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Experimental Infection of Pregnant Ewes with *Chlamydia pecorum*

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Pregnant ewes were infected in midpregnancy with three isolates of *Chlamydia pecorum* derived from the feces of healthy lambs from three different farms. Oral infection, alone or together with *Fasciola hepatica*, did not result in tissue invasion, since all placental and fecal samples were negative for chlamydiae. Intravenous infection resulted in placental infection in 16 of 18 ewes in that chlamydiae were cultured from placentas or vaginal swabs. Two ewes bore dead lambs after a shortened gestation time. The chlamydiae isolated were all *C. pecorum*. There were no significant differences between the weights of the lambs from the infected groups and those from uninfected control ewes. Most ewes showed no serological evidence of infection by the complement fixation test; therefore, it is unlikely that the enteric subtype of *C. pecorum* is responsible for the cross-reactions sometimes seen in flocks being tested for *C. psittaci* infection.

Until recently, it was believed that all the chlamydial diseases in sheep, including abortion, polyarthritis, and conjunctivitis, were caused by *Chlamydia psittaci* and that the organism could also be found in the intestines of sheep with no clinical signs of disease. Fukushi and Hirai (5) differentiated a new species, *C. pecorum*, from *C. psittaci*, as the species responsible for diseases other than enzootic abortion. Both species have been isolated from the feces of sheep, although the majority of these isolates were probably *C. pecorum*. Prior to their separation as independent species, *C. psittaci* and *C. pecorum* were frequently referred to as abortion or invasive types or strains and enteric, intestinal, or noninvasive types of *C. psittaci*, respectively.

Clarkson and Philips (4) isolated *C. pecorum* from the feces of a high proportion of lambs on all 26 farms sampled in Britain, and some of these isolates were shown to be antigenically heterogeneous in contrast to the homogeneity seen in abortion isolates of *C. psittaci* (2, 6). Jones (9) has recently referred to subtypes of *C. pecorum*, depending on host and clinical significance, but these subtypes have not yet been differentiated by precise techniques. Examples of subtypes found in sheep are the arthritis/conjunctivitis subtype and the enteric subtype although the intestine may act as a reservoir for other invasive subtypes.

Rodolakis et al. (12) used two mouse models to compare the invasiveness of 10 intestinal isolates and 27 pathogenic isolates of *C. psittaci*, the majority of which were derived from abortion matter. Although the intestinal isolates were noninvasive when they were injected subcutaneously, they were capable of becoming established in the placentas of pregnant mice after intravenous (i.v.) injection; however, these isolates produced fewer chlamydiae than did the invasive abortion strains.

Little experimental work has been done to investigate whether *C. pecorum* can invade the placentas of pregnant sheep. Wilson and Dungworth (15) injected 12 pregnant ewes subcutaneously with an isolate from sheep feces, but none developed placental infection. Anderson and Baxter (1) injected four pregnant ewes subcutaneously with an isolate from the feces of a 3-month-old lamb, which also failed to infect the placentas. The circumstances of the isolation of these chlamydiae strongly suggest that they were *C. pecorum* whereas work in which isolates from the feces of sheep have resulted in placental infection (1, 14) strongly suggests that the species involved was *C. psittaci*.

The work described here was undertaken to clarify the virulence of several enteric subtypes of *C. pecorum* isolated from the feces of healthy sheep by their administration to pregnant ewes by oral and i.v. routes. Buzoni-Gatel and Rodolakis (3) suggested that intestinal lesions induced by other bacteria or parasites could promote penetration of the intestinal barrier by intestinal chlamydiae and thus provide the chlamydiae with circumstances for reaching the placenta. If this scenario were possible, abortion could result or, at least, it could be responsible for the cross-reactions which occur during testing for antibody to *C. psittaci* in sheep health schemes (9, 10). These possibilities were investigated by infecting pregnant ewes with *Fasciola hepatica* at the same time as *C. pecorum* since this helminth parasite penetrates the intestinal mucosa for a few days after infection.

MATERIALS AND METHODS

Chlamydial isolates. Three isolates were obtained from the feces of lambs on three different farms in Britain and were designated T22, T23, and T25. Isolates T22 and T23 were from farms where it was known that enzootic abortion did not occur, but T25 originated from a farm on which enzootic abortion occurred and *C. psittaci* had been isolated from fetal membranes. In all three cases, cultures derived from several infected lambs were pooled to establish the isolates.

Cultural methods. The methods used to isolate chlamydiae from rectal fecal samples from lambs have been described previously (4). In order to produce sufficient material to infect sheep, chlamydiae were grown in specific-pathogen-free fertile hens' eggs. Harvested yolk sacs were mixed with an equal volume of transport medium and frozen at −80°C. A small amount was inoculated onto a monolayer in order to calculate the titer (8). For use, the egg-grown material was thawed, sonicated, and centrifuged at 1,000 × *g* for 10 min, and the sediment and the upper fatty layer were discarded.

Experimental procedure. Ewes, purchased from farms where enzootic abortion did not occur, were bled, and their sera were tested by the complement fixation test for chlamydial antibodies at the Edinburgh Veterinary Investigation Centre. The majority of ewes had titers of 1 in 8, and the remainder had titers of 1 in 16, titers which are considered negative under the regulations of the official Sheep and Goat Health Scheme in Britain. The majority of the ewes were the Welsh Mountain breed, and a few were Welsh Mountain crosses. The ewes were estrus synchronized with intravaginal medroxyprogesterone sponges (Veramix; Upjohn Ltd.), and pregnancy was confirmed by ultrasound scanning 70 days after tupping.

The pregnant ewes were infected at 70 to 90 days of gestation.

Oral infection was done by means of a 5-ml syringe with a short length of

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TABLE 1. Plan of experiments and summary of main results

Ewe no.	Isolate used for infection	Dose (IFU, 10^6)[a]	Route of infection	*Fasciola* infection	Lambing results	Wt of lamb(s) (kg)	Gestation (days)	Isolation of chlamydiae[b]
1	T22	2.6	i.v.	No	2 live	4.0/4.0	148	+
2	T22	1.7	i.v.	No	1 live	3.0	142	+
3	T22	2.0	i.v.	No	1 live	2.8	142	+
4	T22	2.2	i.v.	No	1 live	2.3	140	+
5	T23	84	i.v.	No	1 live	3.2	144	+
6	T23	84	i.v.	No	2 live	2.0, 1.9	139	+
7	T23	84	i.v.	No	1 live	2.0	139	+ (VS)
8	T23	84	i.v.	No	1 live	2.6	140	+
9	T23	84	i.v.	No	1 live	2.2	140	−
10	T23	84	i.v.	No	1 abort	1.9	138	+
11	T23	84	i.v.	No	1 live	2.7	143	+ (VS)
12	T23	84	i.v.	No	1 dead	2.4	141	+ (VS)
13	T23	460	i.v.	No	1 live	3.2	146	+
14	T23	460	i.v.	No	1 abort	2.3	137	+
15	T25	100	i.v.	No	1 live	2.5	147	+
16	T25	100	i.v.	No	1 live	2.7	148	− (VS)
17	T25	100	i.v.	No	2 live	2.3, 1.9	139	+
18	T25	100	i.v.	No	1 live	3.0	147	+
19	T22	280	Oral	No	2 live	4.0, 2.9	147	− (VS)
20	T22	180	Oral	No	1 live	4.8	149	−
21	T22	1.7	Oral	No	2 live	2.8, 2.6	143	−
22	T22	2.0	Oral	No	1 live	2.9	143	−
23	T23	93	Oral	No	1 live	2.9	143	−
24	T23	93	Oral	No	1 live	3.5	147	−
25	T22, T23	100, 34, 67	Oral	No	1 live	2.8	145	−
26	T22, T23	100, 34, 67	Oral	No	2 dead	2.0	143	−
27	T22, T23	100, 34, 67	Oral	No	1 live	4.5	148	−
28	T22, T23	100, 34, 67	Oral	No	2 live	3.4, 2.8	146	− (VS)
29	T22, T23	87, 57, 63	Oral	Yes	1 live	2.8	143	−
30	T22, T23	87, 57, 63	Oral	Yes	1 live	2.9	146	−
31	T22, T23	87, 57, 63	Oral	Yes	2 live	3.4, 2.8	143	−
32	T22, T23	87, 57, 63	Oral	Yes	2 live	2.5, 2.5	146	−
33	T22, T23	87, 57, 63	Oral	Yes	1 live	3.3	147	−
34	T22, T23	87, 57, 63	Oral	Yes	2 live	2.8, 2.6	142	−
35	Uninfected sac		i.v.	No	1 live	3.8	147	− (VS)
36	Uninfected sac		i.v.	No	1 dead	3.8	147	−
37	None			No	1 live	3.6	143	− (VS)
38	None			No	1 live	3.4	148	− (VS)
39	None			No	1 live	4.5	146	−
40	None			No	1 live	2.4	147	−
41	None			No	1 live	2.8	147	−
42	None			No	1 live	4.0	148	−
43	None			No	2 live	3.5, 3.4	145	−
44	None			No	1 live	2.6	144	−
45	None			No	2 live	2.8, 2.2	142	−

[a] IFU, inclusion-forming units. Three doses indicated infection on day 0 with T23, on day 4 with T22, and on day 8 with T23, respectively.
[b] Fetal membranes were examined except where vaginal swab (VS) is indicated. +, chlamydiae isolated in culture; −, no chlamydiae isolated.

plastic tubing into the back of the mouth. i.v. infection was done by means of a 5-ml syringe and an 18-gauge needle into the jugular vein. After the ewes had been infected, they were housed in pens which held two ewes on straw bedding in an isolation unit. Because of the limited number of pens in the unit, the work was carried out as four experiments, but since the results were similar in each experiment, they have been combined.

For details on infection of the ewes, see Table 1. Briefly, 18 ewes were infected i.v.: 4 with T22, 10 with T23, and 4 with T25; 16 ewes were infected orally: 4 with T22 alone, 2 with T23 alone, and 10 with both T22 and T23. Four of the 10 ewes infected with both T22 and T23 were infected on day 0 with T23, on day 4 with T22, and on day 8 with T23. The other six were infected similarly and, in addition, were given 700 metacercariae of *F. hepatica*, each in a gelatin capsule on day 0. There were 11 uninfected controls, 2 of which were injected i.v. with yolk sac membranes from eggs which had not been infected with chlamydiae.

Blood samples, taken from the jugular veins of the ewes immediately before infection and 14 days after lambing, were tested for chlamydial antibody by a complement fixation test, which involved the use of a chlamydial group antigen, at the East of Scotland College of Agriculture.

Fecal samples were taken from the rectums of ewes and cultured for chlamydiae at twice weekly intervals from 1 week before infection to 30 days after lambing from four ewes infected i.v. and from four infected orally. Two controls were tested in a similar manner. Seven lambs from these ewes were also sampled and tested at approximately weekly intervals from birth to 70 days old.

The ewes were allowed to lamb normally. Placentas were collected, placed in individual sterile plastic bags, and cultured for chlamydiae. When placentas could not be found, vaginal swabs were cultured for chlamydiae.

Identification of chlamydiae. Chlamydiae were identified as *C. psittaci* or *C. pecorum* initially by their rate of growth, by the type of inclusion in primary culture in McCoy cells (1, 13), and by their ability to multiply in a sheep fibroblast cell culture (11). Further identification of selected isolates was done by restriction endonuclease profiles of DNA amplified by PCR as described previously (7).

RESULTS

Lambing results. The lambing results are summarized in Table 1. The 11 uninfected ewes produced 13 lambs, one of which was dead at birth, with a gestation time of 147 days. The 18 ewes which were infected i.v. with *C. pecorum* produced 21 lambs, 2 of which were regarded as abortions, since they were

born dead at gestation times of 137 and 138 days, respectively, and 1 was dead at birth, with a gestation time of 141 days. The 10 ewes which were infected orally produced 13 lambs, 1 dead at birth with a gestation time of 143 days. The six ewes infected orally and also with *F. hepatica* produced nine live lambs.

The mean gestation times for the groups were not significantly different: 145.7 ± 2.2 (standard deviation), 142.2 ± 3.6, 146.0 ± 2.4, and 144.5 ± 2.1 days for the uninfected ewes, i.v. infected ewes, orally infected ewes, and ewes infected orally and with *F. hepatica*, respectively.

The lamb mean weights for the groups were not significantly different from each other: 3.29 ± 0.68, 2.61 ± 0.62, 3.40 ± 0.95 and 2.84 ± 0.32 kg for the lambs from the uninfected ewes, i.v. infected ewes, orally infected ewes, and ewes infected orally and with *F. hepatica*, respectively. Macroscopically, the placental membranes from all ewes except those infected i.v. were normal, with bright red cotyledons and no intercotyledonary thickening. Placentas were obtained for 13 of the 18 ewes infected i.v., and 12 of which showed some intercotyledonary thickening and necrosis of the cotyledons. Most of the intercotyledonary thickening was localized to small areas immediately around the cotyledons, but placentas from five ewes, including the two which aborted, showed more-extensive thickening.

Isolation of chlamydiae from reproductive tracts. No chlamydiae were isolated from the placentas or vaginal swabs from the uninfected ewes or from any of those infected orally. Placentas were obtained from 13 of the 18 ewes infected i.v., and vaginal swabs were obtained from the remaining 5. Twelve placentas and four vaginal swabs were positive for chlamydiae by culture.

Identification of chlamydiae. The chlamydiae possessed cultural characteristics of *C. pecorum* in that they produced mature diffuse inclusions within 48 h and failed to infect a sheep fibroblast cell line. The genomic DNA of isolates from the placentas of sheep 3 and 4 infected i.v. with isolate T22, from placentas or vaginal swabs from sheep 5, 6, 7, 8, 10, 11, and 12 infected i.v. with T23, and from placentas or vaginal swabs from sheep 15 and 17 infected i.v. with T25 were analyzed as described previously (7). Amplified DNA fragments were digested with the enzyme *Alu*I, and the fragments were separated by polyacrylamide gel electrophoresis. All 11 isolates showed bands characteristic of *C. pecorum*, which were distinct from the pattern seen for *C. psittaci* (2). The isolated chlamydiae were similar to those used to infect the ewes.

Isolation of chlamydiae from feces. No chlamydiae were isolated from the feces of four ewes infected orally, four ewes infected i.v., or two uninfected ewes sampled twice weekly from 1 week before infection to 30 days after lambing. Chlamydiae were isolated from the feces of two of seven lambs sampled over the first 70 days of life. These lambs were twins from sheep 1, which had been infected i.v. with T22. One lamb had chlamydiae in its feces 30 and 38 days after birth, and the other was positive for chlamydiae 38, 42, and 52 days after birth. The chlamydiae were identified as *C. pecorum* by their growth characteristics in McCoy cells and their inability to grow in lamb fibroblast cells.

Serology. All the ewes had reciprocal titers of chlamydial complement-fixing antibody of 8 or 16 prior to infection. The majority showed no increase in titer after lambing, even when the ewe had a placental infection. After lambing, one i.v. infected ewe had a reciprocal titer of 32 and two i.v. infected ewes, one of which aborted, had titers of 64.

DISCUSSION

This study of the infection of pregnant ewes with *C. pecorum* produced clear-cut results. i.v. infection resulted in placental colonization whereas oral infection, even with high numbers of chlamydiae, did not. The ewes showed no evidence that the chlamydiae had become established in the alimentary tracts or in any other tissues. Since Wilson and Dungworth (15) and Anderson and Baxter (1) were unable to infect pregnant ewes with fecal isolates of *C. psittaci* (probably *C. pecorum*) by subcutaneous injection, enteric isolates of *C. pecorum* do not appear to be normally invasive in sheep. Similar results have been seen for subcutaneous and i.v. infections in mice (12).

It has been suggested that concurrent infection with other organisms may cause enteric chlamydiae to be able to colonize the placenta (3, 12); Jones et al. (10) speculated that serologically false-positive reactions for *C. psittaci* may be caused by intestinal infections with parasites, such as coccidia or helminths, which allow the enteric subtype of *C. pecorum* to change from a localized to a systemic infection. *F. hepatica* is a helminth which penetrates the small intestine and migrates through the peritoneal cavity to reach the liver, but concurrent infection with this parasite and oral infection with *C. pecorum* did not result in placental colonization. These results strongly suggest that the enteric subtypes of *C. pecorum* are unlikely to be invasive. Since most sheep which were infected i.v. did not show a serological response, even though placental invasion occurred, it also seems unlikely that enteric isolates of *C. pecorum* are responsible for cross-reactions in serological tests for enzootic abortion. Of 18 ewes infected i.v., only 2 showed a reciprocal titer of 64 and another had a titer of 32, which would be considered positive and questionable, respectively, by the official Sheep and Goat Health Scheme in Britain. It should be noted that the ewes were infected i.v. with chlamydiae grown in eggs and that egg proteins contribute to these titers, since the chlamydial group antigen used in the complement fixation test is also egg derived. Jones et al. (10) did not detect cross-reactions to enzootic abortion antigens in specific-pathogen-free lambs which had been vaccinated with an enteric subtype of *C. pecorum*. They suggested that false seropositivity in field samples for enzootic abortion may be associated with the arthritogenic/conjunctival subtype, since cross-reactions did occur with sera from specific-pathogen-free lambs vaccinated with this subtype.

Although 16 of 18 ewes injected i.v. showed placental infection, only 2 aborted whereas in other experiments with much lower doses of *C. psittaci* derived from aborting ewes, 70% of pregnant ewes aborted (unpublished observations).

Although no systemic infection resulted from oral infection, probably because these isolates are not invasive, the ewes were from farms on which enteric chlamydiae were common (4). It is possible that the inability to cross the intestinal barrier was a manifestation of immunity in the ewes rather than an intrinsic property of *C. pecorum*.

Despite frequent examination of the feces of the ewes which had been infected with large numbers of *C. pecorum*, no chlamydiae were isolated from the feces of any of the ewes, indicating that the bacteria must have been destroyed rapidly. This result may be evidence of a strong immune response, supporting the finding that chlamydiae could not be isolated from adult ewes from farms on which chlamydiae were detected in fecal samples from a high proportion of the lambs (4). No conclusions can be drawn from the finding that twin lambs from an infected ewe tested positive for chlamydiae in their feces on a few occasions since the infection may have been acquired from chlamydiae in the environment and not in the

uterus. There is a need for further work on the natural history of *C. pecorum* in lambs and on the relationship between the subtypes in the intestine and those responsible for diseases such as conjunctivitis and arthritis.

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