Studies on the Response of Ewes to Live Chlamydiae Adapted to Chicken Embryos or Tissue Culture

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ABSTRACT

Ewes infected before gestation with chicken embryo or tissue culture adapted chlamydial strain B-577 were challenge inoculated with the homologous strain at four to 18 weeks of gestation. The ewes responded with group specific complement fixing antibody titers of 1:8 to 1:256 by the second week after initial infection. A secondary antibody response in the surviving challenge inoculated ewes occurred at the time of lambing and reached titers of 1:32 to 1:256 by the second week after parturition. Group specific complement fixing antibodies did not appear to play a significant role in resistance to chlamydial infection.

Ewes infected with the chicken embryo adapted strain B-577 excreted chlamydiae in their feces 60 days after inoculation. However, chlamydiae were not recovered from feces of ewes infected with the tissue culture adapted strain B-577.

Placentas of ewes challenge inoculated by the intravenous route were consistently infected. Chlamydiae were recovered from placentas, some fetuses and lambs. In two instances when challenge inoculation was given by the intramuscular route, infection was detected only by the direct fluorescent antibody method.

RÉSUMÉ

Les auteurs ont inoculé des brebis avec la souche de Chlamydia B-577, adaptée à l'embryon de poulet et à la culture tissulaire, quelques semaines avant de les faire saillir. Ils les inoculèrent une seconde fois, avec la même souche, entre la quatrième et la 18ième semaine de gestation. Au cours de la deuxième semaine ultérieure à l'infection initiale, ces brebis recelaient des anticorps spécifiques fixant le complément et dont le titre variait de 1:8 à 1:256. Les brebis qui survécurent à la seconde inoculation manifestèrent une autre réaction immunologique, au temps de l'agnelage; au cours de la deuxième semaine après la parturition, la teneur de leur sérum en anticorps variait de 1:32 à 1:256. Les anticorps spécifiques fixant le complément ne semblèrent pas jouer un rôle important, relativement à la résistance à l'infection à Chlamydia.

Les brebis infectées avec la souche B-577 adoptée à l'embryon de poulet excrétèrent des Chlamydia dans leur fumier pendant 60 jours, contrairement à celles qu'on avait inoculées avec la même souche adaptée à la culture tissulaire.

Le placenta des brebis ayant reçu la deuxième inoculation par la voie intra-veineuse s'avéra constamment infecté; on recouvra le micro-organisme du placenta, de certains foetus et des agneaux. On ne réussit à déceler l'infection que par la méthode directe d'immunofluorescence, chez deux brebis qui avaient reçu la deuxième inoculation par la voie intramusculaire.

INTRODUCTION

Immune mechanisms functioning in preventing chlamydial abortion in animals are not fully understood (17). Earlier investigators (9, 10, 14) reported that killed and live vaccines effectively controlled chlamydial infection leading to abortion in ewes. However, failures of killed and live chlamydial vaccines also were reported (11, 13). It is known that sheep with intestinal chlamydial infection are susceptible to experimental superinfection (15). Resistance

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to chlamydial infections of ewes may well be influenced by previous exposure and inapparent infections but the mechanisms determining this relative resistance are not known.

We infected ewes prior to service with a fully virulent live chlamydial abortion strain adapted to chicken embryos or with its tissue culture adapted variant. These studies were designed to compare the immunogenic properties of these chlamydiae and to trace infectious events following challenge inoculation in order to identify mechanisms in the host response functioning during reinfection.

MATERIALS AND METHODS

Experimental Ewes

Twelve chlamydia free yearling ewes were divided into groups A and B. Ewes of group A were infected with the yolk sac adapted and ewes of group B with the tissue culture adapted strain B-577 four weeks before service. After breeding, infected ewes were kept in separate pens in a Disease Security Building where their immunity was challenged during gestation.

Infection and Challenge Inoculation

Ewes of group A were infected by the intramuscular (I.M.) route with 2.5 ml of yolk sac propagated chlamydial strain B-577 with an infectivity to $10^{8.16}$ chicken embryo lethal dose ($CELD_{50}$) per 0.5 ml. At between four and 18 weeks of gestation the immunity of these ewes was challenged by intravenous (I.V.) inoculation with 4.0 ml of a similar chlamydial preparation with an infectivity ranging from $10^{6.83}$ to $10^{8.83}$ $CELD_{50}$ per 0.5 ml. This strain was in its 13th to 15th passage in chicken embryos after isolation from an aborted lamb (16).

Ewes of group B were infected I.M. with 2.0 ml of tissue culture adapted strain B-577 in its 54th passage in mouse L cells (B-577 T.C. 54). Trypsin detached infected L cells from two monolayers each containing about 1.0 x 10^7 cells were harvested 72 hours after inoculation. The sonicated cell suspension and the infectious tissue culture fluids were combined and centrifuged at 7000 x G for 30 minutes to sediment chlamydiae. The pellet was suspended in 15.0 ml of Bovarnick's sucrose phosphate buffer (4) pH 7.2 containing 500 μg streptomycin per ml. The infectivity titer of the inoculum used for infecting the ewes was $10^{7.5}$ $CELD_{50}$ per 0.5 ml. At 14 and 17 weeks of gestation the immunity of five ewes was challenged by I.V. or I.M. inoculation with 2.0 ml of the yolk sac propagated, partially purified strain B-577 in its 21st passage in chicken embryos (B-577 Y.S. 21). This partially purified inoculum was prepared as described previously (2) and its infectivity titer was $10^{7.5}$ $CELD_{50}$ per 0.5 ml. The sixth pregnant ewe was not challenge inoculated but used as a contact control.

Clinical Examinations and Testing for Fecal Chlamydial Shedding

Animals were observed daily for clinical signs of disease throughout the duration of the experiment and their rectal temperatures were recorded for seven days following infection and challenge inoculation. Total and differential white blood cell counts were determined for seven days after initial infection and challenge inoculation of ewes in group B.

About 60 days after infection of ewes in group A and three, six, 15 and 21 days postinfection of ewes in group B, fecal samples were tested for chlamydiae. Procedures for decontamination of the fecal material and chlamydial isolation were described elsewhere (15).

Serological Determinations

Group specific chlamydial complement fixing (CF) antibody titers were determined by the microtiter technique in the serum of ewes of group A and B. Twofold serum serial dilutions were reacted with two units of a known chlamydial antigen in the presence of two units of complement. The highest serum dilution fixing the two units of complement (4+reaction) was taken as the end point.

Blood samples for collecting the serum were taken before and at seven day intervals following infection and challenge inoculation. In ewes of group A, sampling concluded the second week and in those of

TABLE I. Antibody Response of Ewes in Group A First Infected and Challenge Inoculated with Chicken Embryo Adapted Chlamydial Strain B-577

Ewe No.	State of Gestation	CF Titers[a] 0	10D[b]	14D	Pre-chall.	7D	14D	Fecal Excretion of Chlamydiae after Infection
527	4W[c]	8	16	16	8	32	N.T.[d]	+[e]
NN	6W	8	32	32	8	32	N.T.	+
590	8W	8	32	32	16	32	N.T.	−[f]
633	12W	16	64	16	8	32	N.T.	+
550	16W	8	16	16	16	64	32	+
522	20W	8	16	8	16	64	N.T.	+

[a]Titers are expressed as the reciprocal of the highest serum dilution with a 4+ reaction
[b]D = days
[c]W = weeks
[d]N.T. = Not tested (ewes were killed after seven days post challenge inoculation)
[e]+ = Chlamydiae isolated
[f]− = Chlamydiae not isolated

group B the 16th week postchallenge inoculation. Serum from fetuses and newborn lambs was not collected because chlamydial infection was traced by direct isolation.

Distribution of Chlamydial Infection After Challenge Inoculation

Because we wanted to identify the sites of chlamydial infection all ewes of group A were necropsied seven to 15 days after challenge inoculation.

Samples of liver, spleen, kidneys, lungs, mammary glands and lymph nodes from the dam were taken for chlamydial isolation in chicken embryos. Portions of fetal and maternal placentomes were taken for chlamydial isolation and preparation of impression smears to be examined for the presence of chlamydiae after staining by the Gimenez method (7) using basic fuchsin and malachite green stains. Fetal liver, lungs, kidneys, spleen, thymus, testicles, brain and intestine were also tested for chlamydiae by culturing in chicken embryos. Parallel samples from these tissues were then sectioned with a cryostat and stained by the Gimenez method. In the embryonic state the whole embryo was taken for isolation studies.

Ewes from group B first infected with the tissue culture adapted variant and challenge inoculated with the chicken embryo adapted strain were allowed to lamb and their newborn lambs were necropsied. Specimens of spleen, liver, kidneys, lungs, thymus, testes, brain, mesenteric lymph nodes and ileum were taken for chlamydial isolation and cryostat sectioning. Representative cotyledon and intercotyledonary samples were taken for isolation of chlamydiae, impression smears and sectioning. Specimens were stained by the direct fluorescent antibody (FA) technique (5) and then processed further for relocation checks (3). The FA stained preparations were unmounted, washed in phosphate buffer saline and restained by the Gimenez method. Thus previously fluorescent structures were relocated to confirm the presence of chlamydiae. Fluorescein isothiocyanate conjugated B-577 antiserum was adsorbed with ovine spleen tissue as described (8).

Isolation procedures for chlamydiae from noncontaminated tissue samples were previously reported (6). Samples from the intestinal mucosa and contaminated placentas were freed of bacteria by methods described for decontaminating fecal material (15).

RESULTS

Clinical Response

Ewes infected with live chicken embryo or tissue culture adapted chlamydiae developed temperatures of 41.9 to 42.1°C within 24 hours postinoculation and remained febrile for two to three days. Some ewes infected with chicken embryo adapted chlamydiae had a biphasic temperature response which levelled off by the sixth day. Challenge inoculated ewes responded with

TABLE II. Antibody Response of Ewes in Group B First Infected with the Tissue Culture Adapted and Challenge Inoculated with Chicken Embryo Adapted Chlamydial Strain B-577

Weeks after Challenge Inoculation	CF Titers[a] in Serum of Ewes				
	A265[b]	A285	A 290	A298	A299
1	<4	<4	<4	<4	<4
2	<4	<4	<4	4	<4
3	<4	<4	16	4	<4
4	<4	<4	8	4	<4
		Lambing		Lambing	
5	<4	<4	4	4	<4
6	<4	64	8	256	<4
	Lambing		Lambing		Lambing
7	<4	4	4	32	<4
8	<4	<4	32	4	<4
12	<4	<4	<4	<4	<4
16	<4	<4	<4	<4	<4

[a]T iters are expressed as the reciprocal of the highest serum dilution with a 4 + reaction
[b]Contact control was not challenge inoculated

fever ranging from 41.1 to 41.7°C for about two days.

Most ewes infected with tissue culture adapted chlamydiae (group B) developed a transient leukocytosis with neutrophilia in parallel to the temperature rise. After challenge inoculation total white blood cell counts remained within the normal range even though a transient neutrophilia occurred.

Antibody Response

Group specific chlamydial CF antibody titers of ewes infected and challenge inoculated with chicken embryo adapted strain B-577 are given in Table I. The antibody response to the challenge inoculation was higher than that following initial infection in ewes nos. 522, 527 and 550.

Ewes infected with strain B-577 T.C. 54 responded with antibody titers ranging from 1:4 to 1:64 a week postinfection. Titers increased by a factor of four in serum of ewes A 265, A 285 and A 290 or by a factor of eight in ewes A 283, A 298 and A 299 a week later. A gradual decline in antibody titer occurred thereafter in all ewes. After challenge inoculation with strain B-577 Y.S. 21, ewes A 285, A 290 and A 298 responded with antibody titers of 1:32 to 1:256 (Table II). The antibody rise in these three ewes occurred after parturition as illustrated by the response in ewe A 298 (Fig. 1). Ewes A 285 and A 298 had the highest antibody titers and their placentas had gross lesions. Challenge inoculated ewe A 299 and the contact control ewe A 265 did not respond with CF antibody titers. Ewe A 283 was excluded since it died at the sixth day as consequence of uterine prolapse and abortion that occurred 24 hours after challenge inoculation.

Shedding of Chlamydiae in Feces after Initial Infection to Stimulate Immunity

Ewes 522, 527, 550, 633 and NN of group A were shedding chlamydiae in their feces

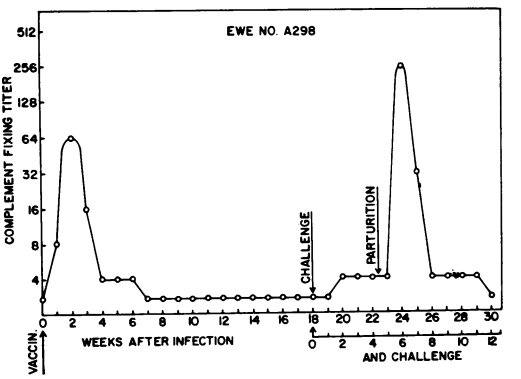

Fig. 1. Group specific CF antibody response in ewe A 298 infected before pregnancy with the tissue culture adapted chlamydial abortion strain B-577. This ewe was challenge inoculated with the yolk sac propagated homologous strain 18 weeks after infection and in the 17th week of gestation.

TABLE III. Distribution of Chlamydial Infection in Placentas and Fetuses or Lambs of Group A and B after Challenge Inoculation

Group	Ewe No.	Placenta	Fetal or Lamb Tissue						
			Liver	Spleen	Kidney	Thymus	Testis	Brain	Ileum
A	522	N.T.[a]	−[b]	−	−	−	F[c]	N.T.	N.T.
	527	+[d]	−	N.T.	N.T.	N.T.	F	−	−
	550	+	+	+	+	+	F	N.T.	N.T.
	590	+	−	N.T.	−	N.T.	F	N.T.	−
	633	+	F_1[e] −	−	−	−	F	N.T.	N.T.
			F_2[f] −	−	−	+	−	N.T.	N.T.
	NN	−	−	N.T.	−	N.T.	F	−	−
B	A265[g]	−	−	−	−	−	F	−	−
	A285	+	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
	A290	−	−	−	−	−	F	−	−
	A298	+	−	−	+	−	−	+	+
	A299	−	−	−	−	−	−	−	−

[a] N.T. = Not tested
[b] − = Chlamydiae not isolated
[c] F = Female
[d] + = Chlamydiae isolated
[e] F_1 = Fetus No. 1
[f] F_2 = Fetus No. 2
[g] = Contact control was not challenge inoculated

when tested about 60 days after initial infection (Table I). Chlamydiae were not recovered from the feces of infected ewes of group B when tested up to the 21st day after infection.

Gross Lesions

Except for ewe 522, no lesions were observed in placentas or fetuses of ewes of group A which were examined seven or 14 days after challenge inoculation. The intercotyledonary areas of the placenta from ewe 522 were congested and the thymus of her term lamb had petechial hemorrhages.

Placentas of ewes A 285 and A 298 from group B had pathological changes. Lesions in placenta from ewe A 285 consisted of cloudy thickening of the peri- and intercotyledonary tissue. The intercotyledonary tissue of the placenta of ewe A 298 was thickened and covered with necrotic yellowish material. The lamb born to this ewe was weak and had enlarged mesenteric lymph nodes and congested meninges.

Distribution of Chlamydial Infection in Ewes and Lambs

Chlamydiae were not isolated from the somatic organs of ewes of group A. Out of five placentas tested, chlamydiae were isolated from four which were also positive in Gimenez-stained impression smears. Chlamydial infectivity in the placentomes ranged from traces in ewe 527 to titers of $10^{2.83}$ $CELD_{50}$ per 0.5 ml in ewe 590. Chlamydiae were isolated from the thymus of one of the two fetuses of ewe 633 and from the liver, kidney, spleen and thymus of one of the two lambs of ewe 550 (Table III).

Among ewes of group B, chlamydiae were isolated from the placenta of ewe A 285 and A298 (Table III) at titers exceeding $10^{2.5}$ $CELD_{50}$. Sections and impression smears from these placentas were FA positive and this reaction was further confirmed in relocation checks after restaining by the Gimenez method.

Chlamydiae were not isolated from placentas of ewes A 290 and A 299. However, sections from these placentas were FA positive. Infection was not confirmed by relocation checks in these preparations. Placental impression smears of these two ewes were FA negative. Chlamydiae were not isolated from the placenta of the contact control, ewe A 265. Her placental sections were FA positive but the corresponding impression smears were FA negative. Infection could not be confirmed by the relocation checks.

Chlamydiae were isolated from the brain, ileum and kidney of the lamb from ewe A 298 (Table III). Except for testes, sections of all the other organs from this lamb were FA positive but infection was confirmed by relocation checks only in spleen sections. Infection was detected by FA in liver, lung and mesenteric lymph nodes of the lamb from ewe A 290 and lung and mesenteric

lymph nodes of the lamb from ewe A 299. No specific fluorescence was observed in sections of different organs of the lamb from the contact control, ewe A 265.

DISCUSSION

Immunity of pregnant ewes to chlamydial infection leading to placental and fetal infection and abortion seems to be a subject that is not completely settled (17). We infected ewes prior to breeding with chicken embryo adapted or tissue culture adapted live chlamydiae to stimulate their immune response. The immunity of these ewes was challenged by intravenous inoculation at different stages of gestation. The infectious events that occurred following challenge inoculation were traced by isolation of chlamydiae in chicken embryos, by examination of properly stained impression smears and by immunofluorescence followed by relocation checks. Our results confirm the findings of Studdert and McKercher (19) and are in disagreement with those of Schoop *et al* (14) who claimed to have induced protection of pregnant ewes with a "live attenuated" chlamydial vaccine. As reported by these workers, their live vaccine prevented or reduced abortions of pregnant ewes under field conditions. They did not determine the infectivity of their live vaccine nor did they attempt to trace chlamydial multiplication in the vaccinated sheep.

In our studies five of six ewes inoculated with live chicken embryo adapted chlamydiae excreted them in the feces by the second month after infection. These ewes and those inoculated with tissue culture adapted chlamydiae did not develop sufficient resistance to prevent placental infection after intravenous challenge inoculation.

Attenuation of chlamydiae is an unsolved problem and it is not known under which conditions this can be accomplished. Adaptation to chicken embryos maintains the pathogenic potential of chlamydiae. Studies on the biological and pathogenic properties of chlamydial strain B-577 during adaptation to mouse L cells clearly indicated that this strain remained pathogenic (3). It infected the placentas and fetuses of pregnant ewes, remained highly virulent for chicken embryos and caused infection in man (3). One should possibly search for naturally occurring chlamydial strains of low pathogenicity and high antigenicity.

Ewes of group A and B responded with group specific CF antibodies to both the chicken embryo adapted or its tissue culture adapted variant. Other workers (9, 10, 14) interpreted the serological response of the host as an indication of a stimulated immunological defense. Group specific CF and neutralizing chlamydial antibodies were used as parameters to evaluate the immunogenicity of killed and live chlamydial vaccines but this aspect clearly needs further analysis.

Mechanisms of immunity and resistance to chlamydial infection seem to be complex. There is clear evidence for an enhanced response and increased resistance to reinfection in sheep that had previously experienced chlamydial infection. Ewes previously exposed cleared chlamydiae from their blood at an enhanced rate regardless of whether they were pregnant or not and secondary chlamydemia was of short duration or did not develop (1). Chlamydial multiplication in cells of somatic organs of ewes was detected at significant levels for one to eight days after initial infection (18). In contrast, chlamydiae were not recovered from the somatic organs of ewes seven days after challenge inoculation. However, there was no clear correlation between clearance capacity and the presence of CF, agglutinating or neutralizing antibodies (1). Enhanced clearance reflects a more efficient uptake of chlamydiae by cells and suppression of chlamydial multiplication in cells of somatic organs characteristic of immune animals resulting in a relative resistance to infection (1). These observations point to mechanisms of cell mediated immunity which were also found by others to be a most important component in the immune response for prevention of chlamydial abortions (12).

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