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Lymphocyte-mediated lysis of sheep chorion: susceptibility of chorionic cells to third-party and maternal cytotoxic lymphocytes and presence of cells in the endometrium exhibiting cytotoxicity toward natural-killer cell targets

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Abstract

In several species, the trophoblast is resistant to lysis by cytotoxic lymphocytes. Such resistance is believed to contribute to survival of the semiallogenic conceptus. We tested whether ovine chorionic cells are susceptible to lysis by specific and nonspecific cytotoxic lymphocytes in peripheral blood (PBL) and whether cytotoxic cells that can lyse target cells for natural-killer cells are present in the endometrium. Primary chorionic cells from pregnant ewes at Days 51–91 of gestation were labeled with ⁵¹Cr and incubated for 20 h at 50:1 and 100:1 ratios with PBL from the pregnant mother or from a third-party ewe. In the absence of interleukin-2 (IL-2), there was no killing of primary chorionic cells by third-party PBL even after infection of chorionic cells with bovine herpes virus-1. Incubation with IL-2-induced cytotoxic action in third-party PBL towards one of six primary chorionic cell preparations only. Primary chorionic cells from two of four placentae were lysed by maternal PBL. Luminal epithelial cells from cyclic ewes and from the pregnant and nonpregnant uterine horns of unilaterally-pregnant ewes were evaluated for the presence of cells capable of killing D17 target cells (a natural-killer cell target). Killing was observed but there was no difference in activity between physiological stages. In contrast, there was intense immunochemical localization of perforin in glandular and luminal endometrial epithelial cells in pregnant ewes, and less intense staining in nonpregnant animals. It is concluded that ovine chorionic cells are generally resistant to killing by natural-killer-like cells and lymphokine-activated killer cells. Generation

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of maternal cytotoxic lymphocytes against trophoblast can occur in some cases and may contribute to pregnancy loss.

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1. Introduction

In allogenic pregnancies, the conceptus is potentially susceptible to destruction by immune cells of the mother that recognize paternally-inherited antigens on the surface of the placenta. While cytotoxic T lymphocytes specific for trophoblast can be generated *in vitro* under certain conditions [1–3], there is little evidence for specific cell-mediated immunity against the placenta in pregnant females [4,5]. Indeed, murine trophoblast can be successfully transplanted across strains without immunological rejection [6,7]. One reason cytotoxic lymphocytes may not be generated against trophoblast is that this tissue has altered expression of major histocompatibility antigens (MHCs). In the human [8] and sheep [9], for example, classic MHC class I and II antigens are not expressed on trophoblast. The absence of these molecules makes trophoblast cells potentially susceptible to lysis by natural-killer (NK) cells which lyse cells without the presence of MHC class-I molecules [10]. However, trophoblast cells from both mice [11,12] and humans [13–15] are resistant to NK cell lysis, although interleukin-2 (IL-2) can induce killing mediated by lymphokine-activated killer cells (LAKs) [12,14,16]. In contrast, the pre-implantation pig trophoblast is susceptible to NK lysis [17].

In ruminants, little is known about the interaction between the conceptus and cytotoxic maternal cells. In early pregnancy, sheep NK and LAK cells can be cytotoxic towards preattachment ovine conceptuses [18]. There is a large increase in numbers of $\gamma\delta$ granulated T lymphocytes in the luminal epithelium at mid to late gestation [19]. These cells may be cytotoxic because they are CD8⁺, granulated, and contain mRNA for perforin and cytokines such as interferon (IFN)- γ and tumor necrosis factor- α [20–22].

In the present study, we tested whether primary cells prepared from the ovine chorion are susceptible to cytotoxic lymphocytes in peripheral blood (PBL) and whether cells capable of nonspecific killing of a NK target, the D17 cell [23], are present in the endometrium of the sheep.

2. Materials and methods

2.1. Materials

Control mouse ascites fluid (clone NS1) was obtained from Sigma (St. Louis, MO). Hybridoma cells producing monoclonal antibody against human perforin (clone 2d4) were a gift from Dr. G. Griffiths (Oxford University, UK). Antibody was obtained as culture supernatant from hybridoma cell cultures prepared by the Hybridoma Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research.

Tissue culture medium-199 (M-199), Eagle's minimal essential medium (MEM), glutamine, bovine serum albumin (BSA), penicillin–streptomycin, red cell lysis buffer, β -mercaptoethanol, trypsin–EDTA and Triton X-100 were obtained from Sigma. Prostaglandin $F_{2\alpha}$ was purchased from Upjohn-Pharmacia (Kalamazoo, MI). Human recombinant IL-2 was from Genzyme (Boston, MA). Fico-Lite-1077, normal goat serum and fetal calf serum were from Atlanta Biologicals (Norcross, GA). Horse serum was from Hyclone (Logan, UT). Fetal bovine serum was from Intergen (Purchase, NY). $Na^{51}CrO_3$ (specific activity: 571 mCi/mg Cr) was from ICN (Irvine, CA). The D17 cell line (canine osteocarcinoma) and bovine herpes virus-1 (BHV-1) (tissue culture infectious dose (TCID) for 0.2 ml bovine turbinate cells, 10-day culture; $TCID_{50} = 10^{6.5}$) were from American Type Culture Collection (Rockville, MD). The T75 cell culture flasks were from Sarstedt (Newton, NC), 96-well Falcon culture plates and cell strainers (100 μ m) were from Becton Dickinson (Franklin Lakes, NJ) and glass chamber slides were from Nalgene (Nunc, Naperville, IL). H_2O_2 , paraformaldehyde, 12 mm \times 75 mm polyethylene culture tubes and glass microscope slides were from Fisher Scientific (Pittsburgh, PA). The HistoScan Monoclonal Detector kit for performing immunohistochemistry was obtained from Biomedica (Foster City, CA). Embedding medium (Tissue-Tek OCT Compound) was obtained from Miles Diagnostic (Elkhart, IN).

2.2. Experimental design for lysis of chorionic cells

Pregnancies were established in adult cycling ewes of predominantly Rambouillet genotype ($n = 7$). Estrous cycles were synchronized by two treatments i.m., of 2 mg prostaglandin $F_{2\alpha}$ at 11-day intervals. Ewes were bred at estrus to fertile Rambouillet rams to establish pregnancy. Ewes were slaughtered by captive-bolt stunning between Days 51 and 91 of gestation and the placenta recovered. Chorionic cells, prepared as described below, were labeled with ^{51}Cr and used as target cells in a chromium release assay using PBL from the pregnant mother (maternal cells) and from third-party ewes. Third-party ewes included pregnant ewes ($n = 3$) and cyclic ewes ($n = 7$). All animals were multiparous. Each preparation of chorionic cells was tested separately using PBL from one or two third-party ewes. As a control, each preparation of PBL was also evaluated for ability to lyse D17 cells, which are a target for lysis by NK-like cells. For both chorionic cells and D17 cells, the killing assay was performed with unstimulated PBL and PBL cultured with IL-2, and with untreated target cells and target cells infected with BHV-1.

2.3. Experimental design to evaluate presence of NK-like cells in endometrial epithelium

Multiparous ewes that were predominately of Rambouillet breeding ($n = 7$) were surgically made unilaterally pregnant to limit pregnancy to only one horn as described elsewhere [24]. Briefly, one uterine horn was ligated surgically and the ovary ipsilateral to the ligated horn was also removed. After >30 days of recovery, estrous cycles were synchronized as described above and ewes were bred at estrus to fertile rams to establish pregnancy. Ewes were slaughtered by captive-bolt stunning at Day 140 of gestation and the pregnant uterus recovered for the collection of endometrial epithelium as described afterwards. Endometrial epithelium was also obtained from five cyclic ewes slaughtered

at random days of the estrous cycle. Each preparation of endometrial epithelial cells was tested separately for NK-like lysis against BHV-1-infected D17 cells.

2.4. Preparation of effector cells for ^{51}Cr release assay

2.4.1. PBL

Peripheral blood lymphocytes were isolated from jugular blood. Briefly, peripheral blood mononuclear cells were purified by density gradient centrifugation on Fico-Lite-1077 at $450 \times g$ for 30 min. The cell pellet was resuspended with 4 ml red cell lysis buffer, mixed with a Pasteur pipette for ~ 20 s to remove erythrocytes, and diluted with 4 ml double-strength Dulbecco's phosphate buffered saline (DPBS). Cells were washed twice with 2 ml modified M-199 by centrifugation at $110 \times g$ for 5 min, and resuspended with 2 ml modified M-199 (Tissue Culture Medium-199 containing 5% (v/v) horse serum, 200 U/ml penicillin, 0.2 mg/ml streptomycin, 2 mM supplemental glutamine, and 10^{-5} M β -mercaptoethanol).

For the experiment with chorionic cells, maternal PBL isolated on the day of slaughter were incubated in M-199 in modified T75 flasks at 37°C and 5% CO_2 until after the trophoblast cells were cultured with BHV-1. Third-party PBL were collected either on the same day that maternal blood was collected and were processed similarly, or were collected and processed as described above on the next day.

2.4.2. Endometrial epithelial cells

Uterine horns were longitudinally opened along the anti-mesometrial border. Luminal epithelium (which contains a mix of epithelial cells and lymphocytes) [21] was removed from intercaruncular areas of the endometrium by mechanically scraping the inner surface of the endometrium with a sterile surgical blade. Cell scrapings were collected into a 50 ml sterile culture tube containing 5 ml M-199 supplemented with 2 mM EDTA. Cells were incubated at 37°C for 1 h. Cells in suspension were then triturated vigorously for 5 min to break cell clumps, filtered through a sterile $100\ \mu\text{m}$ cell strainer into 50 ml sterile culture tubes and centrifuged at $110 \times g$ for 5 min. The cell pellet was resuspended with 5 ml modified M-199 and cell number was determined using a hemacytometer.

2.5. Preparation of target cells for ^{51}Cr release assay

2.5.1. Chorionic cells

After slaughter, the uterus was opened longitudinally along the anti-mesometrial border to expose chorionic membranes. Using a sterile forceps, the interplacental chorionic membrane layer was separated from the allantois. Small pieces (0.5–2 cm) of chorion collected in this manner were placed into cell culture dishes containing modified M-199 that was also supplemented with 2 mM EDTA, shaken to remove blood, and then placed into new culture dishes containing modified M-199 + 2 mM EDTA. The chorionic tissue was disassociated by scraping with a scalpel blade to produce a mixture of suspended cells and small pieces of tissue. Cells collected in this manner were incubated at 37°C and 5% CO_2 for approximately 1 h. The cells were triturated through a Pasteur pipette and then through an 18 gauge needle. The cell suspension was then filtered through a cell strainer

(100 µm) to remove tissue clumps. Cells were centrifuged at $110 \times g$ for 5 min. The supernatant was discarded and the pellet resuspended with 4 ml of red cell lysis buffer, mixed with a Pasteur pipette for about 20 s to remove erythrocytes, and diluted with 4 ml of double strength DPBS. Cells were centrifuged at $110 \times g$ for 5 min, resuspended with modified M-199, counted and adjusted to 1×10^5 cell/ml in modified M-199.

2.6. ^{51}Cr release assay

A 1 ml aliquot of 1×10^6 target cells (D17 cells or chorionic cells) was centrifuged at $110 \times g$ for 5 min, resuspended with 100 µl modified MEM and 100 µCi $\text{Na}^{51}\text{CrO}_3$ and incubated for 1 h at 37 °C and 5% CO_2 . The labeled cells were washed twice with medium, resuspended to 1×10^5 cells/ml and 100 µl of cells pipetted into individual wells of a 96-well, flat-bottomed culture plate. Twenty microliters of the working BHV-1 solution (a 1:10 dilution of the original BHV-1 solution with modified MEM) was added to half of the wells. Plates were incubated overnight at 37 °C and 5% CO_2 . Cells were then washed twice with 100 µl modified MEM by centrifugation at $80 \times g$ for 3 min and resuspended with 50 µl modified MEM. An aliquot of 100 µl effector cells (1×10^6 , 5×10^5 or 2.5×10^5 PBL or endometrial epithelial cells in modified M-199) were pipetted into wells containing 1×10^4 ^{51}Cr labeled target cells (50 µl) in a flat-bottom, 96-well plate to produce effector:target cell ratios of 100:1 and 50:1. In some experiments, wells also contained either human recombinant IL-2 in DPBS (5 µl to produce a final concentration of 500 U/ml) or an equivalent volume of DPBS as a control. Modified M-199 (45 µl) was added into each well to a final volume of 200 µl. Wells to measure spontaneous release contained 100 µl target cells and 100 µl modified MEM or M-199 only, while wells to measure maximum release contained 100 µl target cells and 100 µl 2% (v/v) Triton X-100.

Plates were centrifuged at $80 \times g$ for 3 min to facilitate contact between effector and target cells before incubation at 37 °C and 5% CO_2 for 20 h. The assay was terminated by centrifugation of plates at $350 \times g$ for 15 min at room temperature. A 100 µl aliquot of each supernatant was removed and counted for radioactivity using a gamma counter (Cobra Auto-Gamma, Packard Instrument Inc. Downers Grove, IL). Results of triplicate wells were averaged and expressed as percent lysis using the formula:

$$\text{Percent lysis} = \left[\frac{\text{sample DPM} - \text{spontaneous DPM}}{\text{maximum DPM} - \text{spontaneous DPM}} \right] \times 100.$$

2.7. Immunohistochemical localization of perforin

Samples of uterine endometrium from unilaterally-pregnant ewes ($n = 6$), ovariectomized ewes ($n = 4$) and a cyclic ewe ($n = 1$) were snap-frozen in Tissue-Tek OCT embedding compound. Tissues from each group were processed in parallel to avoid confounding physiological stage with procedural replicate. Tissue was prepared for immunohistochemistry by preparing 6 µm sections with a cryostat microtome. Sections were placed onto precleaned glass slides, fixed with four to five drops 95% ethanol, and subjected to immunohistochemical staining. Procedures for immunohistochemistry were carried out according to manufacturer's instructions. All steps were performed at room

temperature and cells were washed with staining buffer (10 mM NaPO₄, pH 7.4 containing 0.9% (w/v) NaCl (PBS) supplemented with 2% (v/v) normal goat serum) between all steps. Briefly, cells were sequentially incubated with blocking buffer (PBS containing 2% (v/v) normal goat serum, 0.3% (v/v) H₂O₂) and tissue conditioner supplied in the kit before incubation with primary antibody for 6 h. Primary antibodies used were a 1:500 dilution of anti-perforin in staining buffer or a 1:500 dilution of control mouse ascites fluid as a negative control. Slides were then sequentially incubated with secondary antibody (biotinylated anti-mouse immunoglobulin supplied in the kit) for 30 min, streptavidin-alkaline phosphatase reagent (from kit) for 30 min, and 3-amino, 9-ethylcarbazole chromogen reagent for 10 min. Slides were washed under tap water, cover slips mounted, and slides examined for staining using light microscopy (Alphaphot, Nikon, Japan).

2.8. Statistical analysis

Data were analyzed by least square analysis of variance using the General Linear Models Procedure of the Statistical Analysis System [25]. To determine effects of PBL type (maternal versus third-party) on killing of chorionic cells, two analyses were performed. The first included all data. The statistical model included effects of PBL type, chorion donor, treatment (control, IL-2 and virus), ratio and all two- and three-way interactions. The second analysis was similar except only data from untreated PBL and chorionic cells without virus were analyzed. In addition, subsets of data for third-party PBL only and for maternal PBL only were analyzed using effects of chorion donor, treatment (control, IL-2 and virus), ratio and all two-way interactions. In initial assays of data from third-party PBL, the effect of pregnancy status of the PBL donor was found to be not significant and this variable was not included in subsequent analyses. For analysis of data for D17 lysis by PBL, the mathematical model included main effects of PBL donor (i.e. ewe), pregnancy status of the PBL donor, IL-2 (+ or -), virus (+ or -), ratio, and all possible interactions. Data on lysis by endometrial epithelial cell preparations was analyzed using several models. Differences between groups in lytic activity of endometrial epithelial preparations were analyzed using a mathematical model that included effects of source of endometrium (pregnant horn–pregnant uterus, nonpregnant horn–pregnant uterus, and cyclic uterus), ewe nested within source, ratio and all interactions. In addition, another model was performed in which data from both horns of the pregnant ewes were compared to data from cyclic ewes. A smaller subset of data from pregnant ewes only was also analyzed to compare pregnant and nonpregnant uterine horns.

3. Results

3.1. Lysis of chorionic cells by lymphocytes from third-party donors

Results are shown in Fig. 1. In the absence of cytokine stimulation, chorionic cells were generally not lysed by PBL from third-party donors. Of the six chorionic cell preparations tested, there was no lysis in five cases and only very low lysis ($3.2 \pm 0.93\%$) in the sixth case. Stated differently, of the 10 third-party PBL preparations (3 pregnant and

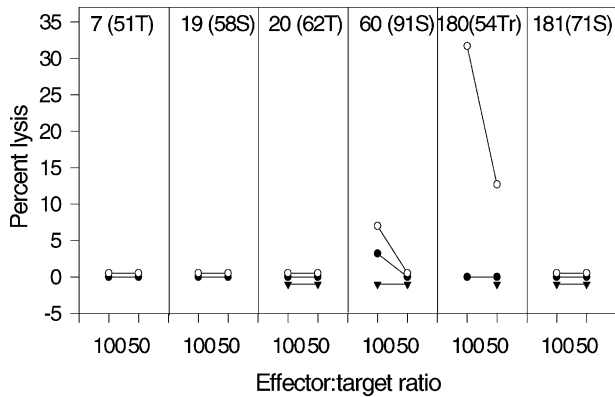


Fig. 1. Lysis of chorionic cells by third-party peripheral blood lymphocytes (PBLs). Chorionic cells prepared from six separate pregnant ewes were subjected to a 20 h ^{51}Cr -release assay using PBL from third-party ewes at effector:target ratios of 50:1 and 100:1. The numbers at the top of each graph are the identification number for the chorionic-cell donor, the day of gestation at which chorion was collected, and type of pregnancy; singleton (S), twin (T) or triplet (Tr). Each chorionic cell preparation was tested in triplicate for lysis using PBL preparations from one (chorion from #60 and #180) or two ewes. Solid circles represent results for untreated PBL, open circles represent results for IL-2-stimulated PBL and solid triangles represent BHV-1-treated chorionic cells. Data represent values averaged across all assays. Percent lysis was affected by donor ($P < 0.001$), IL-2 ($P < 0.001$) and virus \times ratio ($P < 0.02$).

7 cyclic ewes), only 1 (a cyclic ewe) showed any lysis towards chorion. There was no significant difference in lytic activity between pregnant and cyclic PBL.

Chorionic cells remained resistant to lysis even when preincubated overnight with BHV-1 virus. Treatment of PBL with IL-2-induced large LAK-like activity towards one preparation of chorionic cells only (control versus IL-2 at 100:1 = 0% versus 31.7%).

While third-party PBL did not generally lyse chorionic cells, all preparations of PBL used for lysis of chorionic cells possessed cytotoxic activity against D17 cells, a target for ovine NK cells. Results for a subset of PBL donors in which effects of BHV-1 virus and IL-2 were evaluated are shown in Fig. 2. Lysis of D17 cells was increased by incubation of D17 cells with BHV-1 virus, especially at effector:target ratios of 100:1 (virus \times ratio; $P < 0.02$), and by incubation of PBL with IL-2 ($P < 0.001$). The magnitude of lysis of D17 cells was similar for PBL from pregnant and cyclic ewes (results not shown).

3.2. Lysis of chorionic cells by maternal lymphocytes

While third-party PBL were generally unable to lyse chorionic cells, trophoblast cells from two of four preparations tested were lysed by maternal PBL (Fig. 3). There was no significant difference in the degree of lysis between 100:1 and 50:1 effector:target ratios; lysis was numerically greater at 50:1. Treatment with IL-2 increased lysis further for one ewe only (donor \times treatment, $P = 0.05$). When chorionic cells were incubated with BHV-1 virus, there was no increase in lysis for either of the two chorionic cell preparations tested.

The proportion of PBL donors that lysed chorionic cells tended ($P < 0.10$) to be greater for maternal cells (2/4) than for third-party cells (1/10). When comparing results from maternal

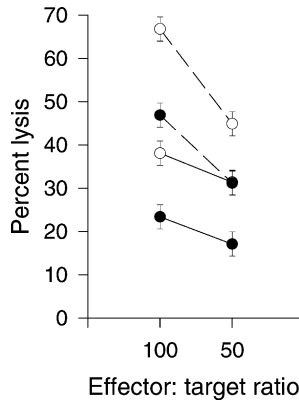


Fig. 2. Lysis of D17 cells by peripheral blood lymphocytes. Effector cells were the same preparations used for chorionic cell lysis (Fig. 1). Data are the least-squares means \pm S.E.M. of the results from six donors. Solid lines represent results for control target cells and dashed lines represent results for BHV-1-infected cells. Solid circles represent results for untreated PBL while open circles represent results for IL-2-stimulated PBL. Lysis was affected by IL-2 ($P < 0.001$), ratio ($P < 0.001$) and virus \times ratio ($P < 0.02$).

effectors with results from third-party effectors (Fig. 1), analysis of variance indicated that lysis was greater for maternal cells (PBL type; $P = 0.07$). There were also effects of PBL type \times donor ($P < 0.0001$), and PBL type \times treatment ($P < 0.04$). When the dataset was restricted to unstimulated cells only (i.e. after excluding IL-2 and virus treatments), there

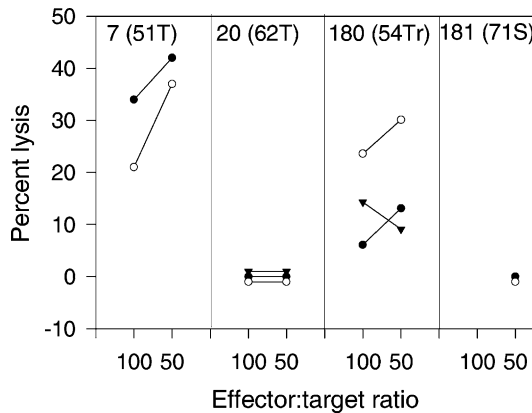


Fig. 3. Lysis of primary cultures of chorionic cells by maternal peripheral blood lymphocytes (PBL). Chorionic cells prepared from four separate pregnant ewes were subjected to a 20 h ^{51}Cr -release assay using PBL from the same pregnant ewe at effector:target ratios of 50:1 and 100:1. The numbers at the top of each graph are the identification number for the chorionic-cell donor, the day of gestation at which chorion was collected, and type of pregnancy; singleton (S), twin (T) or triplet (Tr). Solid circles represent results for untreated PBL, open circles represent results for IL-2-stimulated PBL and solid triangles represent BHV-1-treated chorionic cells. Data represent the means of triplicate determinations. Percent lysis was affected by donor ($P < 0.01$), donor \times treatment ($P = 0.05$) and treatment \times ratio ($P < 0.05$).

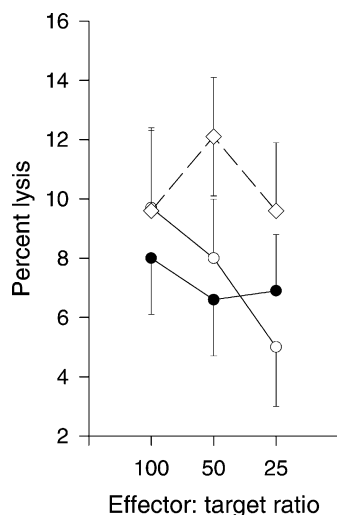


Fig. 4. Lysis of BHV-1-infected D17 cells by cells in preparations of endometrial luminal epithelium. Data are the least-squares means \pm S.E.M. of the results from unilaterally-pregnant and cyclic ewes. Filled circles represent results from the pregnant uterine horn of unilaterally-pregnant ewes ($n = 7$), open circles represent results from the nonpregnant uterine horn of the unilaterally-pregnant ewes ($n = 6$) and open diamonds represent results from cyclic ewes ($n = 5$). There were no significant differences between pregnant and nonpregnant ewes or between the two uterine horns of the unilaterally-pregnant ewes.

were effects of PBL type ($P < 0.0001$), PBL type \times donor ($P < 0.0001$), and PBL type \times ratio ($P < 0.02$).

3.3. Presence of NK-like activity in endometrial epithelial preparations

Preparations of luminal epithelium from the endometrium lysed virus-infected D17 cells (Fig. 4). However, there were no significant differences in the amount of killing between preparations derived from the pregnant or nonpregnant uterine horn of unilaterally-pregnant ewes or between these preparations and epithelial cells from cyclic ewes.

3.4. Immunolocalization of perforin

To further evaluate presence of endometrial cells with characteristics of lytic cells, immunohistochemistry was performed on endometrium using an antibody that recognizes the cytolytic protein perforin (Fig. 5). For tissue from both pregnant and nonpregnant horns of unilaterally-pregnant ewes, the antibody reacted very strongly with glandular epithelium, less intensely with luminal epithelium and, in some animals, weakly to the stratum compactum region of the stroma. Immunoreaction product was located throughout the epithelium rather than being limited to specific, isolated cells. There was no difference in intensity of reaction product between tissues from pregnant and nonpregnant uterine horns of unilaterally-pregnant ewes. Immunoreaction product was also seen in tissue from

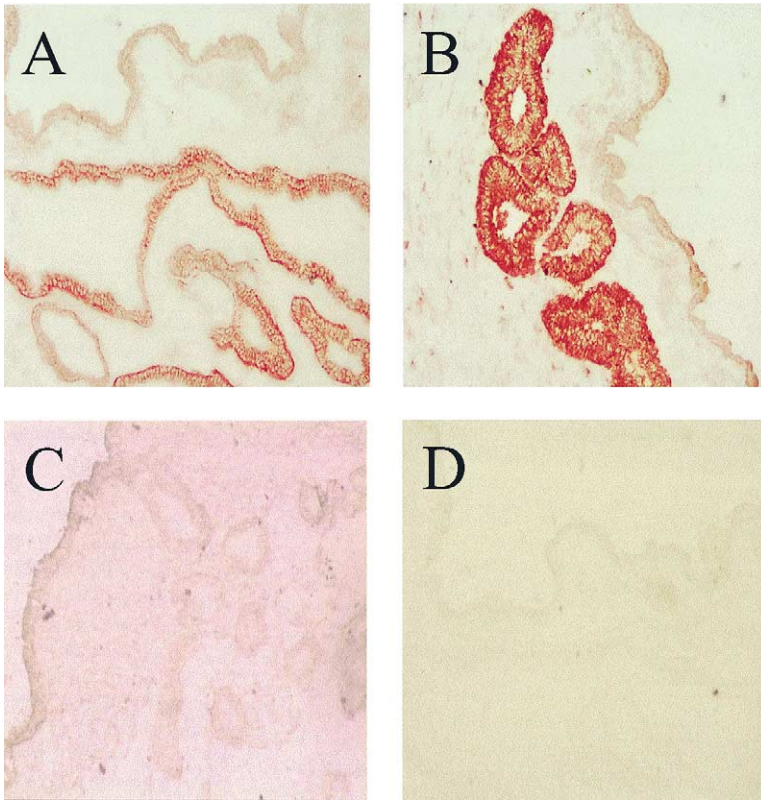


Fig. 5. Immunohistochemical localization of perforin in endometrium using streptavidin-alkaline phosphatase reagent and 3-amino, 9-ethylcarbazole as substrate. Represented are tissues from the pregnant (Panel A) and nonpregnant (Panel B) horns of unilaterally-pregnant ewes and from a cyclic ewe (Panel C). The photomicrograph in Panel D is of a negative control for perforin immunoreactivity using the same tissue block as panel B and in which control mouse ascites fluid replaced antiperforin.

ovariectomized ewes and the cyclic ewe. However, the intensity of staining was reduced compared to samples from pregnant ewes (compare Fig. 5A and B with C).

4. Discussion

Results indicate that chorionic placental cells from sheep are generally resistant to lysis by NK and LAK-like cells. However, the present data, with limited numbers of ewes, provide some evidence for the spontaneous generation of maternal cytotoxic lymphocytes against chorionic cells during pregnancy. These findings illustrate the success at which the chorion can ordinarily evade cytotoxic responses of NK or LAK-like cells, and the potential and occasional susceptibility of the chorion to specific maternal cytotoxic immune responses.

The lack of expression of classic MHC class-I antigens on sheep chorioallantois [9] would be expected to make these cells more susceptible to lysis by NK cells. However, as

has been found in mouse [11,12] and human [13,14] trophoblast, preparations of chorionic cells were generally resistant to lysis by NK cells. The fact that BHV-1 infection did not increase susceptibility of chorionic cells to NK cells probably reflects the fact that MHC class-I molecules are down-regulated even in the absence of virus. In addition, infected chorionic cells may not experience increased secretion of IFN- α or IFN- β to modulate NK cell cytotoxic responses [26].

In contrast to the mouse [12] and human [14,16], where IL-2 increased lysis of trophoblast cells by lymphocytes, IL-2 did not generally augment lysis of chorionic cells by sheep third-party PBL. Perhaps longer preincubation times of PBL with IL-2 would have been more effective in inducing LAK cells. However, the incubation time used was sufficient to increase killing of D17 cells by PBL. Failure of chorionic cells to be killed by NK or LAK cells is not because of the absence of these cytotoxic cells in the sheep, because PBL from third-party ewes that did not kill chorionic cells did kill D17 cells in a virus- and IL-2-dependent manner.

Taken together, results suggest that despite decreased MHC class-I expression, the sheep chorion possesses mechanisms to prevent destruction of chorionic cells from lysis by NK cells. Such mechanisms could include membrane-bound inhibitory receptors or release of soluble inhibitors of NK- or LAK-cell function. In the human, human leukocyte antigen-G has been proposed to function as a trophoblast ligand for NK-cell inhibitory receptors [15] although the proposed role for this nonclassic MHC class-I molecule is controversial [14]. Consistent with the idea that the chorion may produce soluble inhibitory molecules is the observation that sheep chorioallantois secretes molecules that inhibit lymphocyte proliferation [27].

In contrast to our results, tissue from peri-implantation sheep conceptuses was lysed by NK cells and prolonged incubation (5 days) with IL-2 generated LAK activity capable of further lysis [18]. While the different culture times (1 day versus 5 days) with IL-2 may explain some of these differences, it is probable that the peri-implantation conceptus may be more susceptible to lysis by cytotoxic cells. In the pig, too, trophoblast tissue from peri-implantation conceptuses is susceptible to lysis by NK cells [17]. It is likely that placental cells exhibit distinct characteristics at different stages of development that affect their susceptibility to lysis by maternal lymphocytes. For example, trophoblast tissue from preattachment conceptuses might not express inhibitory molecules that suppress cytotoxic cells. The peri-implantation ruminant conceptus may be particularly sensitive to lysis by NK cells because the trophoblast at this stage secretes IFN- τ which can activate NK cells [28].

Given the potential susceptibility of the placenta to NK cells and LAK cells, it is of interest to determine whether such cells exist in endometrium. Such is the case for the mouse [29], human [30] and pig [17]. However, identification of NK cells in the endometrium of sheep has been made difficult by the lack of antibodies against NK cells that react with sheep cells. In the present study, we evaluated whether cells capable of lysing NK-cell target cells are present in the luminal epithelium of the endometrium. This compartment of the endometrium was examined because endometrial lymphocytes in the ewe are almost exclusively located in the epithelium or immediately adjacent to it [9,19,21]. The observation that lytic activity towards virus-infected D17 cells was observed in preparations of endometrial luminal epithelium indicates that cells capable of non-specific killing do indeed exist in the endometrium.

The endometrial cell type responsible for this nonspecific killing is unclear. One possibility is that these cells are $\gamma\delta$ -T cells since, during pregnancy, endometrial $\gamma\delta$ -T cells contain perforin mRNA [22]. However, the number of $\gamma\delta$ -T cells increases in the luminal epithelium during middle and late pregnancy [19,21,31] while, in the present study, there was no difference in lytic activity between epithelial preparations of pregnant versus nonpregnant ewes. Another possibility is that a fraction of the epithelial cells themselves can lyse D17 cells. This idea is based on the identification of immunoreactive perforin throughout the epithelium of the endometrium, particularly in pregnant ewes and in the glands. Here again, however, the increased expression of perforin in the endometrium during pregnancy is in contrast to the lack of difference in cytotoxic activity of epithelial cell preparations from cyclic versus pregnant ewes.

While killing of chorionic cells by nonspecific cytotoxic cells was rare in the present study, two of four chorionic cell preparations were lysed by maternal PBL. Such a result suggests it is possible for generation of maternal cytotoxic lymphocytes against chorionic cells. It is not clear if these maternal cells were reactive against a paternally-inherited antigen. For one chorionic cell preparation (from ewe 7), maternal cytotoxic lymphocytes were possibly allospecific since third-party PBL did not lyse chorionic cells. In the other case (chorion from ewe 180), allospecificity is unlikely since chorionic cells were susceptible to lysis by both maternal and third-party PBL. The limited number of animals studied makes it difficult to know how frequently pregnancies occur in sheep where maternal cytotoxic lymphocytes against chorion are generated. In the two most studied species, the mouse and human, it is difficult to generate lymphocyte responses against the trophoblast [2–5]. The mechanism by which antichorion cytotoxic lymphocytes are produced during pregnancy is unknown but could include aberrant expression of MHC class-I antigens, expression of novel antigenic molecules, or lack of expression of molecules that down-regulate the interaction of maternal lymphocytes with the trophoblast. The two chorionic-cell preparations that were lysed by their maternal PBL were from ewes with multiple pregnancies (one twin and one triplet). It is unknown whether multiple pregnancy might be a contributing factor in development of anti-chorion cytotoxic cells. Unexpectedly, killing of chorionic cells by maternal lymphocytes was generally higher at a ratio of 50:1 than at 100:1. One explanation for this observation is that activity of maternal cytotoxic lymphocytes was inhibited by suppressor cells in the PBL preparation and that effectiveness of these suppressor cells was lost when PBL were more dilute.

It is not surprising that not every chorion examined was killed by maternal PBL, particularly if the generation of antichorion cytotoxic cells represents some aberrant event rather than part of the normal course of pregnancy. Given that chorionic cells were susceptible to maternal cytotoxic cells in two of four pregnancies examined, certain forms of pregnancy loss or reductions in fetal growth may reflect cytotoxic responses mounted by the mother against the placenta. Similarly, women with recurrent spontaneous abortion have been reported to have heightened lymphocyte responsiveness to trophoblast antigens [32]. The preliminary data regarding generation of maternal cytotoxic cells developed in this study should lead to further research to identify how widespread this phenomenon is during pregnancy in sheep and whether development of maternal cytotoxic cells against chorion is related to continued maintenance of pregnancy, placental function, and fetal growth.

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