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Pregnancy Failure following Vaginal Infection of Sheep with *Chlamydia psittaci* prior to Breeding

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Enzootic abortion in sheep, caused by *Chlamydia psittaci*, has been associated with pregnancy failure in most sheep-producing countries. Late-term abortions or the birth of weak low-birth-weight lambs occurred following primary *C. psittaci* infection in pregnant ewes. However, the mode by which *C. psittaci* can be transmitted among sheep has not been established. The present study was designed to determine whether the vaginal tracts of nonpregnant ewes were susceptible to *C. psittaci* infection and whether such infections had an impact during the next pregnancy. At day 0 of the estrus cycle, the vaginal tracts of 10 nonpregnant ewes were inoculated with *C. psittaci* and 10 ewes were exposed by subcutaneous injection. The ewes were bred 6 weeks postinfection. Five ewes from the vaginally infected group and four from the subcutaneously infected group were reinfected by subcutaneous injection at day 60 of gestation. Pregnancy outcomes and antibody responses to infection were compared with that of ewes that were infected with *C. psittaci*, either subcutaneously or intravaginally, for the first time during pregnancy and with that of noninfected control ewes. Subcutaneous infection of nonpregnant ewes did not cause subsequent pregnancy failure; rather, this provided protection against abortion following reinfection during pregnancy. As expected, abortions or the birth of weak lambs was observed in those ewes that received primary *C. psittaci* infection by either route during pregnancy. Similarly, abortion or the birth of weak lambs was a consequence of vaginal inoculation prior to breeding, thereby confirming the susceptibility of the vaginal mucosa to infection and demonstrating the potential for venereal transmission.

Chlamydia psittaci, an obligate intracellular pathogen, is the etiologic agent of enzootic abortion in sheep. The infection generally remains inapparent until the animal aborts late in gestation or gives birth to weak or dead lambs (21). Following experimental primary exposure in pregnant ewes, the organism localizes in the placenta, and the resulting damage to the placenta leads to disease (6). Parturition or abortion in such animals is accompanied by the passage of an infected placenta and/or fetal tissues. Therefore, speculation concerning the transmission of the organism to naive ewes has focused on the periparturient period when the lambing environment may become contaminated with diseased tissues (28). Seroconversion has been used to demonstrate that the organism may be transmitted to sheep housed with ewes that were aborting (5, 18). Furthermore, pregnant ewes exposed to aborted material may also abort the current pregnancy. Oral inoculation of nonpregnant ewes with *C. psittaci* resulted in a greater antibody response to the organism than in similar ewes inoculated by intramuscular injection (27). Subsequent pregnancy in the ewes ended in approximately 20% of the ewes from both orally and intramuscularly infected groups aborting their pregnancies or giving birth to weak lambs.

C. psittaci-induced abortion in sheep was recognized to stimulate an immune response that protected against subsequent abortion (15). Recently, we reported that sheep that experienced abortion as a result of experimental infection with *C. psittaci* maintained an elevated systemic antibody response to the organism and that this was associated with a chronic reproductive tract infection (17). The infection was generally undetectable except during the periovulation period of subsequent estrus cycles. Enhanced excretion of chlamydiae from the reproductive tract during estrus may provide for a venereal mode of transmission. Previous experiments that have attempted to mimic venereal transmission by artificially inseminating ewes with ram semen contaminated with infectious *C. psittaci* were equivocal in demonstrating reproductive failure (2, 28). However, experimental infection of tonsillar mucosae resulted in chlamydial abortion in susceptible pregnant sheep (10). Therefore, we hypothesized that the vaginal mucosae could be similarly susceptible to *C. psittaci* infection and that this infection may result in reproductive disease. This hypothesis was examined by vaginally infecting ewes 6 weeks prior to breeding and then monitoring their reproductive success and serum antibody response to *C. psittaci*. Their responses were compared with those of cohort animals experimentally infected by subcutaneous injection with the organism.

MATERIALS AND METHODS

***Chlamydia* culture and purification.** *C. psittaci* isolate V287 was used to experimentally infect sheep. The organism was originally isolated from an aborted ovine fetus in 1988 by inoculation of infected fetal tissue into the yolk sac of 7-day-old embryonated hen eggs (23). Following initial isolation, the organism was grown in HeLa 229 cell (ATCC CCL2.1) monolayers in 175-cm^2 polystyrene culture flasks (Gibco BRL, Burlington, Ontario, Canada) in 50 ml of Eagle's minimum essential medium (EMEM; ICN Flow, Mississauga, Ontario, Canada) supplemented with 10% fetal bovine serum (Gibco BRL), 100 μg of vancomycin hydrochloride per ml (Sigma Chemical Co., St. Louis, Mo.), and 25 U of nystatin per ml (Sigma). Confluent monolayers were infected with *C. psittaci* isolate V287, suspended in sucrose-phosphate-glutamic acid (SPG; pH 7.2), by shaking on a rocking platform at room temperature for 4 h. Fresh EMEM containing 0.5 μg of cycloheximide per ml (Sigma) was added to the cells, and the flasks were incubated at 37°C under 5% CO_2 for 3 to 4 days. Infected monolayers were gently dislodged with a cell scraper (Becton Dickinson, Lincoln Park, N.J.) and centrifuged at 500 × *g* for 10 min at 4°C. The supernatant was collected, and the pellet was resuspended in SPG. Following a brief sonication, the SPG suspension was centrifuged again as described above. The supernatants were pooled and centrifuged at 30,000 × *g* for 30 min at 4°C (50.2 Ti rotor; Beckman Instruments,

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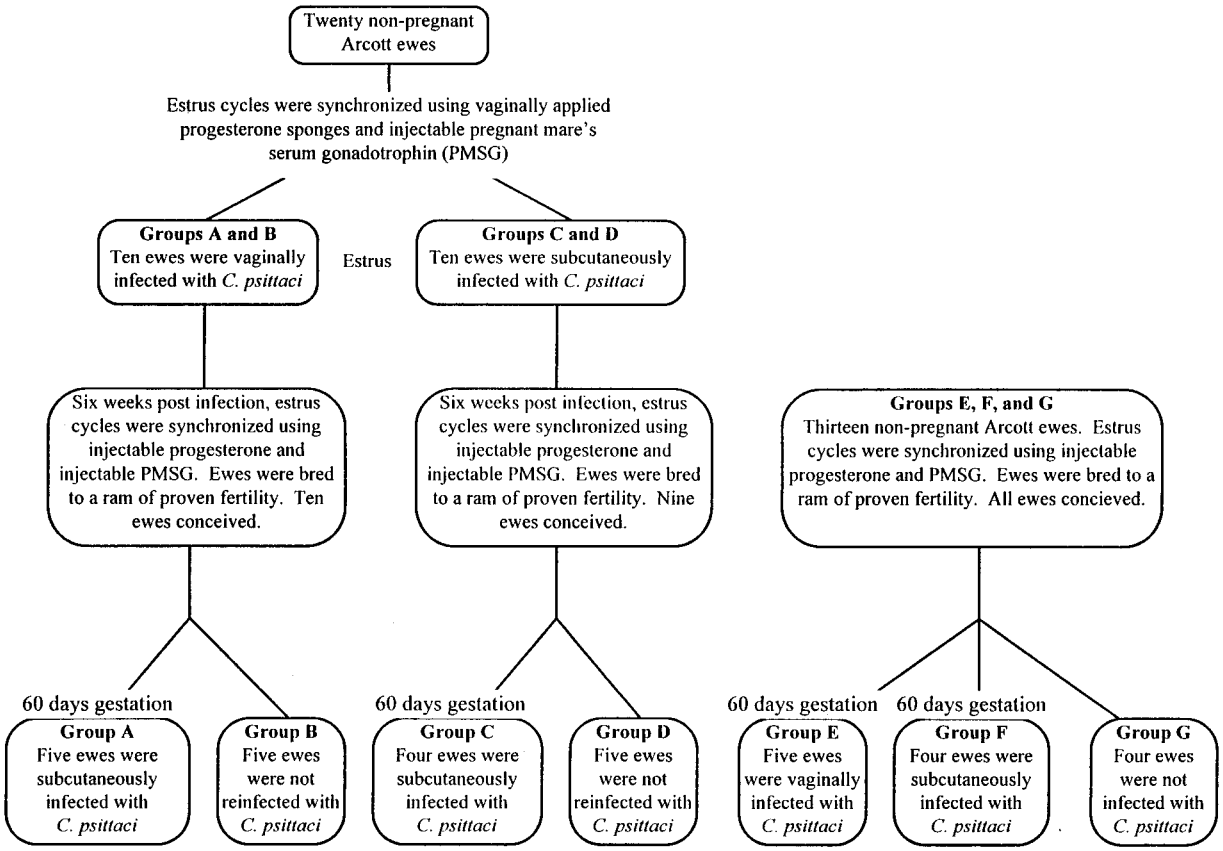

FIG. 1. Experimental design and challenge of sheep with *C. psittaci.*

Inc., Mississauga, Ontario, Canada). The pellets containing infectious chlamydiae were resuspended in SPG to approximately 1/10 the original volume and stored at −135°C. Preparations were confirmed free of viruses, mycoplasmas, or other bacteria by conventional culture (3). The inoculum used to experimentally infect sheep contained an estimated 10^5 50% egg-lethal dose (19).

Chlamydial antigen required for enzyme-linked immunosorbent assay (ELISA) was prepared from L929 cell (ATCC CCL1) monolayers that were grown in EMEM supplemented with 10% fetal bovine serum, 200 μg of streptomycin (Sigma), and 50 μg of gentamicin (Sigma) and inoculated with *C. psittaci* V287 infectious stock. Following 4 days of incubation at 37°C under 5% CO_2, chlamydiae were recovered as described above. The elementary bodies (EBs) were then partially purified by layering over 10 ml of 35% (vol/vol) Hypaque-M (diatrizoate meglumine and diatrizoate sodium, 76% for injection; Sterling Winthrop Inc., Markham, Ontario, Canada) in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma) containing 0.15 M NaCl (Fisher Scientific, Unionville, Ontario, Canada) and centrifuged at 43,000 × *g* for 1 h at 4°C (SW27 rotor; Beckman). The pellet was resuspended in approximately 5 ml of SPG and centrifuged at 30,000 × *g* for 30 min at 4°C. One to 2 ml of SPG was used to resuspend the pellets, and the EB suspension was stored at −135°C. The protein concentration of the partially purified chlamydial EBs (7) was determined by the method of Lowry et al. (12).

Sheep, experimental challenge, and design. Thirty-three 1-year-old Arcott ewes were obtained from a flock free of *C. psittaci*. The sheep were randomly separated into five groups of five animals and two groups of four animals. The groups were housed separately following experimental manipulation (Fig. 1). The estrus cycles of 20 ewes, groups A to D, were synchronized with vaginally applied progesterone sponges (Veramix; Upjohn Company, Orangeville, Ontario, Canada) and injectable pregnant mare's serum gonadotrophin (PMSG [Equinex]; Ayerst Laboratories, Montreal, Quebec, Canada). At day 0 of the estrus cycle, the external os of the vagina was cleaned with 4% chlorhexidine gluconate skin cleanser, and vaginal swabs were collected from each animal. The vaginal swabs were examined for the presence of chlamydia-specific lipopolysaccharide (LPS) with a Clearview Chlamydiae test kit (Unipath, Inc., Nepean, Ontario, Canada). Ten ewes in groups A and B were then vaginally infected by gently placing 1 ml of infectious *C. psittaci* V287 into each vaginal canal with a tuberculin syringe (Becton Dickinson). This procedure was conducted carefully to reduce the risk of abrading the vaginal mucosa. The 10 ewes in groups C and D were injected subcutaneously in the left axilla with 1 ml of infectious *C. psittaci* V287. Twenty-four hours after infection, vaginal swabs were collected aseptically from the ewes and tested for chlamydiae with the Clearview Chlamydiae test kit (Unipath). Rectal temperatures, appetite, and attitude were recorded daily for 5 days preinfection and 10 days postinfection.

Four weeks following infection of groups A to D, the estrus cycles of the ewes were artificially synchronized for a second time but with injectable progesterone, instead of progesterone sponges, followed by PMSG. The sheep received 1 ml of Centra Progestin (Dispar, Joliette, Quebec, Canada) given intramuscularly at 3-day intervals for 9 days followed by four daily intramuscular injections of 0.1 ml of Gesterol in Oil (E. L. Stickley and Co., Brantford, Ontario, Canada). One day after the last progesterone injection, the sheep were given PMSG intramuscularly so that ovulation would typically occur 60 to 70 h later (26). Immediately prior to breeding, vaginal swabs were collected aseptically from the ewes and tested with the Clearview Chlamydiae test kit (Unipath). The ewes were bred to two rams obtained from the *C. psittaci*-free source flock. One ram was mated to ewes in groups A and B, while the other ram was mated to ewes in groups C and D. Pregnancy diagnosis was completed by ultrasound examination at 50 days following ovulation. All of the ewes except for one in group C conceived. The ewe that failed to conceive was excluded from the remainder of the study. When the ewes reached 60 days of gestation, groups A and C were injected subcutaneously in the left axilla with 1 ml of infectious *C. psittaci* V287. At approximately 100 days of gestation, the ewes were moved to a disease containment facility until 1 month following parturition. All of the groups were housed and transported separately throughout the course of the study.

Thirteen ewes in groups E to G were prepared for breeding by synchronization of their estrus cycles with injectable progesterone followed by PMSG approximately 3 months after synchronization of groups A to D. The staggered breeding schedule was necessary so that the animals could be properly accommodated in the disease containment facility following 100 days of gestation. Two additional rams were obtained from the *C. psittaci*-free source flock and mated to these ewes. Pregnancy diagnosis was completed by ultrasound examination at 50 days following ovulation. All of the ewes conceived. At 60 days of gestation, the ewes in group E were vaginally inoculated with *C. psittaci* V287 as described previously. At the same time point, the ewes in group F were inoculated subcutaneously in the left axilla with 1 ml of infectious *C. psittaci* V287. Control inoculum prepared from uninfected HeLa cells was injected subcutaneously into the four ewes in group G. Rectal temperatures, appetite, and attitude were monitored as before.

TABLE 1. Parameters used to assess reproductive efficiency of ewes

Parameter	Numerical score or value
Wt of lamb or fetus at parturition[a]	wt in kg
Gross placental pathology	
0%	1
1–25%	2
26–50%	3
51–75%	4
76–100%	5
Condition of offspring	
Healthy lamb	1
Weak lamb that survived	2
Weak lamb that was euthanized within 24 h postpartum	3
Weak lamb that was euthanized within 1 h postpartum	4
Lamb that was found dead	4
Stillborn lamb	5
Preterm abortion	5

[a] The weight of the lamb or aborted fetus was determined within 8 h of parturition.

All sheep were bled by jugular venipuncture at weekly intervals commencing 1 week prior to primary infection and concluding 6 weeks postpartum. A total of 36 serum samples were collected from groups A to D, and 20 were collected from groups E to G. Sera were tested for anti-*C. psittaci* immunoglobulin G (IgG) antibodies by ELISA. In addition, progesterone levels in serum from nonpregnant ewes was determined with a commercially available progesterone ELISA (Ovusure; Cambridge Veterinary Sciences Ltd., Littleport, Cambs, United Kingdom). The serum progesterone levels were used to determine the number of estrus cycles and the length of each cycle experienced during the interval between primary infection and breeding.

Tissues associated with abortion or parturition were examined for macroscopic lesions, bacterial contamination, and chlamydial antigen with the Clearview Chlamydiae test kit. Vaginal swabs were collected from ewes within 24 h following abortion or parturition and similarly examined for the chlamydial antigen. Each placenta was categorized on the basis of the overall percentage of gross pathology consistent with *C. psittaci* infection. Placental pathology associated with enzootic abortion has been reviewed previously (24). The birth weights and viability of the lambs were also recorded. The criteria used to determine viability was based on the ability of the lambs to suckle. Weak lambs that were unable to suckle received handler assistance for 1 h postpartum or from the time that they were discovered. Lambs that did not improve following handler attention or that were recumbent and unable to stand approximately 24 h postpartum were euthanized by cardiac infusion of sodium pentobarbital (Euthanyl Forte; M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada).

All animal experimentation was in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Guelph's Animal Care Committee.

ELISA for *C. psittaci*. Ovine sera were tested for the presence of chlamydia-specific IgG antibodies by an ELISA. Polystyrene microtitration plates (Gibco BRL) were inoculated with partially purified *C. psittaci* V287 EBs, diluted to 2 μg of protein per 100 μl of phosphate-buffered saline (PBS), and incubated for 4 h at 37°C. The V287 EB antigen did not dry on the microtiter plates during the 4-h incubation period. The plates were washed with block buffer, i.e., PBS containing 0.05% Tween 20 (Fisher Scientific) and 0.5% fish skin gelatin (Fisher Scientific) warmed to 37°C. Uncoated sites in the microtitration plate wells were then blocked by incubation with block buffer for 30 min at 37°C in a humidified incubator. Test sera were diluted in block buffer at a concentration of 1:400 in volumes of 100 μl per well, and the samples were replicated four times on each plate as described by Wright (29). The plates were incubated at 37°C for 2 h in a humidified incubator and then washed three times with block buffer as before. Rabbit anti-sheep IgG (heavy and light chain)-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories, Inc., Bio/Can Scientific, Mississauga, Ontario, Canada) was added at a concentration of 1:5,000, and the plates were incubated for 1 h as before. The plates were washed three times, and color was developed by adding *p*-nitrophenylphosphate (Sigma). The positive control serum was obtained from a ewe experimentally infected with *C. psittaci* and used at a dilution of 1:400. The negative control was serum collected from a noninfected ewe. The optical density (OD) at 405 nm was recorded with a microplate autoreader (Biotechnology Instruments, Winooski, Vt.) when the positive control gave an OD reading of approximately 1. Results were expressed as a ratio of the OD reading of the test sample to the OD reading of the positive control.

Determination of IgM reactivity to *C. psittaci* in the ovine sera was similarly performed except that serum samples were diluted 1/50 prior to testing. The concentration of partially purified *C. psittaci* V287 was increased to 4 μg of protein per 100 μl of PBS and rabbit anti-sheep IgM(μ)-alkaline phosphatase conjugate (Kirkegard & Perry Laboratories, Inc., Gaithersburg, Md.) was used at a concentration of 1:100. The positive control serum was a pool of ovine sera collected approximately 1 week following experimental infection with *C. psittaci* V287. The negative control serum was the same as that for the IgG ELISA, and both sera were used at a dilution of 1/50.

A negative cutoff value for each antibody isotype was determined as the mean plus 3 standard deviations of the average OD readings of serum samples obtained from 79 ewes at the *C. psittaci*-free source flock. Seroconversion was defined as greater than 0.3019 in the IgG ELISA and 0.1549 in the IgM ELISA.

Statistical analysis. The reproductive efficiency of the ewes in the different groups was analyzed using a general linear model procedure (SAS Inc., Cary, N.C.). When significant differences were detected, Duncan's multiple range test was used (22). The parameters considered in the statistical analysis included degree of placental pathology, lamb weight at birth, and lamb viability (Table 1). A P value of <0.05 was considered significant.

Stringency of the *C. psittaci* ELISA was assigned by use of the coefficient of variation between quadruplicate positive control wells on each plate. Data obtained from a plate were used only if the coefficient of variation was less than or equal to 5%.

TABLE 2. Summary of the outcomes of pregnancy following experimental infection with *C. psittaci*[a]

Expt group (no. of ewes)	Summary of expt infection	Ewes negative for infection at parturition			Ewes positive for infection at parturition				
		No. of ewes	Avg length of gestation[b]	No.[c] of lambs	No. of ewes	Avg length of gestation[b]	No.[c] of healthy lambs	No.[c] of stillborn lambs or aborted fetuses	No.[c] of weak lambs
A (5)	Vaginally infected prior to breeding and subcutaneously infected at 60 days of gestation	2	142	2/1	3	140 ± 4	0/0	0/0	0/4
B (5)	Vaginally infected prior to breeding	1	144	1/1	4	142 ± 2	1/1	0/0	4/4
C (4)	Subcutaneously infected prior to breeding and again at 60 days of gestation	4	145 ± 1	6/3	0	NA[d]	NA	NA	NA
D (5)[e]	Subcutaneously infected prior to breeding	4	145 ± 1	2/5	0	NA	NA	NA	NA
E (5)	Vaginally infected at 60 days of gestation	3	141 ± 3	3/3	2	135 ± 6	0/0	2/1	1/1
F (4)	Subcutaneously infected at 60 days of gestation	0	NA	NA	4	132 ± 5	0/0	0/6	4/1
G (4)	Noninfected control ewes	4	145 ± 1	3/5	0	NA	NA	NA	NA

[a] *C. psittaci* infection was determined by the detection of chlamydiae in postpartum vaginal swabs and the presence of placental pathology.
[b] Average gestation in mean number of days ± standard deviation.
[c] Male lambs/female lambs and/or fetuses.
[d] NA, not applicable.
[e] One ewe was euthanized because of pregnancy toxemia following 133 days of gestation. The ewe carried three fetuses that were normally developed and without any evidence of chlamydial infection.

FIG. 2. Photograph of weak *C. psittaci*-infected lamb (left) and healthy noninfected lamb (right) at 2 days of age. The weak lamb was emaciated and depressed.

RESULTS

Experimental infection. Primary subcutaneous infection of ewes with *C. psittaci* V287 resulted in a slight rise in rectal temperature of approximately 2°C for 5 days, mild depression, and anorexia. In contrast, vaginal infection of nonpregnant ewes was not associated with abnormal clinical signs. Serum progesterone levels indicated that *C. psittaci* infection did not alter the course of the 17-day estrus cycle of nonpregnant ewes and that these ewes were bred during their second ovulation postinfection. Reinfection by subcutaneous injection during pregnancy in the five ewes in group A was associated with a slight increase in rectal temperature that lasted for approximately 2 days. There were no abnormal clinical signs associated with the administration of control inoculum to group G sheep.

Chlamydia-specific LPS could not be detected on vaginal swabs collected from the nonpregnant ewes in groups A to D prior to experimental infection. Twenty-four hours following infection, chlamydial antigen was detected on vaginal swabs from the vaginally infected ewes, groups A and B, but not from the subcutaneously infected ewes, groups C and D. Subsequent artificial estrus synchronization was followed by detection of vaginal chlamydiae on swabs from two of five ewes in group A and four of five ewes in group B. There was no detection of chlamydial LPS on swabs from the remaining ewes in groups A and B or from any of the ewes from groups C and D. The ewes that excreted chlamydiae from the reproductive tract subsequently gave birth to weak, infected lambs.

Reproductive efficiency. Experimental vaginal infection of nonpregnant ewes in group B with *C. psittaci* was sufficient to cause placental disease during the next pregnancy (Table 2). Although the disease did not manifest as abortion, four of five ewes gave birth to eight weak, low-birth-weight, chlamydia-infected lambs. The weak lambs were thin, dehydrated, and depressed (Fig. 2). Five of these lambs did not survive. Chlamydial antigen was detected on vaginal swabs obtained from these four ewes postpartum. Placentitis with numerous necrotic cotyledons was the predominant pathologic feature observed in the placentas associated with the weak lambs. These lesions were attributed to *C. psittaci* by gross appearance and antigen detection. Two ewes that gave birth to weak lambs also delivered two noninfected healthy lambs, one from each ewe. The corresponding placentas were not infected with *C. psittaci.*

Decreased lambing performance was observed for the ewes in groups A and E. Three of five ewes in group A that were vaginally infected prior to being bred and reinfected subcutaneously at 60 days gestation gave birth to weak lambs. One ewe in group E, vaginally infected at 60 days gestation, aborted three fetuses near term. Another ewe in group E gave birth to two weak lambs. The postpartum samples collected from these animals were consistent with *C. psittaci* infection. Two ewes in group A and three ewes in group E had healthy lambs without any evidence of *C. psittaci* infection.

Subcutaneous *C. psittaci* infection of ewes before pregnancy, i.e., the ewes in group D, was not associated with subsequent reproductive failure. However, three ewes in group D did exhibit nonchlamydia-related reproductive problems. Following 133 days of gestation, one ewe was found in sternal recumbency with ketonuria. The ewe was diagnosed with pregnancy

TABLE 3. Summary of the statistical comparison of the reproductive efficiency of ewes

Expt group (no. of ewes)	Summary of expt infection	No. of lambs	Avg placental pathology score[a]	Avg lamb and/or fetal score[b] at parturition	Avg lamb and/or fetal wt (kg)[c] at parturition
A (5)	Vaginally infected prior to breeding and subcutaneously infected at 60 days of gestation	7	1.9 ± 1.1A	2.1 ± 1.3D	3.4 ± 0.8G
B (5)	Vaginally infected prior to breeding	12	2.0 ± 0.9A	2.2 ± 1.0D	2.6 ± 0.7H
C (4)	Subcutaneously infected prior to breeding and again at 60 days of gestation	9	1B	1E	4.0 ± 0.7G
D (5)[d]	Subcutaneously infected prior to breeding	7	1B	2.1 ± 1.2D	3.7 ± 1.3G
E (5)	Vaginally infected at 60 days of gestation	11	2.0 ± 1.3A	2.5 ± 1.9D	3.4 ± 1.1G
F (4)	Subcutaneously infected at 60 days of gestation	11	3.0 ± 0.6C	4.0 ± 1.2F	1.8 ± 0.3I
G (4)	Noninfected control ewes	8	1	1E	4.1 ± 0.6G

[a] Each placenta was examined for gross pathology consistent with *C. psittaci* infection and assigned a numerical score as shown in Table 1. Values are average scores ± standard deviations. Scores followed by different letters are different ($P < 0.05$) by a general linear model procedure and Duncan's multiple range test.

[b] The lamb and/or fetal score was calculated from the condition of the offspring within 24 h postpartum and assigned as shown in Table 1. (Lambs that were assigned scores of 2 to 4 plus stillborn lambs may be products of either term or preterm births.) Values are average scores ± standard deviations. Scores followed by different letters are different ($P < 0.05$) by a general linear model procedure and Duncan's multiple range test.

[c] The weight of the lamb or aborted fetus was determined within 8 h of parturition. Values are average weights ± standard deviations. Values followed by different letters are different ($P < 0.05$) by a general linear model procedure and Duncan's multiple range test.

[d] One ewe was euthanized due to pregnancy toxemia following 133 days of gestation. The ewe carried three fetuses that were normally developed and without any evidence of chlamydial infection.

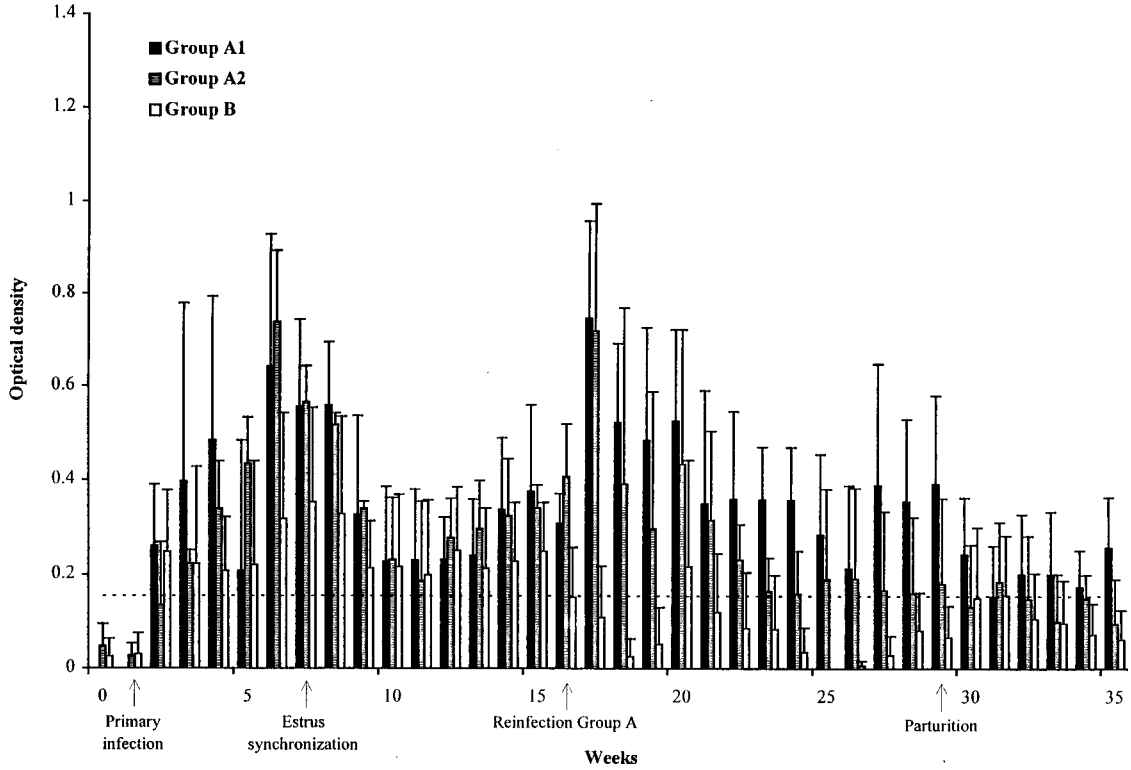

FIG. 3. Mean IgM antibody response to *C. psittaci* ± standard error of the mean. Primary infection of the nonpregnant ewes in groups A and B occurred by vaginal inoculation. The ewes in group A were reinfected with *C. psittaci* by subcutaneous inoculation following 60 days of gestation. The results from the three ewes in group A that gave birth to weak lambs infected with *C. psittaci* are displayed as group A1. The two ewes in group A that gave birth to healthy lambs are represented as group A2. Four ewes in group B that mounted a serum antibody response to *C. psittaci* are represented. The one ewe in group B that did not mount a detectable serum antibody response to *C. psittaci* is not represented. The positive seroconversion value of 0.1549 is represented by a dashed lined.

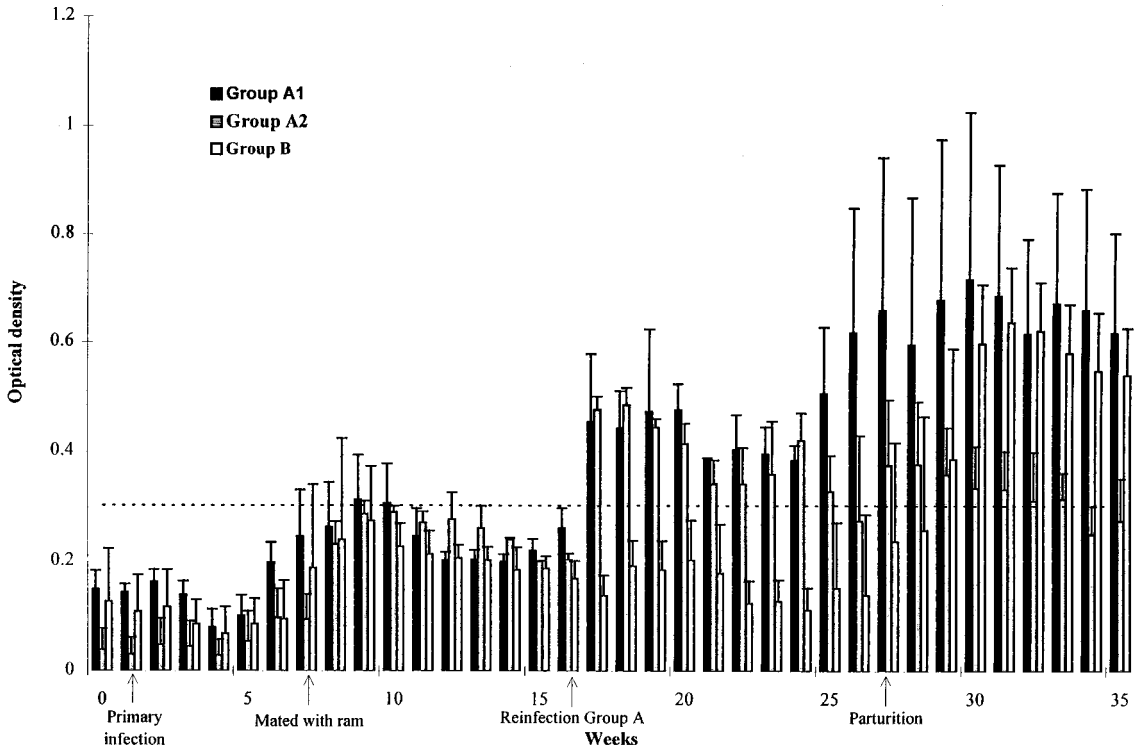

FIG. 4. Mean IgG antibody response to *C. psittaci* ± standard error of the mean. Primary infection of the nonpregnant ewes in groups A and B occurred by vaginal inoculation. The ewes in group A were reinfected with *C. psittaci* by subcutaneous inoculation following 60 days of gestation. The results from the three ewes in group A that gave birth to weak lambs infected with *C. psittaci* are displayed as group A1. The two ewes in group A that gave birth to healthy lambs are represented as group A2. Four ewes in group B that mounted a serum antibody response to *C. psittaci* are represented. The one ewe in group B that did not mount a detectable serum antibody response to *C. psittaci* is not represented. The positive seroconversion value of 0.3019 is represented by a dashed lined.

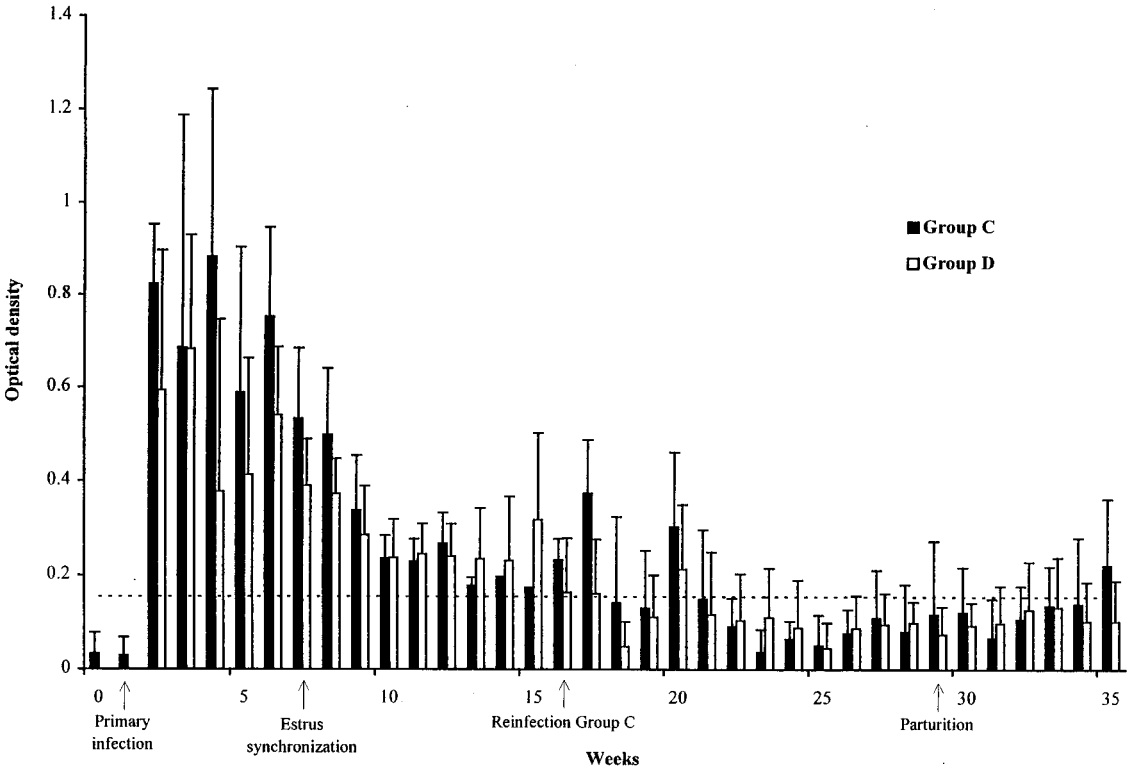

FIG. 5. Mean IgM antibody response to *C. psittaci* ± standard error of the mean. Primary infection of the nonpregnant ewes in groups C and D occurred by subcutaneous inoculation. The ewes in group C were reinfected with *C. psittaci* by subcutaneous inoculation following 60 days of gestation. The positive seroconversion value of 0.1549 is represented by a dashed lined.

toxemia and, because of its severely weakened state, was euthanized by intravenous infusion with Euthanyl Forte. Postmortem examination revealed an enlarged liver with fatty infiltration, ketones in the urine, and three normally developed fetuses. These findings confirmed pregnancy toxemia. Placental and fetal tissues were free of gross pathologic lesions and chlamydial antigen. Another ewe had one lamb that was euthanized within 1 h of birth because it suffered from atresia ani. A third ewe from group D gave birth to three weak lambs, two of which did not survive. Gross pathology was not observed in the placentas, and chlamydial antigen could not be detected in maternal, placental, or neonatal samples. Although the cause of the birth of these weak lambs was not discovered, it was unlikely that *C. psittaci* was responsible.

The four ewes in group C that were subcutaneously infected with *C. psittaci* prior to being bred and again during pregnancy gave birth to nine healthy lambs. Postpartum vaginal swabs were negative for chlamydial antigen, and the placentas were free of disease. In contrast, primary subcutaneous infection of four ewes at 60 days of gestation, i.e., the ewes in group F, resulted in two ewes aborting their pregnancies and two ewes giving birth to weak lambs. Only one lamb from the ewes in group F survived. The placentas contained lesions consistent with *C. psittaci* infection. Chlamydial antigen was detected in all of the samples collected. The ewes that received the control inoculum, i.e., the ewes in group G, gave birth to eight healthy lambs. There was no indication of *C. psittaci* infection in the control group. The statistical comparison of the reproductive efficiency of ewes in various groups is given in Table 3.

Abortion or the birth of weak *C. psittaci*-infected lambs was the only clinical sign observed in 12 of the 13 ewes which were positive for chlamydiae at parturition. One ewe in group F had two retained placentas 48 h postpartum. The ewe was treated with 50 U of oxytocin (Rogar/STB, Dorval, Quebec, Canada) intramuscularly to induce uterine contraction that would aid in the expulsion of the placentas. Unfortunately, this treatment failed, and the health of the ewe declined. Following euthanasia with Euthanyl Forte, the placentas were removed and observed to be infected with *C. psittaci.*

***C. psittaci*-specific serum antibody response.** Vaginal infection of nonpregnant ewes in groups A and B with *C. psittaci* resulted in increased systemic IgM antibodies to the organism approximately 2 to 3 weeks after infection (Fig. 3). The IgM response continued to rise until the ewes were bred, and thereafter the response declined. Reinfection of the ewes in group A was associated with a second rise in IgM antibodies to *C. psittaci*. In both groups A and B, seroconversion by IgM ELISA was transient and typically followed experimental infection. In contrast, the anti-*C. psittaci* IgG response was slower to develop, but the ewes that experienced pregnancy failure as a result of infection remained seropositive after reinfection in group A or parturition in group B (Fig. 4). One of the two ewes in group A that delivered healthy lambs was negative for IgG antibodies to *C. psittaci* by the end of the experiment, while the other ewe was slightly above the cutoff value. The ewe in group B that had two healthy lambs did not mount a detectable serum antibody response to *C. psittaci.*

A marked IgM response to *C. psittaci* was observed in the nonpregnant ewes in groups C and D 1 week following subcutaneous infection (Fig. 5). The ewes were IgM positive up to 9 weeks postinfection. There was a second short-lived increase in IgM reactivity following reinfection in group C. Systemic IgG

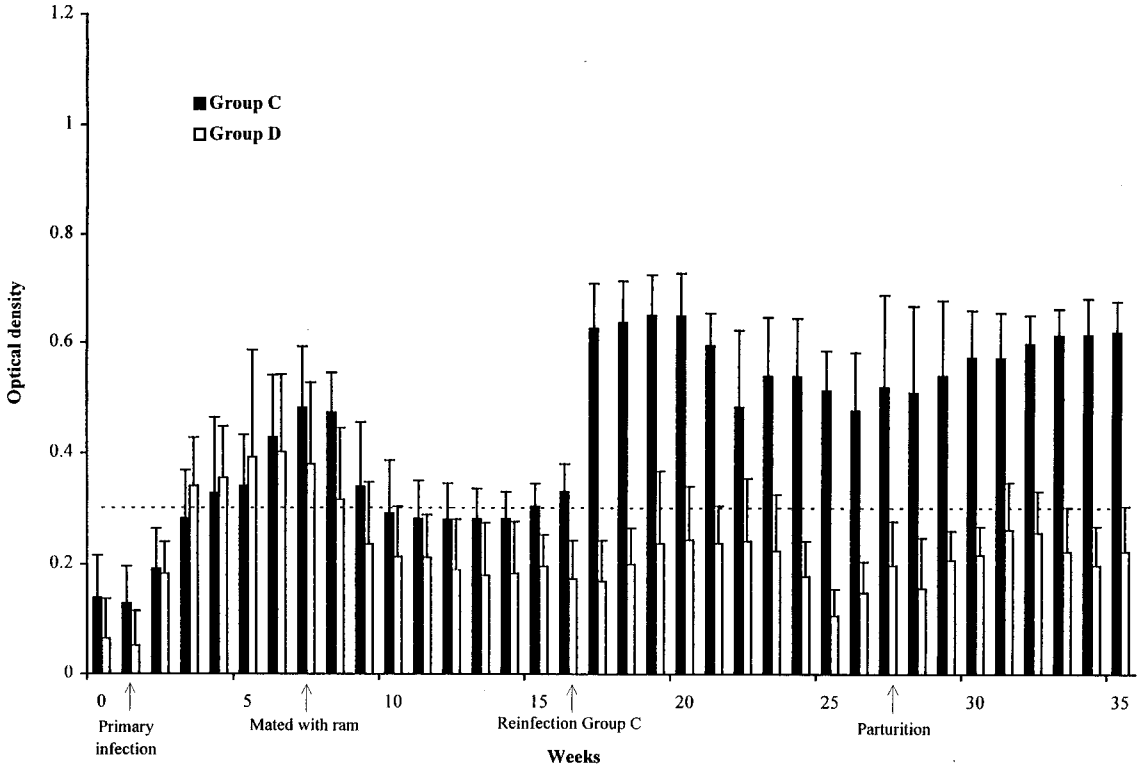

FIG. 6. Mean IgG antibody response to *C. psittaci* ± standard error of the mean. Primary infection of the nonpregnant ewes in groups C and D occurred by subcutaneous inoculation. The ewes in group C were reinfected with *C. psittaci* by subcutaneous inoculation following 60 days of gestation. The positive seroconversion value of 0.3019 is represented by a dashed lined.

antibodies to *C. psittaci* began to rise approximately 2 to 3 weeks after primary infection (Fig. 6). The IgG response declined during pregnancy and only increased in group C ewes following reinfection. The ewes in group D that were not reinfected were considered seronegative by IgG ELISA by the end of the experiment.

Primary infection of pregnant ewes by vaginal or subcutaneous inoculation, i.e., ewes in groups E and F, respectively, resulted in increased IgM and IgG antibody responses to the organism within 1 week (Fig. 7 and 8). The response decreased until a few weeks before abortion or parturition, when there was a second rise in the antibody levels in the ewes that exhibited disease. *C. psittaci*-induced pregnancy failure resulted in IgG seroconversion that persisted for the duration of the study, while IgM seroconversion was transient. Ewes in group G remained seronegative throughout the study.

DISCUSSION

Primary *C. psittaci* infection in pregnant ewes may cause pregnancy failure through abortion or the birth of weak, low-birth-weight lambs. The organism exhibits tropism for placental tissue, where continued multiplication has been associated with inflammation and tissue damage (6). The initial route of infection may influence the outcome of pregnancy since systemic infection of pregnant ewes has been the most effective method for experimentally reproducing the disease. In this study, ewes vaginally inoculated during pregnancy were susceptible to disease, but the extent of placental pathology and effects on lambs were comparatively less severe. The ability of *C. psittaci* to reach the placenta appeared to be a necessary requisite for disease, and systemic exposure may have facilitated this process. Placental infection as a consequence of vaginal inoculation of pregnant ewes may require that the organism move through the mucosae and spread to the target tissue by blood or lymphatic circulation. Another possibility would be a localized ascending cell-to-cell transfer of *C. psittaci* to the uterus.

Progression of *C. psittaci* infection in placentas may vary as indicated by the differences in gross pathology. These differences may be the basis for the variation in lamb health at parturition, which ranged from apparently healthy lambs to aborted or stillborn lambs. Placental infection may impair maternal-fetal transfer of nutrients and waste products, thereby causing decreased fetal growth. Additionally, *C. psittaci* infection may alter placental steroid and prostaglandin release, thus contributing to premature labor (11). In some cases, both weak and healthy lambs may be delivered by the same ewe. Premature labor in these ewes may not be induced if the placenta associated with the healthy lamb remains relatively free of infection and continues to produce sufficient progesterone to maintain the pregnancy.

Although not well documented in sheep, the vaginal epithelium at estrus can be distinguished by a cornified cell layer between cuboidal or columnar surface cells and underlying squamous cells (4, 8). By late estrus and into metestrus, the next phase of the estrus cycle, the superficial cells become desquamated. Successful infection of vaginal mucosae by *C. psittaci* during estrus would require that the organism invade deeper tissues to avoid being exfoliated along with the epithelial cells. Isolates of *C. psittaci* recovered from cases of ovine, caprine, and bovine abortions were shown to invade deeper tissues because they were detected in the spleens of mice after footpad inoculation (20). The cyclic changes, humoral and/or

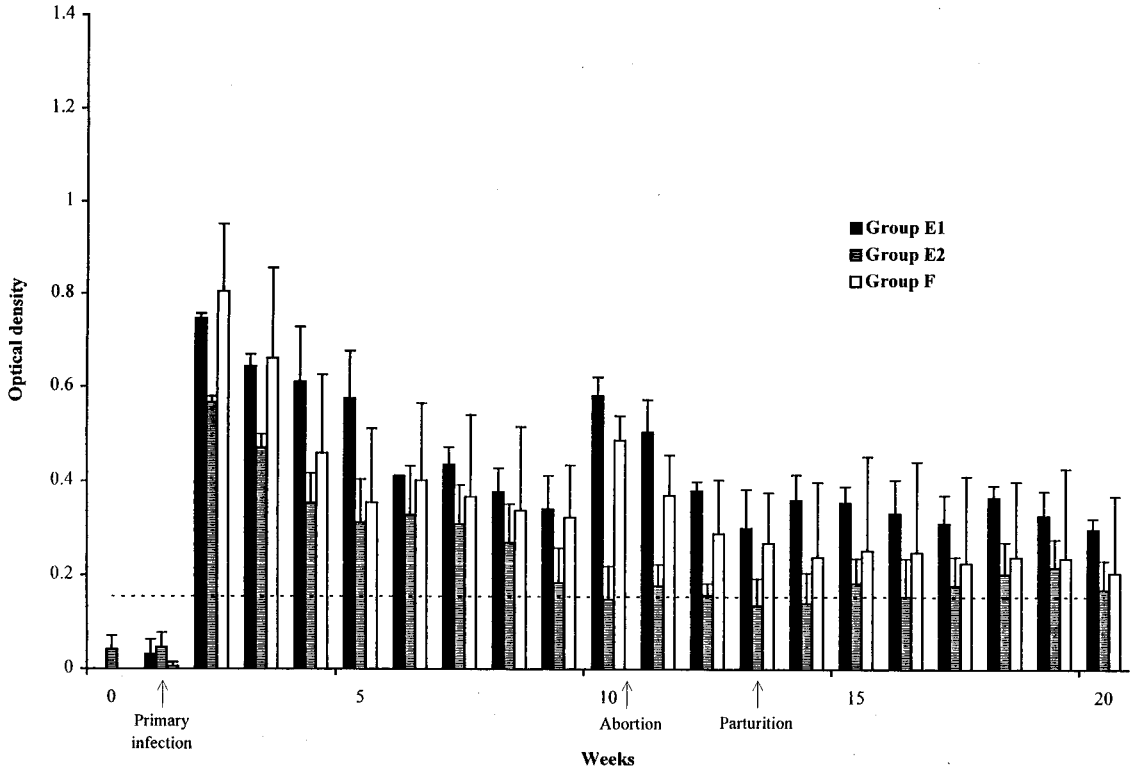

FIG. 7. Mean IgM antibody response to *C. psittaci* ± standard error of the mean. Ewes in group E were vaginally infected with *C. psittaci* following 60 days of gestation. Ewes in group F were infected by subcutaneous inoculation following 60 days of gestation. The two ewes in group E with signs of infection at parturition, one ewe which gave birth to weak lambs and the other one which aborted the pregnancy, are displayed as group E1. Group E2 are the three ewes from group E that gave birth to healthy lambs. The positive seroconversion value of 0.1549 is represented by a dashed lined.

cellular, that occurred during the estrus cycle also appeared to influence the invasive properties of *C. psittaci*. Systemic IgG antibody responses to *C. psittaci*, detected by ELISA, following primary vaginal infection in nonpregnant ewes were weak for at least two estrus cycles. Furthermore, some ewes did not respond serologically until after being bred. It was not clear whether the systemic IgG response was due to hematologic dissemination of *C. psittaci* or enhanced chlamydial replication and further invasion.

A previous study reported a biphasic rise in the IgG antibody response to *C. psittaci*, by ELISA, following subcutaneous infection and parturition (13). Similar fluctuations in antibody responses were observed in our vaginally and subcutaneously infected ewes. The decline in specific IgG during pregnancy may be related to sequestration of *C. psittaci* in the placenta, whereas reexposure to *C. psittaci* following parturition and placental membrane rupture may induce an anamnestic rise. The anti-*C. psittaci* response may be useful for the detection of infected ewes and therefore provide a method by which producers could identify infected animals for treatment or removal. However, serum antibody tests may be limited in their ability to detect primary vaginal infection since there was not a strong systemic response associated with vaginal infection. Ewes that were vaginally infected were seronegative by IgM ELISA until breeding 5 weeks postinfection. The IgM response was transient in both systemically and vaginally infected sheep. The IgG response was strong following parturition of infected lambs or fetuses, and therefore, the utility of IgG tests may be restricted to identifying ewes which had experienced previous pregnancy failure due to chlamydiae.

An early publication suggested that nonpregnant ewes become infected during the periparturate period when other ewes expel contaminated placental and fetal tissues (16). It was further suggested that chlamydiae may remain latent in lymphoid tissue since abortion was induced in a small number of pregnant ewes injected with lymphoid tissue suspensions prepared from systemically infected nonpregnant ewes. More recently, *C. psittaci* was isolated for a short time from lymph nodes that drained the site of subcutaneous infection in 3 of 10 ewes (9). Beyond the transient period during which chlamydiae could be recovered, latent forms of the organism within the lymph nodes were not demonstrated. In the present study, subcutaneous infection of nonpregnant ewes prior to being bred did not cause disease during the next pregnancy. This would seem to indicate that systemic lymphoid tissue may not be a site for the persistence of *C. psittaci* infection in nonpregnant ewes.

Vaccination against *C. psittaci* in sheep has been attempted as a means of disease control. The first produced vaccines were bacterins prepared from chick embryo yolk sacs infected with an abortion isolate of *C. psittaci* (14). These vaccines reduced the number of abortions in endemic areas of the United Kingdom but were unable to eliminate the disease. Slightly more refined vaccines that contained purified chemically inactivated *C. psittaci* EBs protected pregnant ewes against systemic homologous challenge (1). Immunoblot analysis revealed a marked response against the major outer membrane protein of *C. psittaci* following vaccination. This led to the development of a subcellular vaccine enriched with the major outer membrane protein (25). The subcellular vaccine was protective against systemic homologous challenge in pregnant ewes. A surprising result in our study was that systemic infection of

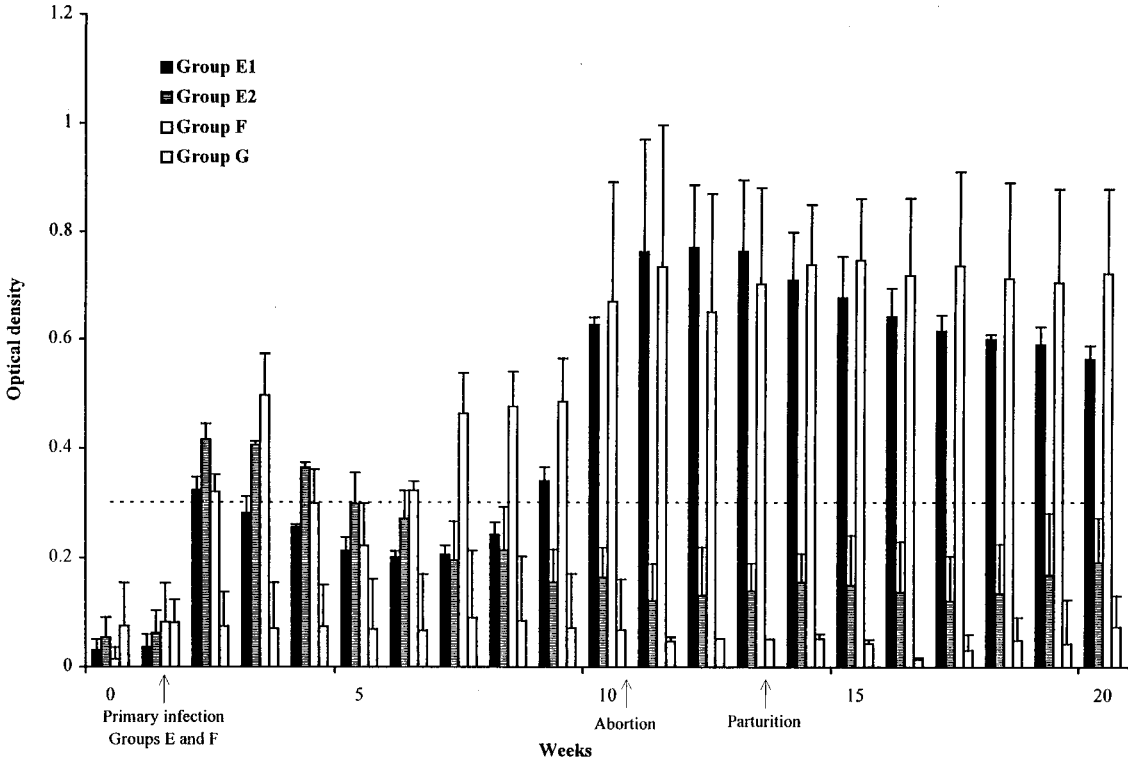

FIG. 8. Mean IgG antibody response to *C. psittaci* ± standard error of the mean. Ewes in group E were vaginally infected with *C. psittaci* following 60 days of gestation. Ewes in group F were infected by subcutaneous inoculation following 60 days of gestation. The two ewes in group E with signs of infection at parturition, one ewe which gave birth to weak lambs and the other one which aborted the pregnancy, are displayed as group E1. Group E2 ewes are the three ewes from group E that gave birth to healthy lambs. Group G ewes were not infected with *C. psittaci*. The positive seroconversion value of 0.3019 is represented by a dashed line.

nonpregnant ewes with virulent *C. psittaci* had no apparent adverse effect and appeared to protect against subsequent systemic homologous challenge during pregnancy. Similarly, the reported success of previous vaccines which stimulated systemic immunity may be partially related to the fact that pregnant ewes were subsequently challenged by systemic inoculation. However, if mucosal tissues are proven to be the initial site of infection, vaccines that generate a mucosal immune response may be more practical for protection against natural exposure.

The mechanism by which *C. psittaci* can be transmitted to naive sheep under natural conditions has not been established. In this study, experimental *C. psittaci* infection of the vaginal mucosae of nonpregnant ewes was sufficient to cause pregnancy failure during the next pregnancy. The organism did not elicit a strong systemic antibody response following mucosal challenge, and infection was not associated with abnormal clinical signs until parturition, when weak lambs were delivered. The previous observation that ewes which experienced *C. psittaci*-induced abortion became carriers and excreted the organism from the reproductive tract during estrus implicates venereal transmission in perpetuation of the infection within a flock (17). The current finding that vaginal infection was possible during estrus further supports the hypothesis that *C. psittaci* may be sexually transmitted among sheep. Venereal transmission to naive ewes may result from coitus with a ram that previously mated with a ewe that was shedding *C. psittaci* from the reproductive tract. The ram may become infected and shed chlamydiae in seminal fluids or may simply transmit the organism as a mechanical carrier.

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