Veterinary Research Communications, 29(Suppl.1) (2005) 81–98

Rechallenge of Previously-infected Pregnant Ewes with *Chlamydophila abortus*

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Sammin, D.J., Markey, B.K., Bassett, H.F. and McElroy, M.C., 2005. Rechallenge of previously-infected pregnant ewes with *Chlamydophila abortus*. *Veterinary Research Communications*, **29(Suppl. 1)**, 81–98

ABSTRACT

In an attempt to ascertain the means whereby previous exposure to *Chlamydophila (C.) abortus* can protect against the re-occurrence of enzootic abortion of ewes (EAE), ten previously-exposed ewes were intravenously rechallenged with a large infective dose of *C. abortus* during pregnancy. The patterns of development of chlamydial placentitis and its sequelae closely resembled that observed following first-time challenge of previously-naïve ewes, although placentitis appeared to develop more slowly following rechallenge infection and none of the rechallenged ewes aborted. Chorioallantoic and foetal pathology and foetal immune responses were qualitatively similar whilst the local maternal response to *C. abortus* infection of the endometrium did not appear to differ in rechallenged and first-time challenged sheep. This demonstrates that if *C. abortus* reaches the foetal side of the placenta, a stereotypical response is elicited, regardless of the status of maternal immunity. Therefore it appears that in natural circumstances, acquired immunity of the dam protects against the re-occurrence of EAE by preventing the causative agent from reaching the susceptible foetal trophoblast.

Keywords: *Chlamyophila abortus*, enzootic abortion of ewes, foetus, ovine, pathology, placentitis, rechallenge infection

Abbreviations: CD, cluster determinant; *C. abortus*, *Chlamydophila abortus*; d.g., days gestation; d.p.c., days post-challenge; EAE, enzootic abortion of ewes; ELISA, enzyme-linked immunosorbent assay; IFU, inclusion-forming units; IEL, intra-epithelial lymphocyte; Ig, immunoglobulin; LPS, lipopolysaccharide; p.i., post-infection; PP value, percent positivity value; rLPS, recombinant (chlamydial) lipopolysaccharide; WC, workshop cluster

Presented at the 2nd Workshop of COST Action 855 "Animal Chlamydioses and the Zoonotic Implications", Budapest, September 1, 2004

INTRODUCTION

Enzootic abortion of ewes (EAE) is a major cause of reproductive loss in intensively-managed lowland sheep flocks (Anonymous, VLA, 2002). The disease which is caused by *Chlamydophila abortus*, occurs in late pregnancy and is associated with severe and extensive pathological changes in the foetal membranes (Stamp *et al.*, 1950). Previous studies have elucidated the sequence of events which occur when pregnant ewes are experimentally challenged with *C. abortus* (Studdert, 1968; Novilla and Jensen, 1970; Buxton *et al.*, 1990). Chlamydial inclusions were first apparent within trophoblasts lining chorioallantoic villi at the hilus of placentomes. The subsequent loss of these trophoblasts from the chorionic surface provoked intense inflammatory changes in the underlying chorioallantois (Novilla and Jensen, 1970; Buxton *et al.*, 1990). As the disease advanced, occasional chlamydial inclusions in the endometrial epithelium at the edge of placentomes were associated with intense focal endometritis (Novilla and Jensen, 1970; Buxton *et al.*, 1990). In addition to pathological changes in the placenta, haematogenous dissemination of chlamydial infectivity to the foetus elicited multifocal necrosis in foetal organs and a foetal systemic response to *C. abortus* (Studdert, 1968; Buxton *et al.*, 1990; Sanderson *et al.*, 1994).

A characteristic epidemiological feature of enzootic abortion of ewes is that ewes which have aborted following infection with *C. abortus* do not abort again in subsequent years (Stamp *et al.*, 1950; Littlejohn, 1950). This holds true despite evidence for the persistence of *C. abortus* infection in the ovine endometrium post-abortion (Papp and Shewen, 1996). It is probable that immune responses in ewes that have aborted prevent abortion from re-occurring by preventing *C. abortus* from reaching the susceptible trophoblast in subsequent pregnancies. In an attempt to investigate this hypothesis, ewes which aborted following challenge with an Irish isolate of *C. abortus* were rechallenged when again pregnant ten months later. The outcome of rechallenge was compared with the outcome of first-time challenge in previously-naïve ewes. Lesion distribution patterns in placentae and foetal organs were compared for both groups as were foetal systemic responses and maternal serological responses to *C. abortus.*

MATERIALS AND METHODS

Experimental animals

Crossbred ewes were sourced from a commercial flock with no known history of enzootic abortion. Oestrus was synchronised using intravaginal progestagen sponges ('Chronogest'; Intervet, Boxmeer, Netherlands) and pregnancy was diagnosed by ultrasound-scanning 70 to 90 days after introduction of the rams.

Preparation of inoculum

The C95-27 strain of *C. abortus* isolated from an outbreak of EAE in Ireland and typed by restriction endonuclease analysis of the 60 kDa *omp-2* gene (Markey *et al.*, 1996), was propogated by inoculation into yolk sacs of 6-day-old embryonated hen eggs. A suspension of infected yolk sacs from embryos that died between five and seven days after inoculation was prepared as described by McClenaghan and colleagues (1984) and its infectivity was established by titration on cultured McCoy cells treated with cycloheximide (1 μg/ml). Suspensions of yolk sac material for inoculation of control ewes were prepared in a similar manner from 13-day-old, uninfected embryonated hen eggs.

Experimental challenge

Ten ewes (group A), which had aborted ten months previously, following experimental challenge with *C. abortus*, were rechallenged with *C. abortus* when again pregnant; on both occasions, an infectious dose of 10^9 IFU of yolk sac-grown *C. abortus* was given by intravenous injection at 91–98 days gestation (d.g.). On the same day that group A were rechallenged, nine previously-naïve ewes at the same stage of pregnancy (group B) were given 10^9 IFU of yolk sac-grown *C. abortus* by intravenous injection, and three control ewes at the same stage of pregnancy (group C) were injected intravenously with 1 ml of uninfected yolk sac suspension. Serum was collected from each ewe before intravenous injection.

Clinical observation and management of ewes post-challenge

Ewes were clinically examined at 24-h intervals from two days before challenge until five days post-challenge. Rectal temperature was recorded and a subjective assessment was made of appetite and demeanour.

Protocol for euthanasia of ewes and examination of uterine contents

Ewes were euthanased by intravenous overdose of sodium barbitol ('Euthatal'; Rhone-Merieux, Harlow, UK) at specific timepoints after challenge/rechallenge infection (Table I). As each ewe was killed, the pregnant uterus was removed without delay from the abdominal cavity and opened by incision along its greater curvature to expose the uterine contents. After the uterus was opened, a portion of placentome was collected into medium prepared according to Spencer and Johnson (1983) for chlamydial isolation. Macroscopic evidence of placentitis was recorded and two placentomes were selected at random from each placenta. A portion of each of these placentomes, encompassing the placentomal limbus, was mounted on an aluminium disc in Tissue-Tek OCTTM compound (Sakura, The Netherlands) and snap-frozen in

TABLE I
Number of placentae examined

Group	Subgroup	Timepoint[a]	Maternal placentae[b]	Foetal placentae[c]
A - Rechallenged	A1 (n = 5)	21	5	11
	A2 (n = 5)	35	5	11
B - Challenged	(n = 6)	28	5	6
C - Control	(n = 3)	28	3	6

[a]Number of days post-challenge/rechallenge at which ewes were killed

[b]Number of ewes from which placenta was examined

[c]Number of foetuses from which placenta was examined

liquid nitrogen. Placentomes and intercotyledonary foetal membranes from each placenta and intercaruncular portions of the uterine wall from each ewe were immersed in 4% buffered formal saline. Blood was collected from the umbilicus of each foetus (into EDTA-coated and uncoated tubes for haematology and serology, respectively). The foetus was then weighed. During necropsy, the entire liver and spleen of each foetus were separately weighed and these organ weights were expressed as a percentage of foetal bodyweight. Portions of the liver, spleen, lung and lymph node (mesenteric and hepatic) of each foetus were immersed in 4% buffered formal saline.

Chlamydial isolation

Chlamydial isolation was performed in McCoy cells treated with cycloheximide (1 μg/ml) using the method described by Markey and colleagues (1996).

Histopathology

Formalin-fixed tissues were trimmed, dehydrated through graded alcohols, cleared in xylene and embedded in paraffin wax. Five μm sections were stained with Meyer's haematoxylin and eosin.

Immunohistochemistry

Sequential cryostat sections of each frozen placentome were immunolabelled for chlamydial lipopolysaccharide (LPS); GM1, a cell surface marker expressed by granulocytes and cells of the monocyte/macrophage series; cluster determinant (CD)

TABLE II
Monoclonal antibodies used for immunohistochemistry

Clone	Source	Specificity
DH59B	VMRD Inc., Pullman, WA, USA	GM1
AC1	Progen Biotechnik GmbH, Heidelberg, Germany	Chlamydial LPS
Gc50A1	VMRD Inc.	CD4
Cact 80c	VMRD Inc.	CD8
73B	A gift from Dr. L. Dudler, Institute for Immunology, Basel, Switzerland	CD45R
CC15	Serotec Ltd, Oxford,, UK	WC1

4; CD8; CD45R and workshop cluster (WC) 1. Cryostat sections (5 μm) were mounted on capillary gap glass slides (ChemMate™, DAKO A/S Biotek Solutions, Glostrup, Denmark) and immunolabelled by automated process (TechMate™; DAKO) using one of six different monoclonal antibodies (Table II). Primary antibody binding was detected with biotinylated anti-rabbit/mouse immunoglobulin followed by streptavidin conjugated to horseradish peroxidase (HPO) and diaminobenzidine (DAB) solution (ChemMate™ Detection Kit, DAKO). Immunolabelled slides were then lightly counterstained for 1 min with Meyer's haematoxylin.

Cell counts

Immunolabelled lymphocytes (CD4, CD8, WC1 and CD45R) were counted separately in the chorioallantois and the endometrial subepithelial stroma, using an eyepiece graticule. For each anatomical area in each tissue section, counts were performed in at least five fields; mean cell counts were expressed as cells per square millimetre (mm^2).

Foetal haematology

A diluted sub-sample of whole blood was analysed using a Sysmex F800 micro-cellcounter (Toa Medical Electronics Co. Ltd., Kobe, Japan) to give a total count for leucocytes whilst differential counts of nucleated blood cells were performed on air-dried, Leischman-stained smears. The leucocyte count obtained by automated analysis was 'corrected' by subtracting the number of nucleated red blood cells obtained in the differential cell count. Total plasma protein concentration was estimated by refractometer. A subsample of plasma was heated in a water bath at 60°C for 5 min to denature fibrinogen and the protein concentration of this subsample was estimated by refractometer after centrifugation. The difference in these refractometer readings was taken as the plasma fibrinogen concentration.

Serology

rLPS ELISA for *Chlamydia*-specific IgG

Microtitre plates, coated with an artificial glycoconjugate antigen prepared using recombinant chlamydial LPS (rLPS), were sourced from Medac (Hamburg, Germany). Positive control serum, from sheep experimentally challenged with *C. abortus*, was obtained from the Moredun Research Institute, Edinburgh. Plates were incubated for 1 h at 37°C with phosphate-buffered saline containing 0.05% Tween 80 (PBST) and 15% foetal calf serum (PBST-FCS) followed by washing in PBST to block non-specific binding. The positive control serum and test sera were diluted (1:500 in PBST-FCS) and added to duplicate wells on the test plate. PBST was added to two wells on each plate as a 'blank' and the plate was incubated for 1 h at 37°C followed by six washes in PBST. Diluted rabbit anti-sheep IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA; 1:15 000 in PBST-FCS) was then added to each well and the plate was again incubated for 1 h at 37°C. After thorough washing of the plate, bound conjugate was detected by adding a proprietary preparation of tetramethylbenzidine substrate (Idexx Laboratories, ME, USA) to each well for 10 min at room temperature. The reaction was stopped with 0.5 mol/L sulphuric acid and spectrophotometric absorption was determined at 450 nm. The mean optical density (OD) value obtained for each test serum (corrected by subtraction of the mean OD value obtained for blank wells) was expressed as a percentage of the corrected mean OD value of the positive control serum to give a percent positivity (PP) value. A cut-off PP value (13.6) was determined from the mean PP value plus three standard deviations for sera from 85 ewes in an EAE-free, commercial flock.

rLPS ELISA for *Chlamydia*-specific IgM

Chlamydia-specific IgM in foetal sera was assayed by the same method but using a serum dilution of 1:100 and substituting as anti-globulin, peroxidase-labelled, anti-ovine IgM (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) at a working dilution of 1:2000.

RESULTS

Clinical outcome of challenge

Group A

All ten ewes had an elevated rectal temperature (>40°C) at 24 h post infection (p.i.), but fever had subsided by 48 h p.i. All were clinically normal thereafter and none of the ewes aborted. Each ewe was pregnant with two or more live foetuses when killed (at 21 or 35 days after rechallenge).

Group B

All nine ewes exhibited a marked febrile response, including an elevated rectal temperature for 2–4 days and systemic signs of illness such as lethargy and inappetence. Intrauterine foetal death was apparent in five of the six ewes killed at 28 days post-challenge (d.p.c.); the other three ewes had aborted before this timepoint.

Group C

All three ewes were clinically normal and afebrile throughout the period of observation; none of the ewes aborted and all were pregnant with twin foetuses when killed.

Chlamydial isolation

Chlamydiae were isolated from placentae of all ewes in groups A and B but not from any of the ewes in group C.

Placental pathology

Subgroup A1 (placentae from five ewes and 11 foetuses; 21 days after rechallenge):

Macroscopic changes in placentae consisted of: diffuse gelatinous thickening and opacity of the foetal membranes due to chorioallantoic oedema ($n = 3/11$) and aggregates of pale, friable material surrounding placentomes due to periplacentomal necrosis of the chorioallantoic membrane ($n = 1/11$).

Chorioallantois: Chorioallantoic pathology was apparent in three of the five ewes. Intracytoplasmic inclusions immunopositive for chlamydial LPS were observed in trophoblasts (within the hilar zone of placentomes) and sloughing of inclusion-bearing trophoblasts was associated with neutrophil infiltration of the exposed chorionic surface. Occasional immunopositive lymphocytes (CD4, CD8, WC1 and CD45R) were scattered throughout the chorioallantoic stroma.

Endometrium: Periplacentomal endometritis was apparent in one of the five ewes. In that ewe, numerous inclusions immunolabelled for chlamydial LPS were visible in the endometrial epithelium and the inflammatory response in the endometrial subepithelial stroma featured lymphocytes of all four subsets in addition to neutrophils and macrophages.

Subgroup A2 (placentae from five ewes and 11 foetuses; 35 days after rechallenge)

Macroscopic changes consisted of chorioallantoic oedema (n = 11/11) and periplacentomal necrosis (n = 9/11).

Chorioallantois: Pathological changes were observed in the chorioallantois in all five ewes although there was considerable variation in lesion severity between ewes and even between placentomes in the same placenta. Immunolabelling for chlamydial LPS was evident at the chorionic surface and there was moderate to severe inflammatory infiltration in the underlying chorioallantoic stroma. Chorioallantoic arteries were frequently involved in the inflammatory process and thrombosis was occasionally evident. Marked neutrophil infiltration of the chorionic surface was observed where chorioallantoic villi (within placentomes) and the chorioallantoic membrane (at the edge of placentomes) were denuded of trophoblasts (Figure 1). A less intense, diffusely-distributed infiltrate of macrophages, neutrophils and lymphocytes occurred throughout the deeper chorioallantoic stroma. Randomly-distributed, immunolabelled lymphocytes only constituted a small proportion of this cellular infiltrate; $CD8^+$ and $CD4^+$ cells were more numerous than either $WC1^+$ or $CD45R^+$ cells.

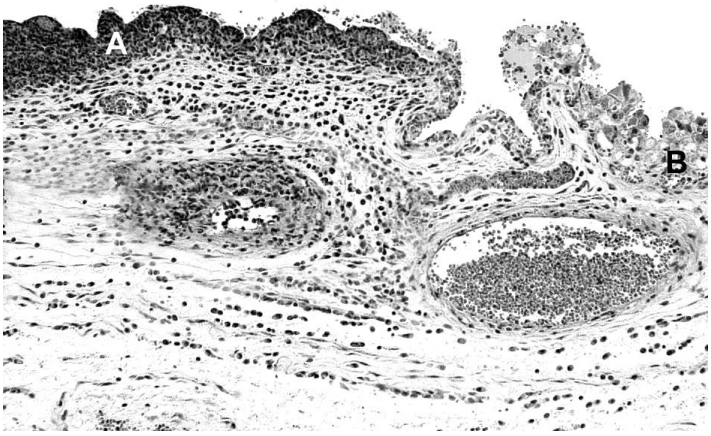

Figure 1. Chorioallantoic membrane, 35 days post-rechallenge. (A) loss of the trophoblast layer and neutrophil infiltration of the chorioallantois and (B) adjacent chorionic surface where the trophoblast layer is intact; the arrow indicates arteritis beneath the inflamed chorionic surface ($\times 20$)

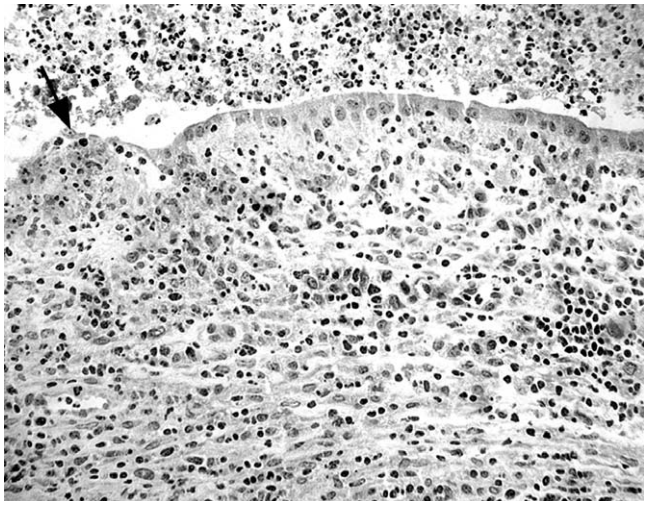

Figure 2. Placentomal limbus, endometrium, 35 days post-rechallenge; attenuation and focal ulceration of epithelium; subepithelial infiltration by neutrophils, macrophages and lymphocytes ($\times 40$)

Endometrium: Immunopositive chlamydial inclusions were apparent in the endometrial epithelium from three of the five ewes whereas focal endometritis was apparent at the placentomal limbus in all five ewes. Attenuation and ulceration of the luminal epithelium was accompanied by intense subepithelial infiltration of neutrophils, macrophages and lymphocytes (Figure 2). All lymphocyte subsets were present in appreciable numbers in the subepithelial stroma. $CD8^+$ cells were consistently more numerous than other lymphocyte subsets (Figure 3).

Group B (placentae from five ewes and six foetuses; 28 days after challenge)

Macroscopic changes included both chorioallantoic oedema ($n = 6/6$) and periplacentomal necrosis ($n = 4/6$).

Chorioallantois: Histopathological changes consisted of extensive loss of the trophoblast layer, marked infiltration of the ulcerated chorionic surface by neutrophils and a diffuse infiltrate of macrophages, neutrophils and occasional lymphocytes deeper within the chorioallantois. Chorioallantoic arteritis was particularly prominent in severely-inflamed areas of the chorioallantoic membrane at the edge of placentomes. Cellular debris overlying the chorionic surface was heavily-immunolabelled for chlamydial LPS. Although only comprising a small proportion of the cellular infiltrate, immunolabelled lymphocytes of each subset were present within chorioallantoic lesions; the relative proportions of $CD4^+$ and $CD8^+$ T cells varied considerably.

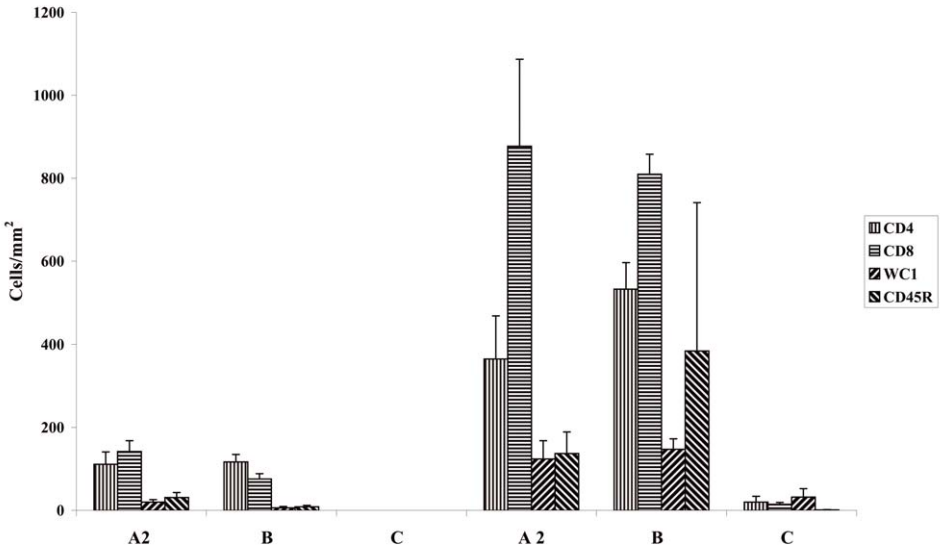

Figure 3. Intralesional lymphocyte subset counts (mean ± standard error) in the fetal chorioallantois and maternal endometrium

Endometrium: Chlamydial inclusions were infrequent in the endometrium. However marked periplacentomal endometritis was evident even where inclusions were not seen in the endometrial epithelium. Attenuation and in some cases, ulceration of the luminal epithelium was accompanied by marked focal inflammation of the subepithelial stroma; focal inflammatory infiltrates were composed of lymphocytes, macrophages and neutrophils. Although immunolabelled lymphocytes of each subset were present, intralesional T-cells were particularly prominent and $CD8^+$ cells were consistently more numerous than $CD4^+$ cells.

Group C (control placentae from three ewes and six foetuses)

No significant macroscopic or histopathological changes were observed in placentae.

Foetal Pathology

Subgroup A1

No significant macroscopic changes were seen. Randomly-distributed, multifocal areas of hepatic necrosis were apparent in four foetuses and in one of these foetuses, similar

TABLE III
Foetal organ weights as percentage bodyweight (mean ± standard error)

(Sub)Group	Foetal	Foetal liver weight (% foetal b.w.)	Foetal spleen weight (% foetal b.w.)
A1	1905 ± 62	Not done	Not done
A2	3063 ± 631	4.7 ± 0.4%	0.45 ± 0.04%
B	2160 ± 64	5.2 ± 0.2%	0.56 ± 0.05%
C	2457 ± 92	3.9 ± 0.1%	0.22 ± 0.01%

b.w, body weight

necrotic foci were also observed in the lung and spleen. Hyperplasia of periarteriolar lymphocytic sheaths was evident in the spleen in two foetuses.

Subgroup A2

Hepato-splenomegaly and grossly-enlarged lymph nodes (hepatic and mesenteric) were apparent in all foetuses (Table III). Multifocal hepatic granulomas were observed in one foetus (Figure 4), in which multifocal granulomatous thickening and necrosis of the alveolar walls was also apparent. Occasional granulomatous foci occurred in the liver of three other foetuses. Lymphoid hyperplastic changes in spleen and lymph node occurred in eight foetuses whilst hepatitis featuring periportal aggregation of mononuclear cells was marked in six foetuses.

Group B

Hepato-splenomegaly and an increase in the size of hepatic and mesenteric lymph nodes was grossly-apparent in all six foetuses. Multifocal necrosis and/or granulomatous foci were randomly-distributed in the livers ($n = 6/6$), spleens ($n = 3/6$) and lungs ($n = 2/6$); occurring in all three organs in one foetus. In addition to hepatic granulomas (many of which exhibited central areas of necrosis infiltrated by neutrophils), periportal aggregation of mononuclear cells was another prominent feature in the liver. In the spleen, foci of coagulative necrosis were heavily-infiltrated by neutrophils and hyperplasia of periarteriolar lymphocytic sheaths was evident. The typical pulmonary lesion consisted of multifocal thickening of alveolar walls caused by interstitial granulomatous inflammation whilst occasional small foci of necrosis occurring within alveolar walls were infiltrated by neutrophils. Cortical hyperplasia with formation of secondary follicles was prominent in the pre-hepatic lymph node whilst medullary hyperplasia was also evident in lymph nodes, causing loss of

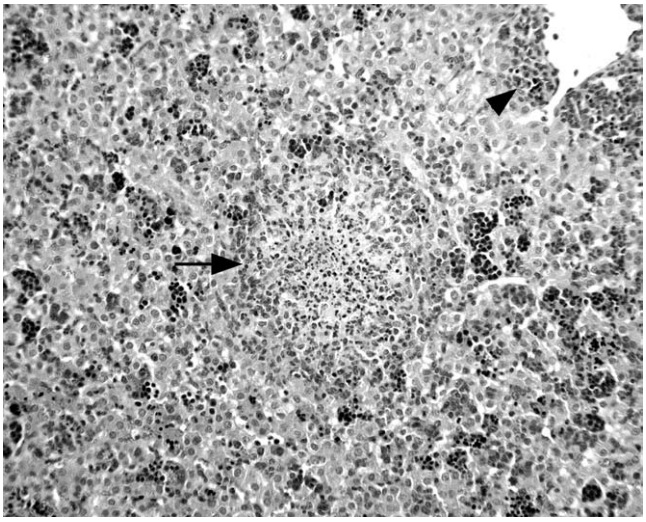

Figure 4. Fetal liver, 35 days post-rechallenge; multifocal granulomas (arrow) and periportal aggregates of lymphocytes (arrow head) (× 10)

distinction between cortex and medulla; focal aggregates of immature neutrophils were apparent in the medullary cords of some of these lymph nodes.

Group C

No significant macroscopic or histopathological changes were observed in any of the six foetuses.

Foetal haematology

Group A

Moderate leucocytosis was evident in subgroup A1 whilst marked leucocytosis with pronouced neutrophilia and elevated plasma fibrinogen were apparent in subgroup A2 (Table IV).

Group B

All six foetuses displayed marked leucocytosis with pronouced neutrophilia, lymphocytosis and elevated plasma fibrinogen levels.

TABLE IV
Mean (±standard error) for foetal blood cell counts, plasma fibrinogen concentrations and serum PP values for IgM and IgG

(Sub)Group	Corrected leukocytes ($\times 10^9$/mm^3)	Neutrophil count ($\times 10^9$/mm^3)	Lymphocyte count ($\times 10^9$/mm^3)	Plasma fibrinogen (g/L)	rLPS ELISA	
					IgM (PP value)[a]	IgG (PP value)[a]
A1	6.5±1.4	4.1±1.2	1.6±0.3	1.8±0.2	2.2±0.8	0.2±0.1
A2	29.9±4.3	20.9±4.1	6.4±1.2	4.5±0.5	60.6±5.0	4.6±1.0
B	46.2±13.3	29.9±9.5	12.1±3.4	3.3±0.4	60.5±14.1	7.2±2.2
C	2.7±0.3	0.5±0.1	2.1±0.1	1.0±0.0	1.1±0.3	0.1±0.1

[a]Percent positivity value

Group C

Leucocytic counts were within the expected range for near-term ovine foetuses (Al Salami and Filippich, 1999).

Maternal serology – rLPS ELISA for Chlamydia-specific IgG

Group A

Five of the ten rechallenged ewes were seropositive prior to rechallenge and eight were seropositive when sampled pre-slaughter; mean PP values prior to rechallenge (n = 10), 21 days after rechallenge (n = 5) and 35 days after rechallenge (n = 5) were 20.7±7.2, 38.6±11.4 and 30.2±10.1, respectively.

Group B

All six ewes were seronegative prior to challenge and all had seroconverted by 28 days post-challenge; mean PP values were 2.6±2.1 prior to challenge and 37.4±12.1 pre-slaughter.

Group C

All three ewes were seronegative at the beginning and end of the 28 day 'control' period; mean PP values were 2.8±2.8 initially and 2.8±1.6 pre-slaughter.

Foetal serology

Subgroup A1

Chlamydia-specific IgM (but not IgG) was barely detectable in sera from two foetuses.

Subgroup A2

Chlamydia-specific antibody, both IgM and IgG, was detectable in all foetuses.

Group B

Chlamydia-specific IgM detectable in sera from all six foetuses whilst *Chlamydia*-specific IgG was only detectable in four foetuses.

Group C

Chlamydia-specific serum antibody was not detected with either ELISA.

DISCUSSION

Ewes which aborted when initially challenged with *C. abortus* developed placental lesions typical of EAE following intravenous rechallenge. *C. abortus* was isolated from placentae of all ten rechallenged ewes and chlamydial placentitis was apparent in ewes whether they were killed at 21 or 35 days after rechallenge. A similar pattern of cellular infiltration was evident in the chorioallantois and endometrium following rechallenge as was observed in first-time challenged ewes. Serological and haematological responses of foetuses from rechallenged ewes closely resembled responses of foetuses from first-time challenged ewes.

Rechallenged ewes were febrile only on day 1 after inoculation whereas when first challenged with *C. abortus*, these ewes had elevated rectal temperatures for a two to three day period (results not shown but equivalent to the group B response). This was despite the fact that the same strain of *C. abortus*, the same infective dose, the same method of inoculum preparation and the same route of challenge were used for both first-time challenge and rechallenge. This suggests some degree of protection against the acute phase ill-effects of experimental intravenous challenge and was possibly related to the presence of circulating antibody. Although some of the ewes in group A were no longer 'seropositive' when serum antibody was assayed by rLPS ELISA immediately before rechallenge infection, all had seroconverted post-abortion the previous year as assayed by rLPS ELISA (Sammin, 2004). Studdert and McKercher (1968) have also reported that ewes with pre-existing chlamydial antibody titres

exhibited less severe clinical responses than seronegative sheep following experimental challenge with *C. abortus.*

In addition and in marked contrast to the first time they were challenged with *C. abortus*, none of the rechallenged ewes in the present study aborted. Nevertheless, *C. abortus* did reach the placenta and caused placentitis in rechallenged ewes. This was consistent with previous studies in which pre-existing *Chlamydia*-specific, serum antibody did not prevent the development of placentitis or abortion in ewes that were experimentally challenged with chlamydiae during pregnancy (Storz, 1963; Studdert and McKercher, 1968). It should however be emphasised that in the present study, ewes were rechallenged intravenously with a very large infective dose of *C. abortus.* Studdert and McKercher (1968) rechallenged three ewes which had previously aborted with three different doses of *C. abortus* and in that study, only the animal receiving the largest infective dose (by the intravenous route) showed any evidence of placental infection, aborting 32 days after challenge (Studdert and McKercher, 1968). It is also worth noting from vaccine development studies, that vaccination with *C. abortus* reduced the incidence of abortion in ewes when they were subsequently challenged by the subcutaneous route with *C. abortus* during pregnancy (McEwen *et al.*, 1951; Aitken *et al.*, 1986; Chalmers *et al.*, 1997) but it did not lower the incidence of abortion in ewes which were subjected to high dose intravenous challenge (Rodolakis and Souriau, 1979).

Pathological changes in the placentae of rechallenged ewes were largely confined to the foetal chorioallantois and exhibited the same distribution pattern as that seen following challenge of previously-naïve ewes. However, the development of placentitis appeared to be slower in rechallenged ewes as chorioallantoic pathology in rechallenged ewes killed at 35 days was similar in extent and severity to that observed in previously-naïve ewes, 28 days after first-time challenge with *C. abortus.* This may be a further manifestation of protective humoral immunity. In rechallenged ewes, *Chlamydia*-specific serum antibody might hasten the removal of chlamydiae from the circulation and a shorter duration of chlamydiaemia might reduce the numbers of viable chlamydiae reaching the placenta.

Endometritis was apparent at the edge of placentomes in rechallenged ewes at 35 days p.i. The pattern of intralesional lymphocytic infiltration in the endometrium of rechallenged ewes was similar to that observed in ewes killed 28 days after first-time challenge, featuring an increase in all lymphocyte subsets and a predominance of $CD8^+$ T cells. Therefore, $CD8^+$ cells were presumed to play a pivotal role in the local immune response of sheep to *C. abortus* infection as has been demonstrated for *C. abortus* infection in mice (Buzoni-Gatel *et al.*, 1992; Montes de Oca *et al.*, 2000). In a previous study, Papp and Shewen (1996), reported an increased number of plasma cells and intraepithelial lymphocytes (IELs) in the endometrium of ewes one year after they had aborted due to an experimental challenge with *C. abortus.* However B-lymphocytes, although present in appreciable numbers, were not a predominant component of the endometrial infiltrate in any of the rechallenged ewes in the present study. Furthermore, the morphology, immunophenotype and distribution pattern of IELs in the endometrium of rechallenged ewes in the present study were as previously described in control and *C. abortus* challenged ewes (Sammin, 2004).

Multifocal necrosis in the liver and other organs of foetuses from experimentally-challenged ewes has been ascribed to haematogenous dissemination of infectivity from the placenta (Studdert, 1968; Buxton *et al.*, 1990). Necrotic foci were less frequent in the liver, spleen and lung of foetuses from rechallenged ewes at 35 days p.i. than in the same organs of foetuses from first-time challenged ewes killed at 28 days p.i.. However where evident, the morphological appearance of hepatic granulomas and their random distribution in the parenchyma of the foetal liver were similar in both groups. Furthermore, lymphoid hyperplasia in the foetal spleen and lymph nodes was of identical pattern following first-time challenge and rechallenge infection. The slower development of placentitis in rechallenged ewes might account for the paucity of embolic lesions in organs of foetuses, 35 days after rechallenge infection. Alternatively, the foetal immune response which may be capable of eliminating *C. abortus* infection, might allow resolution of lesions in foetal organs when given sufficient time (Kennedy *et al.*, 2001).

Foetal leucocytosis and in particular neutrophilia was evident in both subgroup A2 and group B at a time when there was massive influx of foetal-derived neutrophils into the damaged foetal component of the placenta (the chorioallantois). In addition in both (sub)groups, elevated levels of fibrinogen in foetal plasma was coincident with thrombosis of inflamed chorioallantoic blood vessels.

Chlamydia-specific serum IgM was barely detectable and there was no evidence of a specific serum IgG response in foetuses from subgroup A1 whilst specific serum antibody of both isotypes was detected in foetuses of subgroup A2. Not surprisingly the PP values obtained for foetal sera with the rLPS (IgG) ELISA were markedly lower than PP values obtained for maternal sera with the same ELISA. Apart from age-related differences in immunocompetence, the antibody response of the foetus was a primary response and would not be expected to develop before placentitis was established and chlamydiae gained access to the foetal bloodstream. In contrast, ewes were mounting a secondary response to *C. abortus* and bacteraemia would have been immediate following intravenous rechallenge.

Although the normal ovine placenta is impermeable to immunoglobulins (Griebel, 1998), transplacental transfer of immunoglobulin from the dam to the foetus has occurred where certain types of placental lesion existed (Poitras *et al.*, 1986). However, even with grossly-visible placentitis, antibodies to *Toxoplasma gondii* in foetal serum were ascribed to foetal antibody production as maternal and foetal sera differed with respect to titres of antibody specific for other pathogens (Dubey *et al.*, 1987). Furthermore, Kennedy and colleagues (2001), detected *Chlamydia*-specific antibody in serum and thoracic fluid of aborted foetuses but found very few foetuses to have detectable antibody to adenovirus despite a seroprevalence of 70 to 90% in adult sheep. In the present study, when chlamydial LPS-specific IgG levels of individual foetuses and their dams were compared (subgroup A2 and group B), foetal antibody levels were found to be independent of maternal antibody levels and also appeared to be independent of the extent and severity of placentitis. Thus the presence of *Chlamydia*-specific antibody in sera of foetuses from challenged or rechallenged ewes suggests a foetal humoral response to *C. abortus* and not transplacental transfer of antibody from the seropositive dam.

This study provides further evidence that ewes which have previously aborted due to infection with *C. abortus*, are protected to a certain extent against the re-occurrence of chlamydial abortion. Immunity associated with previous experimental challenge was sufficient to significantly reduce the acute phase response of ewes to rechallenge infection and to prevent the early occurrence of abortion. Yet it was insufficient to prevent *C. abortus* from reaching the placenta. The patterns of chlamydial antigen distribution, tissue injury and resulting leucocytic infiltration of the chorioallantois and the foetal systemic response to *C. abortus* after rechallenge were as for first-time challenge although placentitis may have been slower to develop in rechallenged ewes. Therefore, in previously-aborted ewes, the principal effect of maternal immunity is most likely to prevent, or failing that, to limit the numbers of *C. abortus* reaching the susceptible trophoblast. However, if maternal immune defences are overcome such that viable organisms reach the foetal side of the placenta, uncontrolled replication will occur in the trophoblast and a stereotypical pattern of pathological changes will ensue.

ACKNOWLEDGEMENTS

Robert Fisk, Louis Pentony, Martin Hill, Alan Kelly, Anne Church, Peter White, Eileen Dillon, Tom O'Donovan, Eoin Monks and Vincent Geraghty (Veterinary Research Laboratory, Abbotstown). Mary Gleeson, Brian Cloak and Christy King (Faculty of Veterinary Medicine, UCD). Dr L Dudler (Institute for Immunology, Basle) for kindly providing gifts of monoclonal antibodies and Ms B. Behnke (Medac GmbH) for providing rLPS-coated microtitre ELISA plates.

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