD25 in  $\gamma\delta$ -TCR<sup>-</sup> cells but not  $\gamma\delta$ -TCR<sup>+</sup> cells. Stimulation of PBML for 14 hr with ConA resulted in an increase in steady state amounts of interleukin-2 (IL-2) mRNA that was not inhibited by OvUS. Ovine US was also inhibitory to lymphocyte proliferation induced by human IL-2. Results suggest that OvUS acts to inhibit lymphocyte proliferation by blocking the upregulation of the IL-2 receptor and inhibiting IL-2-mediated events. Lack of an effect of OvUS on ConA-stimulated CD25 expression in  $\gamma\delta$ -TCR<sup>+</sup> cells may reflect a different mechanism of activation of these cells or insensitivity to inhibition by OvUS.

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Ovine uterine serpin is a member of the serpin superfamily of serine proteinase inhibitors that is expressed in high amounts in the sheep uterus during pregnancy in response to progesterone (1-4). Similar proteins are also produced in pigs (5, 6) and cattle (4, 6, 7). The majority of serpins, such as  $\alpha$ 1-antitrypsin, inhibit proteinases by forming a tight tetrahedral complex with their target proteinase through interaction of the protein with the reac-

tive center loop (8, 9). However, OvUS is distinct from the prototypical serpin because it has no antiproteolytic activity to any of the serine proteinases tested (1) and only weak inhibitory activity against pepsin A and pepsin C (6), which does not seem to be dependent on an intact reactive center loop (10).

Functionally, OvUS acts as an inhibitor of lymphocyte proliferation. It inhibits a wide variety of events including mixed lymphocyte reactions, mitogen-stimulated lymphocyte proliferation (11-14) and Poly I · Poly C-induced NK-cell activity and abortion in mice (15). In fact it is believed that OvUS mediates the inhibitory effects of progesterone on uterine immune function that leads to prolonged skin graft survival (16) and decreased lymphocyte numbers in the endometrium (17). The mechanism by which OvUS acts to inhibit lymphocyte function is unknown. Concentrations of OvUS from 125 to 500  $\mu$ g/ml are required for inhibition of lymphocyte proliferation (11, 12). Although these concentrations are physiological (concentrations in uterine fluid are 5 mg/ml or greater), it is likely that OvUS may not function through a classical high-affinity receptor. Nonethe-

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<sup>1</sup> To whom requests for reprints should be addressed at P.O. Box 110920, University of Florida, Gainesville, FL 32611-0920. E-mail: Hansen@dps.ufl.edu

<sup>2</sup> Present address: Howard Hughes Medical Institute, Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

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less, OvUS binds specifically to the lymphocyte surface (18). It is possible that OvUS binds proteins on the surface of the lymphocyte and that such binding either activates an inhibitory response or blocks events required for proliferation.

Stimulation of the T-cell receptor with antigen or a mitogenic lectin induces a signal transduction pathway involving protein kinase C, which stimulates IL-2 secretion and upregulation of the  $\alpha$  subunit of the IL-2 receptor, CD25 (the  $\beta$  and  $\gamma$  subunits are constitutively expressed). Secreted IL-2 functions in an autocrine and paracrine manner by binding to the full IL-2 receptor to initiate mitogenesis of the antigen-stimulated lymphocytes. To understand how immunity may be suppressed in the uterus during pregnancy, several experiments were performed to determine at what steps OvUS acts to inhibit lymphocyte proliferation. Specifically, effects on PMA-stimulated lymphocyte proliferation, mitogen-stimulated CD25 expression, mitogen-stimulated IL-2 gene expression, and responsiveness to human IL-2 (Hu-IL-2) were tested.

## Materials and Methods

**Materials.** Tissue Culture Medium 199, bovine serum albumin (BSA), concanavalin A (ConA), phytohemagglutinin (PHA), Taq polymerase, glutamine, penicillin-streptomycin, biconinonic acid (BCA), and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). Trizol reagent for RNA purification was purchased from Life Technologies (Gaithersburg, MD). Kits for RT-PCR (Retroscrip) were purchased from Ambion (Houston, TX) and included a pair of PCR primers for the housekeeping gene coding for the ribonucleoprotein S-15 (forward: 5'-TTCCGCAAGTTCCTACC-3'; reverse: 5'-CGGGCCGGCCATGCTTTACG-3'; 361-bp product). The primers for PCR of OvIL-2 (forward: 5'-CTT-GTC-TTG-CAT-TGC-ACT-AAC-TCT-TG-3'; reverse: 5'-TCC-ATT-TGT-TCA-GAA-ATT-CTA-CAG-CG-3'; 413-bp product) were designed to anneal to regions of the bovine sequence (19) that are 100% homologous to the ovine sequence; these primers were manufactured by Research Genetics (Huntsville, AL). Recombinant human IL-2 was purchased from Genzyme (Boston, MA). Molecular weight markers (100 bp) were purchased from Promega (Madison, WI). Fluorescein isothiocyanate (FITC) monomer for conjugation of antibodies was purchased from Molecular Probes (Eugene, OR). Horse and mouse sera were purchased from Atlanta Biologicals (Norcross, GA). Falcon tissue culture-treated 96-well plates, Falcon 12 mm  $\times$  75 mm polystyrene tubes, and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

**Antibodies.** Mouse ascites containing antibody to ovine CD25 (Clone 9-14) was generously provided by Dr. Andrew Nash (Center for Animal Biotechnology, University of Melbourne, Australia) and was used at a 1:200 dilution in PBS for all experiments. Hybridoma cells that secrete an antibody to the ovine  $\gamma\delta$ -T cell receptor (Clone 86D) were obtained from the European Animal Cell Col-

lection (Salisbury, UK). Ascites for clone 86D was produced by the Hybridoma Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research and stored at  $-20^{\circ}\text{C}$  until use. For indirect labeling of lymphocytes, an FITC- or phycoerythrin (PE)-labeled sheep anti-mouse IgG F(ab)<sub>2</sub> fragment was purchased from Sigma.

For some experiments, antibody to the  $\gamma\delta$ -TCR was purified by Protein A affinity chromatography using the FPLC system (Pharmacia, Piscataway, NJ). Briefly, ascites produced from clone 86D was diluted 1:10 in 3 M glycine, 1.5 M NaCl, pH 8.9, and 2-6 ml were loaded onto a Protein A-Sepharose column (1  $\times$  5 cm; BioRad, Hercules, CA). The column was washed with 25 ml of 3 M glycine, 1.5 M NaCl, pH 8.9, and then antibody was eluted with 0.5 M citrate-phosphate buffer, pH 5.5. Column fractions (0.5 ml each) were collected into 12  $\times$  75-mm tubes that contained 0.1 ml of 1 M Tris-HCl, pH 8.8. Fractions corresponding to the protein peak were pooled and concentrated using centrifugal ultrafiltration devices (Amicon, Beverly, MA). Antibodies were then buffer exchanged into DPBS using Sephadex G-25 desalting columns (Pierce, Rockford, IL). Protein concentration was determined using the BCA modification of the Lowry procedure (20).

For FITC labeling, antibodies purified as described above were buffer-exchanged into 0.1 M carbonate pH 9.6, and 1 mg of 3.7 mg/ml antibody was incubated with 120  $\mu\text{l}$  of 10 mg/ml FITC in DMSO. After 2 hr in the dark at room temperature, labeled protein was separated from excess FITC by desalting on a G-25 Sephadex chromatography column. The fluorescence:protein ratio was then estimated by UV absorbance, and the protein concentration was determined by the BCA assay.

**Purification of OvUS.** The ovine uterine serpin was purified from uterine fluid collected from unilaterally pregnant ewes. Prior to breeding, one uterine horn was surgically ligated, and the ipsilateral ovary was removed to restrict the subsequent pregnancy to one uterine horn (21). At approximately Day 140 of pregnancy, ewes were sacrificed, and fluid was collected from the nonpregnant uterine horn. Uterine fluid was clarified by centrifugation (10,000g for 30 min at  $4^{\circ}\text{C}$ ) and stored at  $-20^{\circ}\text{C}$  until purification of OvUS by a combination of cation-exchange and gel-filtration chromatography as previously described (2). After purification, OvUS was buffer-exchanged into DPBS and concentrated using Centricon ultrafiltration devices (Amicon, Beverly, MA). Protein purity was confirmed (>95%) by SDS-PAGE under reducing conditions on 10% (w/v) polyacrylamide gels and staining with Coomassie Blue R-125. Concentration of the protein was determined by the BCA modification of the Lowry protein assay using BSA as the standard (20).

**Lymphocyte Proliferation Assays.** Procedures for purification of peripheral blood mononuclear leukocytes (PBML) using Ficoll-Paque and mitogen-stimulated lymphocyte proliferation were as previously described (13, 22). Cells were plated at  $10^5$  cells/well in 100  $\mu\text{l}$  TCM-199 that

was supplemented with 2 mM extra glutamine, 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 5% (v/v) horse serum (TCM-S) in 96-well tissue culture plates. Test proteins were added to each well in a volume of 50  $\mu$ l DPBS. Proliferation was induced by adding PMA (6 nM), PHA (2  $\mu$ g/ml), ConA (5  $\mu$ g/ml), or HuIL-2 (80 U/ml) in 10  $\mu$ l DPBS. Cells were placed in a humidified incubator and cultured at 37°C in a 5% CO<sub>2</sub> atmosphere for 60–72 hr and then pulsed with [<sup>3</sup>H]thymidine (0.1 or 0.5  $\mu$ Ci/well) in 40  $\mu$ l TCM-S for an additional 12–18 hr. Cells were then harvested, and the incorporation of [<sup>3</sup>H]thymidine was determined by liquid scintillation counting. Treatments were done in quadruplicate, and each experiment was replicated two to six times using PBML from a separate ewe for each replicate.

**Effect of OvUS on PHA-Induced CD25 Expression.** Peripheral blood mononuclear cells were placed in 96-well plates (10<sup>6</sup> cells/well) in 100  $\mu$ l TCM-S. Treatments (control, 2  $\mu$ g/ml PHA, 0.5 mg/ml OvUS, and OvUS + PHA) were then added in a total volume of 60  $\mu$ l DPBS. A total of eight wells were prepared for each treatment. Cells were cultured for 72 hr in a humidified incubator at 37°C and 5% (v/v) CO<sub>2</sub>. After culture, cells were washed with flow cytometry buffer [DPBS + 2 mM EDTA + 0.1% (w/v) BSA], harvested by vigorous pipetting and resuspended in 100  $\mu$ L flow cytometry buffer. This experiment was replicated two times with PBML collected from three ewes.

For staining, PBML were incubated in 1% (v/v) mouse serum for 30 min on ice, and then 10  $\mu$ l of a 1:200 dilution of ascites fluid containing antibody to ovine CD25 or 10  $\mu$ l of a 1:200 dilution of control mouse ascites fluid (Sigma) was added. After incubation on ice for 30 min, PBML were washed twice, resuspended in 100  $\mu$ l flow cytometry buffer, and an FITC-labeled sheep anti-mouse F(ab)<sub>2</sub> fragment (10  $\mu$ l) was added to each tube. Cells were incubated on ice for 30 min, washed twice, resuspended in 1 ml DPBS + 0.5% (w/v) paraformaldehyde, and stored at 4°C in the dark until analysis within 3 days of staining.

Flow cytometric analyses were performed on a FACScan flow cytometer (Beckton-Dickerson, Franklin Lakes, NJ). The lymphocyte subpopulation was selected for all analyses of CD25 expression by forward and side scatter, and 10,000 events were counted for each analysis. The percentage of CD25<sup>+</sup> lymphocytes was determined using the WinMDI program version 2.5 (The Scripps Research Institute, La Jolla, CA).

**Effect of OvUS on ConA-Stimulated PBML.** Purified PBML were cultured in sterile polystyrene culture tubes (10<sup>6</sup> cells/tube in 140  $\mu$ l TCM-S) with 60  $\mu$ l DPBS or treatments (control, 5  $\mu$ g/ml ConA, 5  $\mu$ g/ml ConA and 8.9  $\mu$ M OvUS, or 5  $\mu$ g/ml ConA and 8.9  $\mu$ M OVA) for 24 or 72 hr at 37°C in a humidified incubator under 5% CO<sub>2</sub>. All treatments were prepared in quadruplicate and replicated using PBML from three ewes. After culture, PBML were washed twice with ice-cold flow cytometry buffer, resus-

ended in 100  $\mu$ l cold flow cytometry buffer, and stained for CD25 expression as described above.

**Effect of OvUS on CD25 Expression for  $\gamma\delta$ -TCR<sup>+</sup> and  $\gamma\delta$ -TCR<sup>-</sup> Lymphocytes.** Ovine PBML were purified as described above and placed in sterile 12  $\times$  75-mm flow cytometry tubes (10<sup>6</sup>/cells per tube) in 140  $\mu$ l TCM-S. Mitogen and test proteins were added in 60  $\mu$ l DPBS to each tube, and the tubes were cultured in a humidified incubator for 24 hr at 37°C. Cells were then washed twice with flow cytometry buffer, and the pellet was resuspended in 100  $\mu$ l flow cytometry buffer. Cells were stained for CD25 as described above except that PE-labeled sheep anti-mouse F(ab)<sub>2</sub> fragment was used as the second antibody. After the final wash, cells were resuspended in 100  $\mu$ l of flow cytometry buffer, and 10  $\mu$ l of FITC-coupled antibody to ovine  $\gamma\delta$ -TCR (Clone 86D; 1  $\mu$ g) was added. After cells were incubated for 30 min on ice, they were washed three times with flow cytometry buffer, resuspended in 1 ml 0.5% (v/v) paraformaldehyde in DPBS, and stored at 4°C in the dark until analysis within 72 hr. To evaluate the background fluorescence, an equivalent amount of FITC-labeled mouse IgG1 (Sigma Chemical Company, St. Louis, MO) was added to control tubes.

For analysis, the lymphocyte population was gated by forward scatter and side scatter. The gated lymphocytes were further subdivided into  $\gamma\delta$ -TCR<sup>-</sup> and  $\gamma\delta$ -TCR<sup>+</sup> subpopulations based on the intensity of staining for FITC. The expression of CD25 was then evaluated for each of these gated subpopulations by the intensity of staining for PE. For all analyses, compensation between PE and FITC channels was adjusted using labeled flow cytometry beads (Beckton-Dickinson, Franklin Lakes, NJ), and 50,000 cells were counted in each analysis.

**Reverse Transcriptase-PCR for Interleukin-2.** Peripheral blood mononuclear leukocytes were cultured at 10<sup>6</sup> cells per well in 140  $\mu$ l TCM-S. Proteins were added to a final concentration of 8.9  $\mu$ M (i.e., 0.5 mg/ml OvUS) in 50  $\mu$ l DPBS, and ConA was added to a final concentration of 5  $\mu$ g/ml in 10  $\mu$ l DPBS. After 14 hr at 37°C and 5% CO<sub>2</sub>, cells were collected, homogenized in Trizol reagent (Life Technologies, Gaithersburg, MD), and stored at -85°C until further extraction. Total RNA was extracted from the Trizol homogenates according to the manufacturer's instructions, and RNA concentration was determined by measuring A<sub>260</sub>.

All RT-PCR analyses were conducted in 200- $\mu$ l PCR tubes, and reactions were assembled on ice. For the reverse transcription reaction, 2.5  $\mu$ g of total RNA was diluted to 10  $\mu$ l with DEPC-treated water and 2  $\mu$ l 10x RT-PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub>), 2  $\mu$ l of oligodT<sub>18</sub> (50  $\mu$ M), and 4  $\mu$ l dNTP mix (2.5 mM ATP, 2.5 mM CTP, 2.5 mM TTP, and 2.5 mM GTP). Samples were then placed in a thermocycler (M.J. Research, Watertown, MA), incubated at 80°C for 3 min, and then cooled to 4°C. Tubes were returned to the crushed ice and 1  $\mu$ l of placental RNase inhibitor (10 U/ $\mu$ l), and 1  $\mu$ l of Moloney-murine tumor virus reverse transcriptase (100

U/ $\mu$ l) was added. Samples were returned to the thermocycler, incubated at 42°C for 1 hr followed by 10 min at 92°C, and cooled to 4°C. Reverse transcription products were either stored at -20°C or used immediately for the PCR step.

For amplification, PCR reactions were prepared on ice in a total volume of 50  $\mu$ l that contained 1  $\mu$ l (S-15 riboprotein) or 5  $\mu$ l (IL-2) cDNA product, 5  $\mu$ l 10X RT-PCR buffer, 2.5  $\mu$ l dNTP mix, 2.5  $\mu$ l primer mixture (5  $\mu$ M each primer), 34.8  $\mu$ l dH<sub>2</sub>O, and 0.2  $\mu$ l Taq polymerase (5 U/ $\mu$ l). Tubes were placed in a programmable thermocycler (MJ Research, Watertown, MA) and preheated at 94°C for 5 min followed by 25 (S-15) or 42 (IL-2) cycles of heating at 94°C for 30 sec, 55°C for 30 s and 72°C for 30 s. Number of cycles was validated to ensure that amplification was in the linear range. The PCR tubes were then heated at 72°C for 10 min and cooled to 4°C until electrophoresis. Reaction products (30  $\mu$ l) were mixed with 6  $\mu$ l gel loading buffer (Ambion, Houston, Tx) and resolved on 8% (w/v) polyacrylamide gels. After electrophoresis, gels were stained with ethidium bromide (5  $\mu$ g/ml) for 15 min, and excess ethidium bromide was removed by destaining with deionized water. Identity of the amplicon was verified by restriction digestion. Intensity of the bands was determined using an Aphaimage 2000 Gel Documentation System (Alpha Innotech Corporation, San Leandro, CA). All gels were corrected for background staining prior to analysis by subtracting the intensity from a region on the gel that contained no visible bands.

**Statistical Analysis.** Data were analyzed by least-squares analysis of variance (ANOVA) using the General Linear Models procedure of the Statistical Analysis System (23). For all analyses, ewe was considered a random effect, and treatment was considered a fixed effect. Differences between individual treatments were evaluated using orthogonal contrasts. For the experiment evaluating the effect of OvUS on IL-2 mRNA gene expression, S-15 gene expression was used as a covariate to adjust for uneven differences in total RNA. In this experiment, the variance of IL-2 differed between treatments, and the data were rank-transformed (24) prior to ANOVA to meet the assumption of homogeneity of variance.

## Results

**Effect of OvUS on PMA-Stimulated Lymphocyte Proliferation.** Ovine US caused dose-dependent inhibition of PMA-stimulated lymphocyte proliferation after incubation for either 24 or 72 hr (Fig. 1). Similar effects were obtained when PHA was used as the mitogen (Fig. 1).

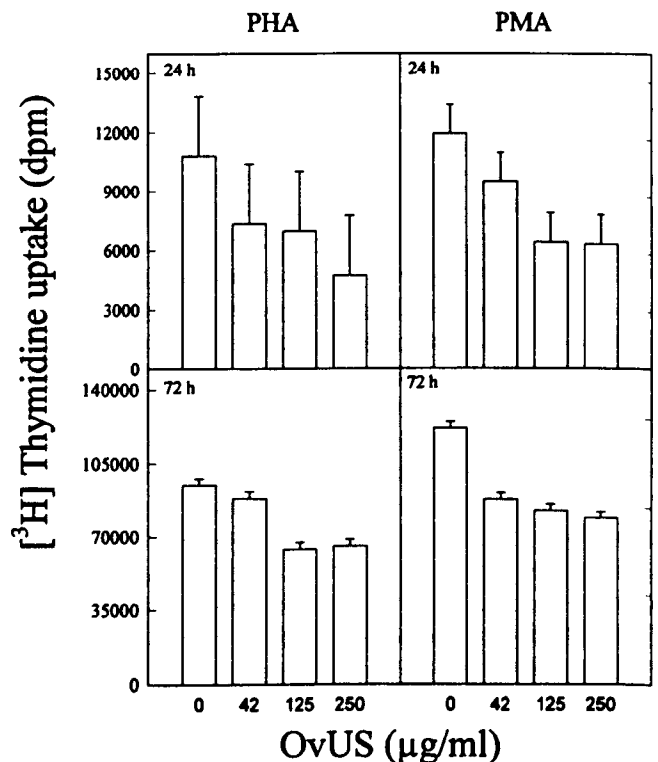
**Effect of OvUS on PHA-Induced CD25 Expression.** Incubation of PBML with PHA for 72 hr caused a significant increase in the number of lymphocytes expressing CD25 ( $P = 0.0223$ ; Fig. 2A). Ovine uterine serpin did not affect CD25 expression in unstimulated lymphocytes but decreased ( $P = 0.0266$ ) the proportion of lymphocytes expressing CD25 on PBML that were stimulated with PHA.

**Effect of OvUS on ConA-Induced CD25 Expression.** Incubation of PBML with ConA for 24 or 72 hr

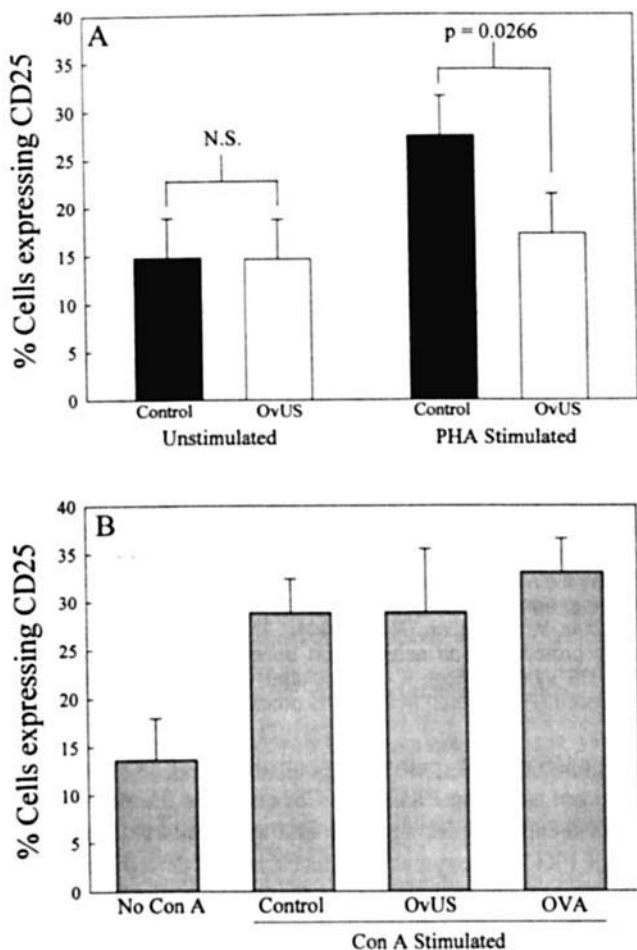
resulted in an increase in the percentage of lymphocytes expressing CD25 ( $P = 0.0211$ ). This increase in CD25 expression was not significantly inhibited by the control protein OVA or an equivalent amount of OvUS.

**Effect of OvUS on ConA-Induced CD25 Expression on  $\gamma\delta$ -TCR<sup>-</sup> and  $\gamma\delta$ -TCR<sup>+</sup> Cells.** Representative histograms illustrating results for an individual ewe are shown in Figure 3, and the least-squares means of overall results are shown in Figure 4. Incubation of ovine PBML with ConA stimulated CD25 expression on both  $\gamma\delta$ -TCR<sup>+</sup> cells ( $P = 0.0001$ ) and  $\gamma\delta$ -TCR<sup>-</sup> cells ( $P = 0.0001$ ). Ovalbumin had no effect on the mitogen-induced increase in percentage of CD25<sup>+</sup> cells gated as  $\gamma\delta$ -TCR<sup>-</sup> or  $\gamma\delta$ -TCR<sup>+</sup>. In contrast, OvUS inhibited the Con-A induced increase in CD25 expression on  $\gamma\delta$ -TCR<sup>-</sup> cells ( $P = 0.0003$ ) but not on  $\gamma\delta$ -TCR<sup>+</sup> cells.

**Effect of OvUS on IL-2 mRNA Production.** Culture of PBML for 14 hr with ConA resulted in a significant increase in steady-state amounts of IL-2 mRNA as measured by RT-PCR ( $P = 0.0007$ , Fig. 5A). This increase was not blocked by either OVA or OvUS. The results of this experiment were highly variable between animals. In five animals, IL-2 mRNA levels were increased in response to OvUS; in another two animals, IL-2 mRNA was decreased.



**Figure 1.** Lymphocyte proliferation stimulated by PHA or PMA is inhibited in a dose-dependent manner by OvUS. Lymphocytes were cultured for either 24 or 72 hr in the presence of OvUS and then pulsed for an additional 16–18 hr with 0.5  $\mu$ Ci <sup>3</sup>H-thymidine. Shown are least squares means  $\pm$  SEM for experiments using four sheep. Unstimulated controls had 219  $\pm$  17 and 627  $\pm$  50 dpm (mean  $\pm$  SEM) of <sup>3</sup>H-thymidine incorporation for cultures stimulated for 24 and 72 hr, respectively.



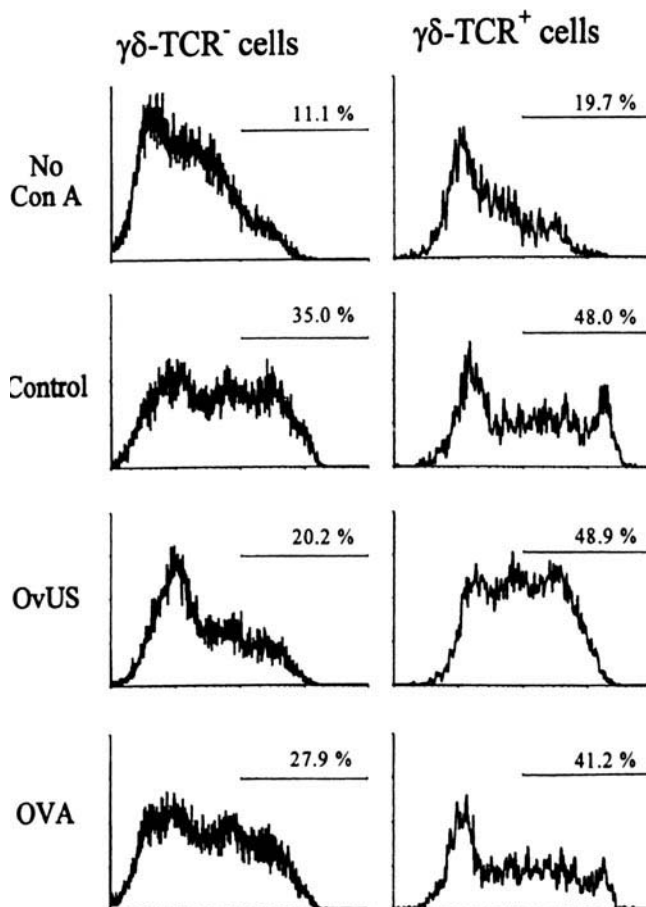
**Figure 2.** Ovine uterine serpin (A) inhibits the increase in CD25 expression on PBML in response to PHA but (B) has no effect on ConA-induced CD25 expression. Shown are least squares means  $\pm$  SEM for three ewes for each experiment.

A representative animal that displayed an increase in IL-2 mRNA is shown in Figure 5B. Steady-state amount of the housekeeping gene, *S-15*, was similar between treatments indicating equal loading of RNA (Fig. 5B). No PCR product was observed in the samples when reverse transcriptase was eliminated from the cDNA synthesis reaction (data not shown).

**Effect of OvUS on IL-2-Stimulated Lymphocyte Proliferation.** Stimulation of lymphocyte proliferation with HuIL-2 resulted in a significant increase in [<sup>3</sup>H]-thymidine incorporation ( $P = 0.0004$ ) that was not inhibited by OVA (Fig. 6). However, OvUS inhibited lymphocyte proliferation ( $P = 0.0057$ ) when compared with these two treatments. Similar results were obtained when the lymphocytes were stimulated with ConA (Fig. 6).

## Discussion

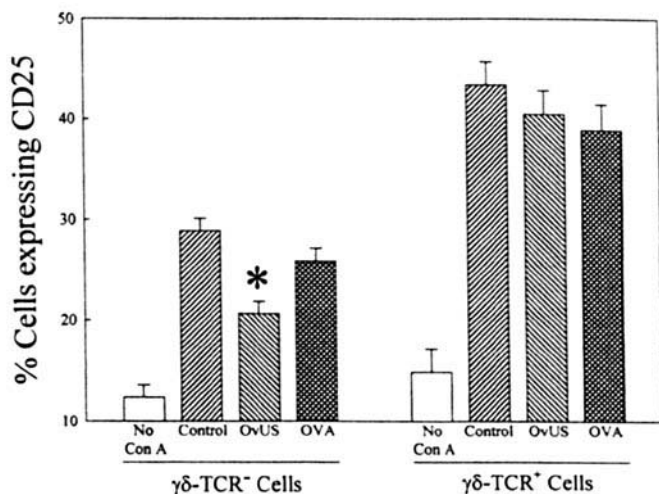
T-cell proliferation and differentiation require a variety of steps including activation of PKC by an appropriately stimulated T-cell receptor, production of IL-2, increased production of an IL-2 receptor including the  $\alpha$  subunit (i.e., CD25), and IL-2 stimulated proliferation. Present results



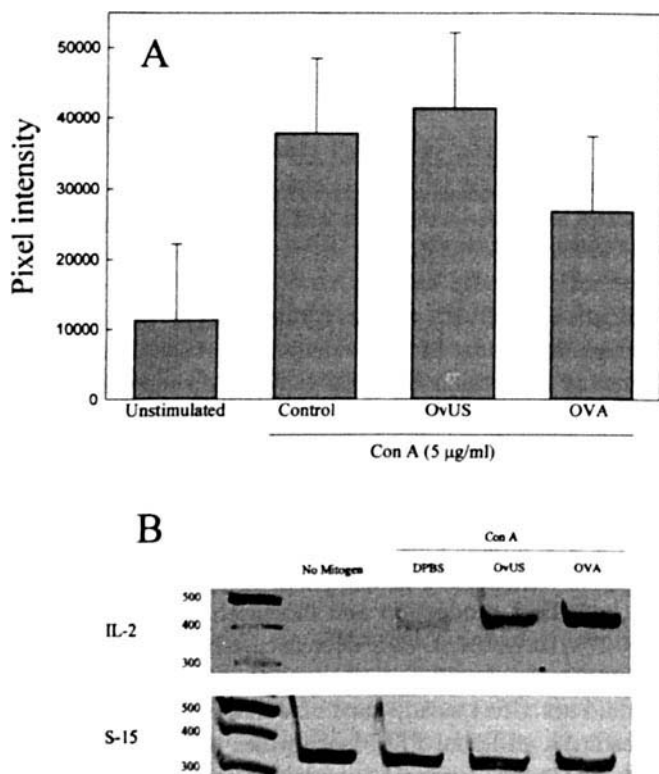
**Figure 3.** Representative histograms illustrating the effect of OvUS on CD25 expression on  $\gamma\delta$ -TCR<sup>-</sup> and  $\gamma\delta$ -TCR<sup>+</sup> lymphocytes. Data were gated by expression of  $\gamma\delta$ -TCR.

indicate that OvUS reduces lymphocyte proliferation through inhibition of PKC-mediated events that include increased expression of CD25 but that do not include increased expression of IL-2.

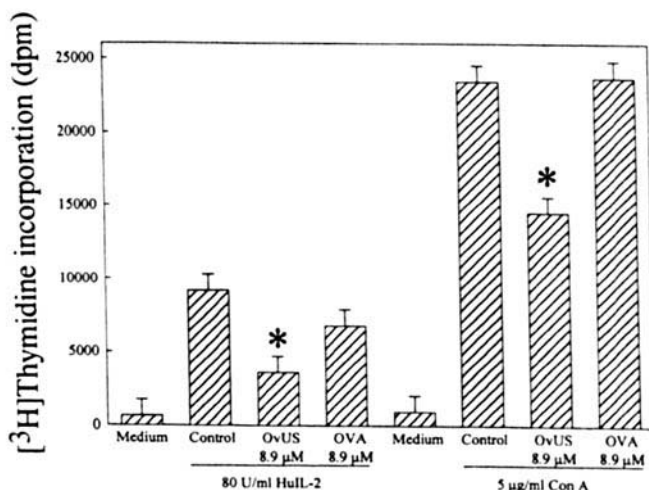
Ovine US inhibited PMA-induced lymphocyte proliferation in a dose-dependent manner, suggesting that OvUS inhibits PKC-mediated events controlling lymphocyte activation. The protein kinase C family of enzymes is involved in signal transduction of the T-cell receptor and is needed for both IL-2 production and increased CD25 expression (25–27). However, OvUS decreased CD25 expression on PHA-stimulated cells and on  $\gamma\delta$  TCR<sup>-</sup> ConA-induced cells but did not affect steady-state amounts of IL-2 mRNA. Interestingly, different PKC isoenzymes are used for CD25 and IL-2 gene expression, and it is possible that OvUS blocks one pathway only. In particular, immunoneutralization of PKC $\alpha$  and PKC $\theta$  blocked the increase in CD25 expression in response to TCR stimulation whereas immunoneutralization of PKC $\beta$ , PKC $\delta$ , and PKC $\epsilon$  blocked the TCR-activated increase in IL-2 synthesis (27). Furthermore, addition of a PKC $\beta$ 1 agonist to resting lymphocytes increased IL-2 production (28). If similar systems are present for the activation of ovine PBML, the failure of OvUS to reduce steady-state amounts of IL-2 mRNA would suggest



**Figure 4.** Ovine uterine serpin inhibits the increase in CD25 expression on  $\gamma\delta$ -TCR<sup>-</sup> lymphocytes but not  $\gamma\delta$ -TCR<sup>+</sup> lymphocytes. Shown are least squares means  $\pm$  SEM for the percentage of cells that are CD25<sup>+</sup> for three ewes (\* $P = 0.0025$ ; orthogonal contrast between OvUS vs Control + OVA).



**Figure 5.** Effect of OvUS on ConA-stimulated changes in IL-2 mRNA. Incubation of PBML for 14 hr increased amounts of IL-2 mRNA ( $P = 0.0007$ ). (Panel A) Least squares means  $\pm$  SEM for pixel intensity for IL-2 adjusted by using S-15 mRNA as a covariate to adjust for loading differences ( $n = 7$  ewes). However, neither ovalbumin nor an equivalent amount of OvUS affected the increase in mRNA for OvIL-2. (Panel B) Representative RT-PCR results for IL-2 and S-15 for PBML cultured with medium alone (Lane 2), ConA (Lane 3), Con A + OvUS (Lane 4) and ConA + OVA (Lane 5). PCR products were separated by acrylamide gel electrophoresis and stained with ethidium bromide. Lane 1 contains DNA standards at 500, 400, and 300 bp.



**Figure 6.** Lymphocyte proliferation stimulated by HuLL-2 or ConA is inhibited by OvUS. Lymphocytes were cultured for 72 hr in the presence of OvUS and then pulsed for an additional 16 hr with 0.1  $\mu$ Ci [<sup>3</sup>H]-thymidine. Shown are least squares means  $\pm$  SEM for results obtained from six ewes. Both HuLL-2 and ConA increased [<sup>3</sup>H]-thymidine incorporation ( $P = 0.0001$ ). There was no effect of the control protein OVA on incorporation, but an equimolar concentration of OvUS inhibited both IL-2-stimulated ( $P = 0.0057$ ) and ConA-stimulated ( $P = 0.0003$ ) lymphocyte proliferation.

that OvUS inhibits pathways controlled by PKC $\alpha$  or PKC $\theta$  while not affecting PKC $\beta$ , PKC $\delta$ , or PKC $\epsilon$ . Further experiments using specific agonists and antagonists of the individual PKC isoenzymes are necessary to determine which isoenzymes are able to induce lymphocyte proliferation in sheep PBML and which isoenzyme-stimulated events are inhibited by OvUS.

Ovine US also inhibited the proliferation of lymphocytes in response to HuLL-2. Signal transduction of the IL-2 receptor is mediated by several enzymes including tyrosine phosphatases, protein kinase A, and members of the protein kinase C family. Administration of antisense RNA to PKC $\zeta$ , PKC $\epsilon$ , and PKC $\beta$  inhibited proliferation of a T-cell clone in response to IL-2 (29). It is possible that OvUS inhibits one or more of these enzymes or down-stream events controlled by these enzymes. Alternatively, inhibition of the upregulation of CD25 by OvUS caused reduced responsiveness to HuLL-2.

The failure of OvUS to inhibit ConA-induced expression of CD25 on  $\gamma\delta$ -T cells was not unexpected. The  $\gamma\delta$ -T cells comprise a major subpopulation of leukocytes in the uterus and appear to become activated during pregnancy despite the high concentrations of OvUS in the uterus. In particular, there is an increase in numbers of  $\gamma\delta$ -TCR<sup>+</sup> in the endometrial epithelium in mid- to late pregnancy (30). Unlike  $\gamma\delta$ -T cells from nonpregnant uteri,  $\gamma\delta$ -T cells from pregnant uteri contained large cytoplasmic granules and had increased metabolic activity (30). In unilaterally pregnant ewes,  $\gamma\delta$ -T cells in the gravid uterine horn contained increased expression of the activation markers CD25, CD44, CD29, and Mel-14 compared with the nongravid horn. Failure of OvUS to inhibit activation of  $\gamma\delta$ -T cells may result from a different mechanism of activation than what is used

for  $\alpha\beta$ -T cells. That there are different activation requirements is indicated by findings that ovine  $\gamma\delta$ -TCR<sup>+</sup> cells express CD25 earlier following mitogen activation than  $\gamma\delta$ -TCR<sup>-</sup> cells (31–33).

In summary, OvUS inhibits lymphocyte activation by inhibiting protein kinase C or protein kinase C-mediated events. This inhibition in turn is probably responsible for decreased CD25 expression and reduced responsiveness to IL-2. The particular PKC isoenzymes affected by OvUS require further study. Cells of the  $\gamma\delta$ -TCR<sup>+</sup> lymphocytes appear to be refractory to the effects of OvUS, which may reflect a different mechanism of activation.

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