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   EPIZOOTIC BOVINE ABORTION: TISSUE
   PREDILECTION AND SEROLOGIC EVALUATION OF
   THE ETIOLOGIC AGENT.

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THESIS

EPIZOOTIC BOVINE ABORTION: TISSUE PREDILECTION AND

SEROLOGIC EVALUATION OF THE ETIOLOGIC AGENT

Submitted by

David E. Reed

In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

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March, 1971

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR

SUPERVISION BY DAVID E. REED ENTITLED <u>EPIZOOTIC BOVINE ABORTION:</u>

<u>TISSUE PREDILECTION AND SEROLOGIC EVALUATION OF THE ETIOLOGIC</u>

<u>AGENT</u> BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

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ABSTRACT OF THESIS

EPIZOOTIC BOVINE ABORTION: TISSUE PREDILECTION AND

SEROLOGIC EVALUATION OF THE ETIOLOGIC AGENT

Twenty-three heifers in the 2nd and 3rd trimesters of gestation
were inoculated with the epizootic bovine abortion (EBA) agent EBA
59-795. Clinical signs of illness in inoculated heifers were first
apparent as febrile responses to maxima of 41.2 to 42.4°C. Eleven
(48%) of the animals showed biphasic temperature responses with the
first peak occurring 4 to 6 hours after inoculation and the second
(higher) peak occurring 12 to 24 hours post inoculation. Nine of
the 23 infected heifers aborted or showed signs of impending abortion
27 to 49 days after inoculation. The abortion of 3 other heifers
between the 5th and 12th post inoculation day was thought to possibly
represent toxic reactions to the inocula. Scheduled euthanasia of
the remaining 11 animals precluded obtaining data on the true abortion
incidence. All heifers sampled later than 6 days after inoculation
responded with significant increases in group-specific Chlamydia CF
titers. The rise in CF titer was generally evident by 6 days post
inoculation and reached a stable maximum by 8 to 9 days after inocu-
lation. The 12 control animals which received placebo inoculations
neither aborted nor showed significant increases in CF titers.

Data obtained from attempts to culture chlamydia from the experi-
mentally infected heifers showed that the EBA agent could be recovered
routinely only from infected placentas. Non-placental organs of the
dams (other than intestines and internal iliac lymph nodes) were
evidently cleared of the infection by 7 days after inoculation. Compar-
ison of the isolation results from 2nd and 3rd trimester fetuses

showed apparent differences in fetal infectability. Inconsistent
isolation results were obtained from the 12 fetuses inoculated in the
2nd trimester. Cultures of these fetuses showed sporadic positive
isolations spread from 5 to 49 days after inoculation. These results
contrast sharply with the isolation results obtained from the 11 3rd
trimester fetuses as no positive isolations were made from these
prior to 29 days after inoculation.

The presence of intestinal chlamydial agents in both control and
infected cattle before and after inoculation prompted studies designed
to differentiate between the intestinal agent and the EBA agent. Using
the complement fixation (CF) test with species-specific antigens (pre-
pared from chlamydial cell walls) and rabbit antisera, complete antigenic
cross reaction was seen between the intestinal isolate and the EBA
agent. The finding that inoculation of the intestinal agent into
a pregnant heifer resulted in abortion and the development of lesions
identical to those produced with the EBA agent, supports the conclusion
that the 2 agents are the same.

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CHAPTER 1

INTRODUCTION

Epizootic Bovine Abortion (EBA) is an abortion syndrome caused by an infectious agent of the genus <u>Chlamydia</u>. It was first described as a separate disease entity in California (Howarth <u>et al</u>., 1956) where the resulting abortions caused considerable economic loss. The disease was subsequently reported in other areas (Storz <u>et al</u>., 1967 and 1968) and other countries (Bargai, 1966; Bassan, 1966; Schoop et al., 1965; Popovici, 1964; and Berberovic, 1961).

The work presented here was designed to (1) achieve a greater understanding of the pathogenesis of experimental EBA through study of the organ predilection of the agent, (2) determine antigenic relationships between EBA and a chlamydial agent found to be preexisting in the experimental cattle, and (3) study the ultrastructural morphology of a chlamydial agent grown in cell culture.

The research project of which this thesis research is a part was the joint effort of the author, in the Department of Microbiology, and Doctors S. D. Lincoln and R. P. Kwapian in the Department of Pathology. Doctors Lincoln and Kwapian confined their studies primarily to the pathology of the Dams, fetuses and intact placentas. Where necessary for clarity, certain results and conclusions obtained by these two co-investigators are incorporated into this thesis with their permission.

REVIEW OF THE LITERATURE

Epizootic Bovine Abortion

History

A unique bovine abortion syndrome from which no etiologic agent could be recovered was first reported in California (Traum and Hart, 1923). The disease was characterized by abortions during late gestation and was thought to be caused by vaginal examination for pregnancy. The syndrome was again investigated by Howarth et al. (1956) who named it epizootic bovine abortion (EBA). They reported a clinical picture of subcutaneous edema, ascites and hemorrhage in fetal tissues and a pronounced fetal hepatopathy. Their report was similar to one case reported by Traum and Hart (1923). Howarth and coworkers (1958) were able to reproduce the disease by fetal inoculation of tissue suspensions from aborted fetuses. Again the etiologic agent could not be recovered. Similar outbreaks of bovine abortion diseases were reported in Germany (Schoop and Kauker, 1956; Beer, 1958, and Beer and Martin, 1958, 1959).

A study of Schoop and Kauker (1956) presented serologic evidence that chlamydial agents might be involved in the abortion syndrome. Adequate precedence for this had already been established by Stamp et al. (1950) and McEwen and Foggie (1955) who had experimentally produced abortion in cattle with the chlamydial agent of enzootic abortion of ewes (EAE). Further evidence of a chlamydial role in abortion came with the work of Enright et al. (1958) on the chlamydial disease of sporadic bovine encephalomyelitis (SBE) in California. One of the herds included in their serologic study experienced a high incidence of abortion. One month after the abortions three dams in the

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herd demonstrated significant complement fixation (CF) titers against SBE antigen. All cattle had negative SBE titers before abortion.

The first actual isolation of a chlamydial agent from a bovine abortion case was made by Giroud (1957) in France. He demonstrated chlamydial CF titers in cattle which had recently aborted and, in one instance, isolated chlamydial organisms which he called neo-rickettsia. Storz et al. (196u) continued the etiologic studies of Howarth in California and were able to recover chlamydial agents from five aborted fetuses. Since 1960, chlamydia have been incriminated in cases of bovine abortion in Yugoslavia (Berberovic, 1961), Bulgaria (Milanov and Chilev, 1963), Romania (Popovici, 1964), Germany (Schoop et al., 1965), Israel (Bargi, 1966 and Bassan, 1966), and in the United States in Utah (Storz et al., 1967) and Colorado (Storz et al., 1968).

Epizootiology

The natural reservoir of infection and the mode of transmission of EBA are presently unknown. Possible methods of spread include insect, venereal, and oral transmission. Infection sources which have been postulated include insects, birds, and mammals.

Arthropod transmission was suggested and generally ruled out through lack of evidence for an insect reservoir and lack of a viremia of sufficient magnitude and duration to infect biting insects (McKercher, 1964 and McKercher et al., 1966). Storz et al. (1967) supported this by reporting an EBA outbreak during the winter when insect transmission was considered unlikely. These observations must be tempered by those of Giroud et al. (1965) and Eddie et al. (1962) who were able to recover chlamydia from ticks and lice.

Venereal transmission was postulated by McKercher (1964) who demonstrated a decreased incidence of abortion with the use of artificial insemination. Later work by McKercher et al. (1966) cast doubt on this possibility through their inability to produce abortion in cows bred with infected semen. Storz et al. (1968) demonstrated chlamydial agents associated with a bovine seminal vesiculitis syndrome (SVS) in Colorado. • With the serum neutralization (SN) test, the agent of SVS was antigenically indistinguishable from EBA. Based on these findings, the possibility of venereal transmission cannot be eliminated.

Intestinal chlamydial infections of calves have been reported by numerous researchers (York and Baker, 1951 and 1956; McKercher and Wada, 1959; French, 1959; Bogel, 1961; Popovici, 1964). Inapparent intestinal infections of adult cattle have been reported by Matumoto et al. (1955), Kawakama et al. (1955) and Wilson (1962). The presence and frequency of these "normal" intestinal inhabitants leads one to suspect a role for them in the epizootiology of EBA and possibly other chlamydial infections of cattle.

Omori et al. (1957) studied similar inapparent intestinal infections of goats. They were able to recover chlamydia not only from feces but also from the dust on body surfaces of the goats. Further studies by Omori et al. (1960) demonstrated excretion of chlamydia by both healthy and clinically ill cattle. They concluded that bovine chlamydial infections were most probably transmitted via contaminated feces. The infection might be acquired by inhalation of contaminated dust or ingestion of contaminated feces.

The sheep abortion syndrome, EAE, is a close counterpart of EBA in cattle (McKercher et al., 1964). The relationships between EAE

infection and sheep intestinal infection were studied by Storz (1966) who showed that the type of intestinal infection depended upon the type of chlamydial disease currently epizootic in the flock. Thus, in an EAE outbreak, unaffected animals could be found to shed chlamydia indistinguishable from the EAE agent. In outbreaks of chlamydial polyarthritis, the fecal agent was identical with the polyarthritis agent. The polyarthritis and EAE agents were readily distinguishable by the SN test (Marriot and Storz, 1966).

Pathogenesis

Storz and McKercher (1962) concluded that, in experimental EBA, the agent established a systemic fetal infection via the umbilical vessels. This conclusion was drawn from the following results:

(1) The agent was found to be distributed throughout the fetus, thus indicating hematogenous spread.

(2) There appeared to be a correlation between development of placental lesions and development of fetal lesions.

The only other possible route of fetal infection is through primary infection of the fetal placenta and secondary infection of the fetus by ingestion of infected amnionic fluid. Doubt was cast on this route by Storz and McKercher (1962) because of their infrequent isolations from the meconium and absence of positive isolations from the stomach contents of infected fetuses.

Abortion of the fetus has generally been attributed to the severity of fetal infection rather than severity of the placental infection (Kennedy et al., 1960; Storz and McKercher, 1962). This was in contrast to EAE where the placentitis seen was often quite severe (Stamp et al., 1950 and Novilla, 1967).

Reference to these agents as "viruses" is evidently incorrect

The general properties of <u>Chlamydia</u> have been summarized by Moulder (1966) and are as follows:

1. Chlamydia are capable of growth in size and division by binary fission. All members of the genus apparently go through the same complex developmental cycle with several different morphologic forms being in evidence. Unlike the viruses, the morphologic integrity of chlamydia are maintained during the infection; that is, the infectious agent is not reduced to its nucleic acid at the time of infection.

2. Chlamydia contain both RNA and DNA. There are ribosome-like RNA bodies and dense DNA nucleoids in the mature infectious forms. Larger, immature forms may contain no dense nucleoid. The DNA in these forms is present as numerous filaments of DNA.

3. Chlamydia have an outer cell wall and an inner cell membrane. They have also been found to contain small amounts of muramic acid.

4. Chlamydia have several enzyme systems but no energy-yielding Lipman System.

With this information, Moulder concluded "....that the micro-organisms of the psittacosis group are obligate intracellular bacteria without relationship to viruses."

Antigenic Relationships

Bedson (1936) first reported a group-specific complement fixing antigen common to all members of the genus <u>Chlamydia</u>. Heat stability experiments with this antigen showed it to be highly stable to boiling temperatures. Bedson's work with this heat stable (hereafter refered to as group-specific) antigen led to discovery of a heat labile component of his crude antigen preparations. He showed that immune

serum could be cleared of all group-specific antibody by repeated
adsorbtion of the serum with boiled group-specific antigen. After
adsorbtion, the serum would no longer react (by the CF test) with
boiled antigen but would still react with unboiled crude group-
specific antigen preparations. Later work by Bedson et al. (1949)
demonstrated that serum adsorbed with boiled group-specific
antigen would show a CF reaction only with homologous, unboiled
antigen. Thus, Bedson concluded that the antigen component which
was destroyed by boiling was species-specific.

Group-Specific CF Antigen. Bedson's work in 1936 showed that the
act of boiling the crude antigen preparations greatly enhanced their CF
properties. Later work by Davis (1949) showed the same enhancement by
addition of 1% phenol to the preparations. Hilleman and Nigg (1948)
purified the antigen from infected chick embryo allantoic fluid by
extraction with ether and precipitation with acetone and alcohol. This
purified antigen had no antigenic activity unless lecithin of egg yolk
or vegetable origin was added.

The properties of the group antigen were summarized by Moulder
(1966) who concluded that group antigen probably was contained in the
cell wall of the chlamydial agent. The resistance to heat and trypsin
and the sensitivity to periodate and lecithinase shown by the group
antigen suggested that the antigenic moiety contained polysaccharide
and lecithin (Moulder, 1966).

Species-Specific CF Antigen. Little work was done with the species
specific (heat-labile) component reported by Bedson (1949) because its
detection required the use of serum which had been reacted with boiled
antigen. Ross and Gogolak (1957) were able to prepare species-specific

CF antigens which could be used to test sera without prior adsorption. This they did by destruction of the group-specific moiety by periodate or lecithinase treatment. Their species-specific antigens from the chlamydial agents of feline pneumonitis and psittacosis showed no cross reactivity while the group-specific antigens (before periodate or lecithinase treatment) showed nearly complete cross reactivity. A comparison of some of the properties of species-specific and group-specific antigens is given in Table 1.

Table 1.--Properties of Group-Specific and Species-Specific Antigens of Chlamydia*

Treatment	Group-Specific	Species-Specific
Boiling	Stable	Labile
Periodate	Labile	Stable
Trypsin	Stable	Stable
Lecithinase	Labile	Stable
SDOC	Soluble	Insoluble
Ether	Soluble	Insoluble
Acetone	Insoluble	---

* Summarized from Hilleman and Nigg (1948), Ross and Gogolak (1957), Jenkin (1960) and Moulder (1966).

Both the species-specific and group-specific CF antigens are probably integral parts of the chlamydial cell walls (Jenkin, Ross, and Moulder, 1961; Fraser and Berman, 1965). Satisfactory species-specific antigen preparations therefore awaited the development of a method for purification of chlamydial cell walls. This method was first devised by Jenkin (1960). The basis of this method came from the work of Schaechter et al. (1957) who prepared rickettsial cell walls by extraction with 11% sodium deoxycholate (SDOC) at 45°C. Jenkin, Ross and Moulder (1961) and Ross and Jenkin (1962) demonstrated that group-specific activity resided in the SDOC soluble fraction and the species

specific CF activity resided in the SDOC insoluble fraction. In CF
tests, their cell wall (SDOC insoluble) preparations of the chlamydial
agents of meningopneumonitis and feline pneumonitis showed no cross
reactivity while group CF antigens demonstrated complete cross reactiv-
ity. Fraser and Berman (1965), using a method similar to that of Jenkin,
Ross and Moulder (1961), were able to group 14 chlamydial strains into
7 antigenic subgroups. It is of interest to note that they assigned the
agents of EBA and EAE to a subgroup distinct from such agents as SBE,
ovine polyarthritis and the normal bovine intestinal isolate of York
and Baker (1951).

Ultrastructural Morphology

Members of the genus Chlamydia have been shown by light microscopy
to have a complex developmental cycle (Bedson and Bland, 1932; Bedson,
1933). More recent ultrastructural studies have shown this cycle in
much detail and were the basis for reclassifying the chlamydial agents
as bacteria (Page, 1966; Moulder, 1966).

The ultrastructural studies of Armstrong and Reed (1964, 1967) and
Higashi (1964, 1965) demonstrated the following developmental cycle:

1. Cells are infected by phagocytosis of the elementary body.
The stage of active phagocytosis lasts approximately two hours after
inoculation.

2. After the elementary body has been ingested, it undergoes a
series of changes which include enlargement of the particle and ex-
pansion of its dense nucleoid. By six hours after inoculation, the
elementary body has expanded to approximately 0.5 to 1.0 microns in
diameter and the dense nuclear material has dispersed. This form
is called the initial body.

3. In the period between 12 and 24 hours after inoculation, the initial body migrates toward the center of the cell and aggregates with other initial bodies in the golgi area. The morphology of this juxtanuclear inclusion apparently varies with the type of fixative used in preparing the culture for study. Some authors (Mitsui et al., 1964; Tajima et al., 1957) have reported the presence of a virus-like matrix in this area. The matrix is described as a reticulated matrix (Mitsui et al., 1964) or an amorphous mass which may or may not have an incomplete membrane bounding it (Tajima et al., 1957). With better fixation techniques, Higashi (1964) demonstrated that this viral matrix might be artifact of preparation and that the presence of "viral" material in the cytoplasm (unbounded by membranes) could be the result of rupture of the vacuole membrane and consequent outflow of the vacuolar contents. This hypothesis was supported by the findings of Armstrong and Reed (1964) who found that the rupture of these membranes was completely prevented by the use of Kellenberger's fixative (Kellenberger et al., 1958). Thus the juxtanuclear inclusion was shown to contain discrete initial bodies instead of a viral matrix (Armstrong and Reed, 1964).

4. From 12 to 72 hours post inoculation, division of the initial bodies occur (Armstrong and Reed, 1967). By 24 hours, dividing initial bodies begin to decrease in size and the intermediate forms are seen. Reduction in size and condensation of the internal components continue until, at 72 hours, the mature elementary bodies predominate in the inclusion.

5. Release of the mature elementary bodies from inclusions may occur either by cell lysis (Becerra, 1969 and Officer and Brown, 1961)

or by rupture of the inclusion without cell destruction (Officer and Brown, 1961).

A summary of chlamydial morphologic forms is as follows:

1. The elementary body (small particle) is a sphere of dimensions 0.25 to 0.30 microns. It is composed of an electron dense nuclear zone and a lighter cytoplasmic zone (Higashi, 1964; Armstrong and Reed 1964, 1967). The nuclear zone is shown in very thin sections to consist of fine filaments (Armstrong and Reed, 1967). The elementary body is bounded by two membranes each of which resembles the typical unit membrane (Armstrong and Reed, 1967; Armstrong, 1968). The outer membrane is considered a cell wall while the inner membrane is the cell membrane Armstrong and Reed, 1967; Anderson et al., 1965; Higashi, 1964).

2. The initial body shows a structure different from that of the elementary body. Its dimensions range from 0.5 to 1.0 microns and its shape is usually nearly spherical (Armstrong and Reed, 1964). Dumbbell shapes suggestive of binary fission are frequently seen (Higashi, 1964; Anderson et al., 1965). The internal structure of the initial body shows a reticular network of low density and numerous ribosome-like granules (Armstrong and Reed, 1967; Armstrong, 1968). Like the elementary body, the initial body has a cell wall and an underlying cell membrane (Armstrong and Reed, 1964).

3. Intermediate bodies are also frequently seen (Higashi, 1964; Armstrong and Reed, 1964; Anderson et al., 1965). These, as the name would suggest are morphologically intermediate between the elementary body and the initial body. Thus, the intermediate forms contain both the dense nucleoid and a reticular matrix of low density.

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

Thirty-eight Hereford yearlings were obtained from the Hartsel ranch, Hartsel, Colorado. All were nonpregnant and had been vaccinated against brucellosis and infectious bovine rhinotracheitis. The heifers were bred artificially with semen from a single Holstein bull. Conception was confirmed 45 days later by rectal examination. During this time two serum samples from each animal were taken two months apart to test for the presence of preinoculation chlamydial antibodies.

Experimental Design

Thirty-five of the pregnant heifers were divided into two groups according to the stage of gestation. Nineteen dams were to be inoculated in the second trimester and the remaining 16 in the third trimester of pregnancy. Ten days before inoculation, heifers were taken to the infectious disease laboratory at the Colorado State University Research Farm. This facility was designed to eliminate disease spread between individual rooms and to prevent contamination from outside. Twelve of the 35 were kept as uninfected controls and housed in rooms separate from the infected animals. The remaining 23 were to be infected and killed at specific times from 5 to 50 days post inoculation. Abortions resulted in the rescheduling of kill dates so that samples could be obtained over a wide range of post inoculation times. Table 2 shows the final grouping of animals after rescheduling.

Preparation of Inoculum and Cattle Inoculation

Inoculum for the 23 principle cattle in this experiment was the California strain of the epizootic bovine abortion agent. The seed

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TABLE 2.--Experimental Design

| Group 1: | Inoculated in Second Trimester Pregnancy | | | |
Cow No.	Status	P.I.D. Killed (days)	Gestation at Inoc. (days)	Gestation at necropsy (days)
56	P	5	125	130
57	P	5	134	139
62	P	6	135	141
72	C	7	135	142
75	C	11	131	142
55	P	12	129	141
69	P	12	129	141
60	P	14	132	146
70	C	18	123	141
77	P	20	135	155
63	C	29	128	157
73	P	33	131	164
86	P	36	128	164
82	C	37	125	162
51	P	44	128	172
85	P	48	132	180
76	C	48	132	180
79	P	49	145	194
68	C	54	129	183
Group II:	Inoculated in Third Trimester Pregnancy			
54	P	6	200	206
65	P	7	201	208
74	C	7	200	207
66	P	14	199	213
52	C	14	210	224
59	P	16	207	223
61	P	19	204	223
84	C	26	200	226
81	P	27	200	227
64	P	29	200	229
53	P	29	204	233
58	P	30	200	230
83	P	30	202	232
87	P	30	204	234
78	C	32	200	232
67	C	41	203	244

P.I.D. = Days post inoculation
P   = Infected principal
C   = Non-infected control

culture, EBA 59-795, was supplied by Dr. J. Storz (Department of Microbiology, Colorado State University, Fort Collins) who isolated it from an aborted bovine fetus (Storz and McKercher, 1962).

Stock cultures of the agent in the 11th yolk passage were kept frozen in sealed ampoules at $-70°C$. Cattle inoculum was prepared freshly, as the 12th passage, in embryonated eggs. Only those yolk sacs with• typical lesions and demonstrable elementary bodies were used for preparation of inoculum. Suitable yolk sacs were homogenized in a TenBroeck grinder with Earle's lactalbumin hydrolysate (L E) medium (Schmidt, 1964). Three ml L E medium was used per yolk sac to give, approximately, a 20% suspension of yolk material. The homogenate was centrifuged at 1,500 X G for 20 minutes and the supernate used as inoculum. Control inoculum was prepared in the same way using the yolk sac membranes of uninfected embryonated eggs.

Each heifer was inoculated intravenously with 5 ml of control or infectious inoculum prepared as above. A portion of the infectious inoculum was retained for infectivity titration and limiting ten-fold dilutions inoculated into the yolk sacs of 7-day developing chicken embryos. The 50% egg infectious dosages (EID50) were determined based on death of the embryos and the presence of elementary bodies in stained yolk sac impression smears. The method of Reed and Muench (1938) was used to calculate end points. In determining the end points, only those yolk sacs with demonstrable elementary bodies were considered positive.

Clinical Observations and Sampling

The temperatures of inoculated cattle were checked daily for 3 days prior to inoculation and twice daily during the febrile response after

inoculation. After temperatures had returned to preinoculation levels, temperature was again recorded once daily.

Blood samples for hematologic determinations were taken daily from 3 days prior to inoculation to 10-14 days post inoculation. Samples were taken thereafter at two-day intervals. Ethylenediamine tetraacetic acid (EDTA) was used as anticoagulant. The same EDTA samples were used from 5 animals, picked at random, to determine if a blood infectious phase could be detected. These 5 samples were each inoculated into the yolk sacs of 7-day chick embryos. The EDTA blood was inoculated undiluted and as 1:10 and 1:100 dilutions in L E medium. The chlamydia reisolation procedures are described later under the heading "Reisolation of EBA Agent". Blood samples were also taken without anticoagulant to obtain serum for complement fixation (CF) test.

Necropsy Procedures

Animals were killed by electrocution. Tissues were collected aseptically in sterile plastic bags and stored at -20°C until isolations could be attempted. Those tissues taken for yolk sac culture are listed in Table 3. Not all tissues listed were cultured from all animals. A complete list of tissues cultured is given in Table 10 of the results.

TABLE 3.--Cattle Tissues Taken for Yolk Sac Culture

Dam	Fetus
Liver, Spleen	Liver, Spleen
Kidney, Lung	Kidney, Lung
Large Intestine	Intestine
Small Intestine	Heart
Placenta, Heart	
Internal Iliac Lymph Node	

Reisolation of the EBA Agent

The methods used for isolating chlamydial agents were adapted from the methods of Hudson et al. (1955). Tissue samples from experimental animals were prepared as 10% suspensions (approximate) in L E medium by homogenizing in TenBroeck tissue grinders. Six to 8-day embryonated eggs were inoculated via yolk sac with these homogenates undiluted and in dilutions of 1:10 and 1:100. Three eggs were inoculated per dilution with each egg receiving 0.5 ml inoculum. Yolk sac inoculation was by syringe and 24 gauge 1.5 inch needle inserted through the air sac end of the egg to a depth of approximately 1.25 inches. Fertile eggs were obtained from the Colorado State University Poultry Farm or from the Meyer Brothers Hatchery in Greeley, Colorado.

Intestinal contents and tissues contaminated with bacteria (e.g. placental membranes obtained after abortion) were treated essentially according to procedures devised by Wilson (1962) and Storz (1963). The modified procedure used was as follows:

1. A 20% suspension of fecal matter or contaminated tissue was prepared as above.

2. Ten ml of the suspension was centrifuged in a swing head centrifuge for 30 min. at 1,200 X G.

3. Five ml of the middle portion of the supernatant fluid was drawn from the tube. Care was taken not to disturb the sediment or the fine flotation film at the top of the tube.

4. The 5 ml sample was diluted with an equal volume of L E medium and again centrifuged 30 min. at 1,200 X G.

5. Seven ml of the supernatant fluid from the centrifugation was removed and centrifuged again for 30 min. at 1,200 X G.

6. Five ml of the supernate from this third cycle of centrifugation was used as egg inoculum.

Inoculated eggs were incubated at 37°C. and candled daily from day 1 to 14 post inoculation. Those embryos that died on the first and second post inoculation days were discarded after cultures or smears were made to determine if bacterial contamination caused their deaths. Embryos dying from day 3 to 14 post inoculation were examined for gross pathologic lesions of congestion of yolk sac vessels (Storz and McKercher, 1962) and for the presence of elementary bodies in stained yolk sac impression smears. Smears were stained by the method of Gimenez (1964). Samples causing typical pathologic changes in embryos and showing elementary bodies in yolk sac impression smears were considered positive for chlamydia. Samples which caused no deaths in the first passage were passed again. If no specific deaths occurred in the second passage, the sample was considered negative for chlamydia. Some samples were passed a third or fourth time where atypical lesions were seen or smears examined for elementary bodies were equivocal.

Infection of Pregnant Cow with I-7666 Intestinal Agent

One cow (#80) in the two-hundredth day of gestation was inoculated intravenously with an intestinal chlamydia, I-7666. This chlamydial agent was isolated by Lincoln (1968) from the feces of one of the experimental heifers. The inoculum was prepared and titrated in the same way as the EBA inocula. The cows temperature was taken daily and blood samples taken at one day intervals for hematology, serology, and reisolation of the agent. The cow was observed until abortion occurred, at which time the dam was killed by electrocution and both dam and fetus subjected to necropsy examination. Tissues were collected in the same

manner as those from EBA infected heifers. Selected tissues (liver, spleen, kidney and gall bladder of the fetus and liver, spleen, kidney and large intestine of the dam) were cultured for the presence of chlamydia.

Group-Specific Complement Fixation (CF) Test

The immunologic responses of inoculated cattle were examined using the CF test. A group-specific CF antigen was prepared essentially according to the method of Storz and McKercher (1962). A summary of their method is as follows:

1) Infected yolk sacs were washed to remove excess yolk material. The wash solution was Dulbecco's phosphate buffer without calcium chloride (Dulbecco and Vogt, 1954).

2) An approximate 10% yolk sac suspension was prepared in 0.4% trypsin[1] in Dulbecco's solution. Homogenization of yolk sacs was accomplished with a TenBroeck tissue grinder.

3) The yolk sac suspension was allowed to digest at 37°C. for 1 hour.

4) The digest was centrifuged at 1,200 X G for 15 minutes to remove debris. The supernatant fluid was removed and centrifuged at 10,000 X G for 1 hour. This yielded a pellet consisting of large numbers of elementary bodies. The pellet of elementary bodies was re-suspended in 0.01M phosphate buffered saline (PBS), pH 7.2 and washed by 4 cycles of low speed (1,200 X G) and 4 cycles of high speed (10,000 X G) centrifugation.

5) The final pellet was resuspended in PBS and heated to boiling temperature for 30 minutes.

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[1]Difco Laboratories, Detroit, Michigan. Assayed potency of 1:250.

Ether extraction was accomplished by methods adapted from the works of Hilleman and Nigg (1948) and Storz and McKercher (1962). The method used was as follows:

1. Equal volumes of purified, boiled elementary bodies and ethyl ether were mixed with vigorous shaking at room temperature for 30 minutes.

2. The mixture was allowed to settle and the ether fraction collected.

3. The ether was evaporated under reduced pressure and the dried film reconstituted in 0.01M PBS. The chlamydial strain used for preparation of the ether extracted group antigen was the bovine intestinal isolate I-7666 (Lincoln, 1968).

Both macro and micro CF tests were used in testing antigens and sera. The general protocols followed for these tests were essentially according to Harris (1964). Volumes of reagents used in the macro CF test were as follows: diluted serum, 0.25 ml; antigen (2 units), 0.25 ml; and complement[1] (2 units), 0.5 ml. The serial dilutions of serum used were generally from 1:8 to 1:2048. The hemolytic system was prepared by incubating for 15 minutes equal volumes 2.8% washed sheep red blood cells and 2 units of rabbit anti-sheep red blood cell hemolysin. After serum, complement and antigen had been incubated for two hours at 37°C, 0.5 ml of sensitized sheep red blood cells (hemolytic system) was added. After 30 minutes of incubation at 37°C, the CF test could be read. End points were read at an estimated 50% lysis.

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[1] Guinea pig complement from Hyland, 4501 colorado Blvd., Los Angles, California.

The micro CF test was run in exactly the same way as the macro test except that the following reagent volumes were used: complement, 0.05 ml; antigen, 0.025 ml; diluted serum, 0.025 ml; and hemolytic system, 0.025 ml. Serial dilutions of sera were made using diluting loops and disposable titrating plates.[1]

Both macro and micro tests were run with the standard complement, antigen, serum and diluent controls. The diluent used was a veronal buffer as described by Harris (1964).

Preparation of Species-Specific CF Antigen: Egg Propagated Chlamydia

The methods used for preparation of species-specific CF antigens were adapted from the methods of Manire (1966), Jenkin (1960), and Frazer and Berman (1965). Species antigens were prepared from the agents of EBA, sheep polyarthritis, and bovine fecal isolate, I-7666. Sources and designations of these agents are summarized in Table 4.

TABLE 4.--Agents used to Prepare Species-Specific Antigens

Agent	Source	Passage Number	Reference
EBA 59-795	aborted bovine fetus	12	Storz and McKercher (1962)
I-7666	feces of a normal Hereford	12	Lincoln (1968)
58 Sp	EBA reisolate from fetal spleen of experimental cow #58	13	
LW-679	joint of sheep with polyarthritis	8	Storz et al. (1965)

[1]Cooke Engineering Co. Distributed by Microbiological Associates, 4773 Bethesda Ave., Bethesda, Md.

Protocol for preparation of these antigens was as follows:

1. Seventy to one hundred 7 - 10 day embryonated eggs were infected with a 10^{-3} dilution of stock agent. Eggs were candled daily and those dying in the first 72 hours discarded.

2. Yolk sac from eggs dying between days 6 and 9 were stored at 4°C until harvested. Only those yolk sacs with lesions typical of chlamydial infection were kept. Care was taken to express excess yolk material from yolk sac membranes. Random yolk sacs were checked for the presence of elementary bodies by microscopic examination of Gimenez-stained impression smears. Yolk sacs thus harvested were directly processed or frozen at -20°C. until used.

3. Fresh or frozen yolk sacs were homogenized with TenBroeck glass homogenizers. Approximately 2.5 ml LE medium was used per yolk sac.

4. The yolk sac homogenate was centrifuged in a swing bucket centrifuge for 30 min. at 1,200 X G. This resulted in both sedimentation and flotation pellets.

5. The flotation pellet was discarded and the supernatant fluid drawn off.

6. The supernate was centrifuged at 10,000 X G for 30 min. in a refrigerated angle-head centrifuge. The resulting pellet and the low speed sedimentation pellet from step 4 above were separately incubated for 1 hour with 0.4% Tris buffered trypsin (0.2M Tris pH 7.4) at 37°C. Approximately 20 volumes trypsin were used per volume of pellet.

7. The tryptic digest of the pellet from step 4 was again centrifuged at 1,200 X G for 30 min. The resulting pellet was discarded and the supernate combined with a tryptic digest of the high speed pellet

from step 6. The combined digests were then centriguged at 1,200 X G

for 30 min.

8. The pellet from high speed centrifugation in step 7 was resuspended in 10 volumes of 20% Tris buffered sucrose (0.033M Tris pH 7.4). Since a uniform suspension could not be achieved by vigorous pipetting, sonification was used to break up the pellet. A Bronson Sonifier[1] was used at a power setting of 2 for approximately 20 sec.

9. The suspension in 20% buffered sucrose was carefully layered over 25% buffered sucrose and centrifuged in a refrigerated angle-head centrifuge for 30 min. at 8,000 X G. Thirty ml 25% sucrose were used for every 10 ml of suspension. Centrifugation yielded a small bottom pellet and a markedly turbid layer at the top of the tube.

10. The pellet was resuspended by sonification at a low power setting of 2 in 15% buffered sucrose. This suspension was layered over a buffered sucrose concentration gradient of 20% to 40% buffered sucrose. The sucrose gradient was prepared by layering equal portions of 40, 35, 30, 25, and 20% 0.033M Tris buffered sucrose in a centrifuge tube. Centrifugation for 1 hour at 3,000 X G in a refrigerated angle-head centrifuge produced a band near the bottom of the tube and a turbid layer near the top. Because of the use of angle-head centrifugation as described by Crawford (1969) rather than the swing head centrifugation normally used for density gradient work, nearly 50% of the sample was contained in a sedimentation pellet. Resuspension of the pellet and centrifugation through another density gradient again yielded a band and a pellet. Because microscopic examination of band and pellet

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[1] Heat Systems Co., 60 Broad Hollow Road, Melville, New York.

showed no apparent difference in purity of the two fractions, they were combined for further purification.

11. The combined fractions were diluted with 40 ml 0.01M phosphate buffered saline (PBS) and centrifuged for 30 min. at 9,000 X G.

12. The pellet from step 11 was resuspended in approximately 20 times its volume of 1% sodium deoxycholate (SDOC) and sonified for 5 minutes at•a power setting of 6. During sonification, care was taken to keep the temperature of the material below 45°C.

13. After sonification, SDOC extraction was carried out by incubation and stirring at 45°C for 4 hours. This suspension was then centrifuged for 30 min. at 10,000 X G and the supernate (SDOC soluble fraction) drawn off and discarded or used as a group-specific antigen.

14. The pellet from above (SDOC insoluble fraction) was resuspended in 0.4% Tris buffered trypsin and incubated with stirring for 1 hour at 37°C. This digest was centrifuged at 10,000 X G for 30 minutes. The resulting pellet was washed by 3 cycles of centrifugation with resuspension in fresh PBS after each centrifugation.

15. The final pellet was resuspended in 10 ml PBS and sonified for 5 min. at a power setting of 6. This was used as the species-specific cell wall antigen.

Preparation of Species-Specific CF Antigens from Tissue Culture Propagated Chlamydia

Cell culture propagation of the EBA agent was attempted to determine if the system could be used to produce cell wall antigen. A cell culture system would be desirable because the agent could be purified from cell cultures far easier than from yolk sac cultures.

Several different cell strains were tested for infectability with EBA. Cell cultures used were second passage bovine embryonic kidney (BEK-2), thirty-fifth passage lamb thyroid (LT-35), sixty-fifth passage bovine embryonic spleen (BES-65), and one hundred-fourteenth passage bovine kidney (MDBK). The high passage LT-35 and BES-65 cells were obtained from primary cultures by the author. The MDBK cell line was purchased from American Type Culture Collection[1] (CCL-22). Cell cultures were prepared essentially according to the method of Madin and Darby (1958). Growth medium used was LE medium with 10% lamb serum. After cultures had reached monolayer proportion, they were changed to a maintenance medium of LE medium with 3% fetal calf serum.

The work of Gordon et al. (1960) and Becerra (1969) showed that infection of cell cultures with yolk sac propagated chlamydia was enhanced by purification of the inoculum. Because of this, inoculum for the cell cultures used in this experiment was purified through step 9 in the yolk sac purification schedule previously given.

Cell cultures were infected by adsorption of a small volume of inoculum (eg. 4 ml per Roux flask of 190 cm^2 cell growth surface) onto cells for 4 hours. After adsorption, fresh medium was added and cultures incubated at 37°C. The pilot study of infectability included BEK-2, LT-35, BES-65, and MDBK cells. Purification and production of cell wall antigen was attempted only with BEK-2 cells.

Protocol used for production of cell wall antigen from infected cell cultures was as follows:

1. Cell cultures infected 72 hours with EBA 59-795 were harvested by trypsin digestion at 37°C. Harvested cells were pelleted by

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[1] 12301 Parklawn Drive, Rockville, Md.

centrifugation at 800 X G for 10 min. Both the cell pellet and super-
nate after trypsinization contained the EBA agent.

2. Pelleted cells were resuspended in LE medium and disrupted by
sonification for 30 sec. at a power setting of 1 to 2. The sonicate
was centrifuged at 800 X G for 10 min. and the supernate drawn off.

3. Supernatant fluids from steps 1 and 2 were centrifuged at
8,000 X G for 30 min. Pellets from this centrifugation were combined
and resuspended in 20% buffered sucrose. The suspension was layered
over 25% buffered sucrose and centrifuged again at 8,000 X G for 30
min.

4. Further purification and preparation of cell wall antigens
followed the previously given protocol for yolk sac propagated agent
steps 9 through 15.

Yields of infectious agent from cell cultures were determined
by titration in 7-day developing chicken embryos. Degree of infection
of cell cultures was determined by a count of infectious centers in
Gimenez stained cell cultures. One inclusion of infectious particles
represents 1 infectious center. An infectivity of 100% is taken as
100 inclusions per 100 cells.

Electron Microscopy: Yolk Sac Preparation

Samples for light and electron microscopy were taken at various
steps in the preparation of cell wall antigen EBA 59-795. The
procedure followed for preparation of samples for electron microscopy
was essentially the same as that of Manire (1966). Details of this
procedure are as follows:

1. One drop of sample was added to the surface of a block of
2% agar.

2. The drop was spread and allowed to dry.

3. Several drops of 0.75% collodion in amyl acetate were added to the surface of the agar and excess drained off.

4. The collodion film was allowed to dry and floated off onto distilled water.

5. Grids were layered on the film and picked up on the non-adhesive side of masking tape.

6. Grids were shadowed with chromium at 27° angle and examined with a Zeiss EM 9A electron microscope.[1]

Electron Microscopy: Infected Cell Culture

A sample of second passage embryonic spleen cells infected with the chlamydial agent of enzootic abortion in ewes (EAE B 577) was obtained from Dr. V. Becerra.[2] The proven similarities between the EAE agent and EBA made this sample useful for study of the structural morphology of a chlamydial agent grown in cell culture. Of particular interest was the morphology of ultrathin sections of chlamydial cell walls. The sample obtained from Dr. Becerra was harvested by trypsinization 72 hours post inoculation and fixed 1 hour in 6.5% buffered glutaraldehyde. After fixation, the cells were washed in PBS and stored overnight at 4°C. The sample was post-fixed in Kellenberger's 1% Osmium tetroxide fixative (Kellenberger, et al., 1958) and embedded in Epon 812 resin.[3] Ultrathin sections were taken with the LKB Ultratome (LKB Produkter AB) and placed on 150 mesh grids for staining.

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[1] Carl Zeiss, Inc., 444 Fifth Avenue, New York, N.Y.

[2] Department of Microbiology, Colorado State University, Fort Collins

[3] Shell Chemical Corporation, Plastics & Resins Div., New York, N.Y.

Specimens were stained at 60°C for 25 minutes with 8% aqueous uranyl

acetate. This was followed by staining for 5 minutes with 0.5% lead

citrate in 0.08% aqueous sodium hydroxide. Observations and electron

micrographs were made with the Zeiss EM 9A electron microscope.

Production of Antisera in Rabbits

White rabbits of mixed breed were obtained locally by the Colorado

State University Reserach Farm. Because of uncertainty regarding the

optimal immunization schedule for chlamydial agents, three different

schedules were used. Based on reports of prolonged immunization sched-

ules (Jenkin et al., 1961), the first rabbits were immunized by three

injections spaced 60 days apart. The results from these rabbits indica-

ted that a shorter immunization schedule might be desirable. The next

group of rabbits was immunized by two injections spaced 24 days apart

and the third group by injections given 14 days apart.

The antigens used, rabbits involved and schedules followed are

summarized in Table 5.

Table 5.--Rabbit Immunization Schedules

Rabbit number	Antigen given	Schedule followed
1 & 2	EBA cell wall	3 injections, 60 days apart
3 & 4	SDOC extract	3 injections, 60 days apart
5 & 6	LW-679 cell wall	2 injections, 24 days apart
7 & 8	I-7666 cell wall	2 injections, 14 days apart
9 & 10	EBA reisolate cow # 58 fetal spleen	2 injections, 14 days apart

All rabbits except numbers 3 and 4 were immunized by intravenous injection into the marginal ear vein. Approximately 1.5 ml were given each rabbit at each inoculation. Rabbits 3 and 4 received the same volume of SDOC extract (step 13 of the species CF antigen preparation procedure) injected subcutaneously. In the course of immunizing these rabbits, it was discovered that the group-specific antigen could be precipitated from the SDOC by addition of acetone (Hilleman and Nigg, 1948). The final injection of rabbits 3 and 4 was therefore given intravenously with an aqueous suspension of acetone precipitated group antigen.

Preinoculation serum samples and samples taken during the course of immunization were obtained by bleeding from the medial ear artery. Final bleedings were by cardiac puncture.

Clinical Response

All 23 cattle inoculated with EBA 59-795 demonstrated marked

febrile responses within 12 to 24 hours post inoculation. The average

temperature reponse is shown in Fig. 1. Eleven of the 23 (48%) demon-

strated a biphasic temperature response with the first peak of 40 to

40.6°C occuring 4 to 6 hours after inoculation. By 12 hours post

inoculation, the temperature had returned to near normal and soon there-

after rose to 41.2 to 42.4°C in the more typical response. Control

animals inoculated with uninfected yolk sac material showed no rise

in temperature after inoculation.

During the febrile reaction, most infected animals were anoretic

and had mild diarrhea. Twelve of the 23 infected animals aborted or

exhibited signs of impending abortion before the scheduled euthanasia

date. Scheduled euthanasia precluded obtaining data on the true abor-

tion incidence. Incidence of abortion is compared with temperature

response and inoculum titer in Table 6.

Hematologic studies disclosed the occurance of a pronounced leuko-

penia beginning on day 1 post inoculation and often lasting until the

seventh day post inoculation. This leukocytic response is graphically

represented in Fig. 2. No significant change was found in the white

cells counts of the control animals.

Immunologic Response of Inoculated Cattle

All 23 cattle infected with the EBA agent developed group-specific

CF titers. Table 7 shows the results of the three preinoculation

titrations and the final titrations of both infected and control cattle.

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Table 6.--EBA Infected Cattle: Titer of Inoculum, Temperature Response and Incidence of Abortion.

Cow No.	PID Killed	Inoculum Titer Log EID50	Maximum Temperature	Abortion	Fetal Age (days)
Group 1: Inoculated in second trimester of pregnancy					
56	5	8.2	41.8°C	yes	130
57	5	8.2	41.8*	yes	139
62	6	8.2	41.4*	---	141
55	12	9.0	42.3*	---	141
69	12	8.2	42.2*	yes	141
60	14	9.0	42.4*	---	146
77	20	8.0	42.4	---	155
73	33	8.2	41.4*	yes	164
86	36	8.2	41.7	---	164
51	44	8.2	41.2	---	172
85	48	8.0	41.4*	yes	180
79	49	9.0	41.7	yes	194
Group 2: Inoculated in third trimester of pregnancy					
54	6	NT	41.8*	---	206
65	7	7.5	41.5	---	208
66	14	7.5	41.2	---	213
59	16	7.5	41.5*	---	223
61	19	4.9	41.6	---	223
81	27	8.0	41.9	yes	227
64	29	7.5	41.2*	yes	229
53	29	NT	41.5	yes	233
58	30	7.5	42.0	yes	230
83	30	NT	42.2*	yes	232
87	30	NT	41.6	yes	234

PID= days post inoculation
NT= not tested
--- refers to heifers killed on schedule before abortion or signs of abortion had occurred
* refers to heifer with biphasic temperature response

More precise testing was done on 7 of the 23 infected cattle with the

finding (Table 8) that the rise in group-specific CF titers began

approximately 6 days after inoculation and reached maxima approximately

8 to 9 days after inoculation. Figure 3 depicts the CF antibody re-

sponses of 2 of these cattle.

Table 8.--Time Required for Group-Specific CF Antibody Responses:
Cattle Inoculated with EBA 59-795.

Cow No.	PID to First Rise in CF Titer	PID to Maximum CF Titer
53	6	9
55	6	8
60	6	8
69	5*	10*
73	6	12*
79	6	8
86	4*	12*

PID = Post inoculation day

* = These animals were bled at 2 to 3 day intervals. All others were
sampled daily during the 0 to 12 PID period.

Reisolation of the EBA Agent

A blood infection was demonstrated in 2 of 5 cattle tested daily

for 6 to 8 days post inoculation. The agent was recovered from the

blood of cow 54 on the 2nd, 3rd, and 5th post inoculation days and

from cow 87 on the 2nd day after inoculation. Cows 83 and 61 were

tested from day 1 to day 8 but no agent could be recovered. Cow 53

was also tested for 6 days post inoculation with no isolations made.

Table 7.—Group-Specific CF Antibody Response of Experimental Cattle.

Group 1:	Inoculated in second trimester of pregnancy			
Cow No.	Preinoc. Sample 1	Preinoc.* Sample 2	Preinoc.** Sample 3	Postinoc.*** Sample
56	1:64	1:32	1:32	1:256
57	1:128	1:32	1:64	1:256
62	1:32	1:16	1:32	1:128
72 c	1:128	1:16	1:32	1:128
75 c	1:64	1:16	1:32	1:64
55	1:16	1:16	1:64	1:4096
69	1:16	1:16	1:64	1:4096
60	1:32	1:16	1:128	1:4096
70 c	1:64	1:64	1:64	1:64
77	1:128	1:16	1:32	1:4096
63 c	1:64	1:16	1:32	1:64
73	1:64	1:64	1:64	1:512
86	1:16	1:16	1:64	1:512
82 c	1:64	1:32	1:64	1:64
51	1:64	1:32	1:32	1:1024
85	1:64	1:32	1:64	1:2048
76 c	1:16	1:16	1:64	1:64
79	1:64	1:64	1:32	1:1024
68 c	1:16	1:16	1:32	1:128
Group 2:	**Inoculated in the third trimester of pregnancy**			
54	1:64	1:16	1:32	1:1024
65	1:16	1:16	1:16	1:256
74 c	1:16	1:64	NT	NT
66	1:16	1:16	1:16	1:2048
52 c	1:64	1:16	1:32	1:64
59	1:32	1:16	1:16	1:2048
61	1:64	1:32	1:32	1:2048
84 c	1:64	1:32	NT	NT
81	1:64	1:16	1:16	1:2048
64	1:64	1:16	1:64	1:2048
53	1:32	1:16	1:32	1:256
58	1:64	1:32	1:32	1:1024
83	1:128	1:32	1:32	1:4096
87	1:64	1:16	1:16	1:1024
78 c	1:128	1:32	1:64	1:32
67 c	1:64	1:16	1:32	1:64

* = second preinoculation sample taken 2 months after 1st preinoculation sample
** = third preinoculation sample taken at time of inoculation, 4 to 6 months after 2nd preinoculation sample
*** = postinoculation sample taken at time of necropsy
NT = not tested
c = control animal inoculated with uninfected yolk sac material

The results of attempts to recover chlamydia from organs of the dams, placentas and fetuses are summarized in Table 9. These results show that chlamydial agents were recovered from the placentas of 18 out of 19 infected cattle tested. Placentas from the remaining 4 cattle (cows 85, 58, 83, and 87) were not suitable for testing because of abortion and subsequent contamination of the placenta. No agent could be recovered from the placenta of cow 65. Chlamydia could not be recovered from placentas of the 12 control cattle.

Of the remaining tissues cultured from the dams (liver, spleen, kidney, internal iliac lymph node and large intestine) chlamydia were recovered from the liver of cow 57, kidney of cow 54, internal iliac lymph node of cow 53, and large intestine of cows 83 and 81. A chlamydial agent was also recovered from the large intestine of a control cow (#70). Collection of the internal iliac lymph node was not in the original protocol but that tissue was collected after gross lesions were noted at necropsy. Thus the internal iliac lymph node was collected from only 18 of the infected and 8 of the control cattle.

The tissue distribution of isolations from the 23 infected fetuses is given in Table 10. Of the 23 infected fetuses cultured, chlamydia were recovered from the livers of 3, spleens of 6, and kidneys of 5. Two other organs were cultured from randomly selected fetuses with the result of chlamydia recovery from the lung of 1 fetus out of 6 tested and the heart of 1 out of 7 fetuses tested. Chlamydia were not re-covered from organs (liver, spleen, and kidney) of the control fetuses.

Infection of Pregnant Cow with I-7666 Intestinal Agent

Inoculation of a pregnant heifer with the I-7666 chlamydial iso-late caused a disease indistinguishable from EBA. Clinical signs were

Table 9.--Incidence of Abortion and Reisolation of the EBA Agent: EBA 59-795 Inoculated Cattle

Cattle Inoculated in Second Trimester of Pregnancy						Cattle Inoculated in Third Trimester of Pregnancy					
Cow No.	P.I.D. Killed	Abortion	Dam	Placenta	Fetus	Cow No.	P.I.D. Killed	Abortion	Dam	Placenta	Fetus
56	5	Yes	-	+	+	54	6	---	+	+	-
57	5	Yes	+	+	-	65	7	---	-	-	-
62	6	---	-	+	+	66	14	---	-	+	-
55	12	---	-	+	+	59	16	---	-	+	-
69	12	Yes	-	+	-	61	19	---	-	+	-
60	14	---	-	+	-	81	27	Yes	+*	+	-
77	20	---	-	+	+	64	29	Yes	-	+	+
73	33	Yes	-	+	+	53	29	Yes	+	+	+
86	36	---	-	+	-	58	30	Yes	-	NS	+
51	44	---	-	+	+	83	30	Yes	+*	NS	+
85	48	Yes	-	NS	-	87	30	Yes	-	NS	+
79	49	Yes	-	+	+						

+ = chlamydial agent recovered from one or more organs tested. NS = not suitable for testing

* Isolated from intestinal contents only.   --- = did not abort before kill date.

- = negative; chlamydia not recovered.   PID = day after inoculation.

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Table 10.--Reisolation of the EBA Agent from Organs of Fetuses from Experimentally Infected Heifers

Cattle Inoculated in Second Trimester of Pregnancy				Cattle Inoculated in Third Trimester of Pregnancy			
Cow No.	PID Killed	Positive Isolations	Negative Isolations	Cow No.	PID Killed	Positive Isolations	Negative Isolations
56	5	Sp	Lu,Li,Ki,My	54	6		Lu,Li,Sp,Ki
57	5		Lu,Li,Ki,My,Sp	65	7		Li,Sp,Ki
62	6	My	Lu,Li,Ki,Sp	66	14		Li,Sp,Ki
55	12	Lu	Li,Sp,Ki,My	59	16		Li,Sp,Ki
69	12		Lu,Li,Sp,Ki	61	19		Li,Sp,Ki
60	14		Li,Sp,Ki	81	27		Li,Sp,Ki
77	20	Ki	Li,Sp	64	29	Li	Sp,Ki
73	33	Ki	Li,Sp,My	53	29	Li,Sp,Ki	
86	36		Li,Sp,Ki	58	30	Li,Sp,Ki	
51	44	Sp	Li,Ki	83	30	Sp	Li,Ki
85	48		Li,Sp,Ki	87	30	Sp	Li,Ki
79	49	Ki	Li,Sp				

PID = day after inoculation
Lu = lung
Li = liver
Sp = spleen
Ki = kidney
My = myocardium

(1) a febrile response which reached a peak of 41.1°C at 36 hours post inoculation and subsided by 72 hours post inoculation, (2) a leukopenic response and (3) abortion occurring 26 days after inoculation.

The chlamydial agent was recovered from the blood on days 2 and 3 post inoculation. Blood samples taken on days 1 and 4 through 8 after inoculation were negative for chlamydia. The agent was recovered from the placenta and from the liver, spleen, kidney and gall bladder of the fetus. Other organs of the dam (liver, spleen, kidney and large intestine) were negative for chlamydia.

A rise in group-specific CF antibody was apparent and is graphically represented in Fig. 4. Two preinoculation titrations of serum samples taken two months apart demonstrated titers of 1:64. The titer at inoculation was 1:32 and the final titer at necropsy was 1:2048.

Preparation of Species-Specific CF Antigen: Egg Propagated Agent

Egg infectivity studies of purified EBA 59-795 elementary bodies (purified through step 11 in the materials and methods section) indicated that mild sonification and density gradient purification did not destroy infectivity. In one experiment where titrations were performed, the infectivity titer before purification was $10^{8.3}$ EID_{50} per yolk sac. After purification, $10^{8.1}$ EID_{50} per yolk sac were recovered.

Light microscopic studies of samples taken at various steps in the preparation of purified elementary bodies showed considerable decrease in debris with sedimentation through a sucrose gradient. Gimenez stained impression smears, however, always showed some malachite green stained debris clustered around the dark, basic fuchsin stained elementary bodies. (Fig. 5). This could be seen despite repeated attempts to remove it with density gradient centrifugation.

Electron microscopic studies were made on three different purified elementary body preparations. The first preparation was from fresh, unfrozen yolk sac material that had not been subjected to sonification during the purification procedure (ie. material from step 11 of the purification procedure but with sonification at steps 8 and 10 omitted). The electron microscopic results of this first preparation are shown in Fig. 6. Elementary bodies are present in Fig. 6 as flattened spheres of dimensions averaging 0.44 microns wide by 0.15 microns high. The prominent feature of the elementary body is the dense, raised center. Several ruptured elementary bodies can be seen. The dense centers are missing from these. The larger, flatter, spheres are presumably the initial bodies. Their dimensions average 0.82 microns wide by less than 0.1 microns high. No dense center can be seen in the typical initial body.

The second elementary body preparation was purified exactly as the first only low power sonification (steps 8 and 10) was used to resuspend the pellets. This preparation, as seen in Fig. 7, contained elementary bodies, initial bodies, and a considerable amount of debris which appeared to be fragments of initial bodies.

The third elementary body preparation was identical to the first except that infected yolk sacs used were stored frozen at -20°C before use. The results of this preparation can be seen in Fig. 8. The only developmental form left intact is the elementary body. Initial bodies are nearly all ruptured leaving a large amount of debris.

A considerable amount of aggregation of elementary bodies was evident in all three preparations. This could be seen grossly as

flocculation when the preparation was allowed to stand overnight at 4°C.

Sonification at high power for 5 min. (step 12) resulted in break-age of approximately half of the elementary bodies (Fig. 9). This sonification was followed by 4 hours of SDOC extraction at 45°C which resulted in complete disruption of all elementary bodies (Fig. 10). The prominent features of Fig. 10 are flattened cell wall fragments and thick aggregates of unidentified material.

The CF properties of both SDOC soluble and insoluble fractions were determined by box titration against homologous rabbit antisera. The titers of the cell wall (SDOC insoluble) fraction varied from 1:2 to 1:10. The titers of the SDOC soluble fraction (group antigen) varied from 1:16 to 1:128. Since the SDOC was harmful to the red blood cells used in the CF test, the group antigen was precipitated with acetone (1:1 v/v) and resuspended in water before use.

Preparation of Species-Specific CF Antigen: Tissue Culture Propagated Agent

The chlamydial agent EBA 59-795 was successfully cultivated in second passage bovine kidney cells (BEK-2), 35th passage lamb thyroid cells (LT-35), and 65th passage bovine spleen cells (BES-65). An approximate input multiplicity of 50 egg infective doses per cell pro-duced mature inclusions in 85% of the BEK-2 cells, 78% of the LT-35 cells and 91% of the BES-65 cells at 60 hours post inoculation. Mature inclusions can be seen in Fig. 12, a Gimenez stained coverslip culture of LT-35 cells 60 hours after infection. The infection was apparently aborted in the MDBK-114 cells since immature inclusions could be seen at 36 hours post inoculation but no mature inclusions were found at

48 or 60 hours post inoculation. At 36 hours after infection, immature inclusions could be seen in all cell strains tested. These inclusions can be seen in Fig. 13 (LT-35 cells 36 hours after inoculation). The immature inclusions were distinquishable from the mature inclusions by the less dense packing and greater size of the particles and the lesser affinity for the basic fuchsin stain.

The yield of infectious agent in a single Roux flask culture of BEK-2 cells was $10^{8.1}$ egg infective dose-50% (EID_{50}). This represents a net decrease as the inoculum contained $10^{9.1}$ EID_{50}. Purification of elementary bodies from infected BEK-2 cells was far easier than from infected yolk sacs. Essentially no increase in purity was seen with centrifugation of the BEK-2 preparation through sucrose (steps 9 and 10 of Materials and Methods). This is in contrast to yolk sac preparations where a significant increase in purity was seen with steps 9 and 10. While preparation purity was high, the yield after SDOC extraction and sonication was low. A test of the cell wall antigen preparation from 1 Roux flask showed no demonstrable CF activity.

The electron microscopic study of the ovine abortion agent (EAE) in lamb spleen cell culture showed chlamydial inclusions similar to those reported by Armstrong (1968) and Anderson et al. (1965). Figure 14 shows a portion of a cytoplasmic inclusion in a lamb spleen cell. The different developmental forms (elementary bodies, initial bodies and intermediate bodies) can be seen.

Figures 15, 16, and 17 show elementary bodies in more detail. The prominent morphologic features of the elementary body are dense center, ribosome-like granules and an outer cell wall. The inner cell membrane (Fig. 17) is less well defined than the cell wall. Many of the

elementary bodies contained light areas (Fig. 15) which were assumed
to be artifacts. The cell in Fig. 16 appears to be in the process
of engulfing an elementary body.

Figure 18 is an electron micrograph of an intermediate body. Both
a cell wall and a cell membrane can be seen and appear to be morpho-
logically identical. The mebranes are of the unit type and have the
same dimensions (50 - 70 A) as the host cell unit membrane. Also pre-
sent is a dense nucleoid with fine 25 to 30 A filaments radiating from
it. Some ribosome-like bodies lie in the cytoplasmic portion of the
intermediate body. Figure 19 shows an intermediate body in the last
stage of binary fission. Separation of the two daughter cells is
nearly complete.

Figure 20 is an electron micrograph of an unusual division of an
initial body. The structure of the initial body is characterized by
its fine filamentous matrix and numerous, scattered ribosome-like
particles. The unusual feature of this division is that complete
cell membrane separation has occurred but cell wall separation has not
yet occurred.

Production of Antisera in Rabbits

The CF antibody response of a single rabbit inoculated with the
SDOC (group antigen) is given in Fig. 21. Group CF antibodies reached
a maximum titer of 1:1024. The base line represents the negative CF
titers obtained with the homologous species-specific antigen (EBA 59-
795). Rabbit number 4, inoculated with the same material, died of un-
determined cause before serum could be collected.

Figures 22 and 23 show the CF antibody response of 2 rabbits to
inoculation with the EBA 59-795 cell wall antigen. Both group and

species antibodies were formed. Rabbit number 1 reached a maximum group titer of 1:256 and a maximum homologous species titer of 1:128. Rabbit number 2 reached maxima of 1:128 (group) and 1:64 (species).

The results of inoculating rabbits 5 and 6 with cell wall antigen prepared from the polyarthritis agent LW-679 are given in Fig. 24 and 25. Maximum group titers obtained were 1:128 (Rabbit 5) and 1:256 (Rabbit 6). Maximum homologous species titers of rabbits 5 and 6 were 1:32 and 1:64 respectively.

Rabbits 7 and 8, inoculated with cell wall antigen from the I-7666 intestinal agent developed maximum group titers of 1:64 and 1:128 respectively while homologous species titers of 1:128 and 1:64 were obtained (Fig. 26 and 27). Rabbit number 7 had a preinoculation group titer of 1:16 and a species titer of 1:4.

Rabbits 9 and 10 were inoculated with the EBA agent reisolated from the fetal spleen of cow 58. Figures 28 and 29 show that this cell wall antigen gave maximum group titers of 1:64 (rabbit 9) and 1:32 (rabbit 10). Homologous species titers obtained for rabbits 9 and 10 were 1:32 and 1:128 respectively. As with rabbit 7, rabbit 10 had a preinoculation group titer of 1:4 and a species titer of 1:2.

Species specificity of the cell wall antigens was tested by multiway comparisons with the known different strains of EBA and LW-679. The CF titers of rabbits 1 (EBA) and 5 (LW-679) against EBA and LW-679 cell wall antigens are given in Table 11. No apparent cross reaction was seen with either antigen.

This preliminary test of species specificity was followed by multiway comparisons of all 4 antigens and all 8 sera. These data are presented in Table 12. A discrepancy was found in the results of
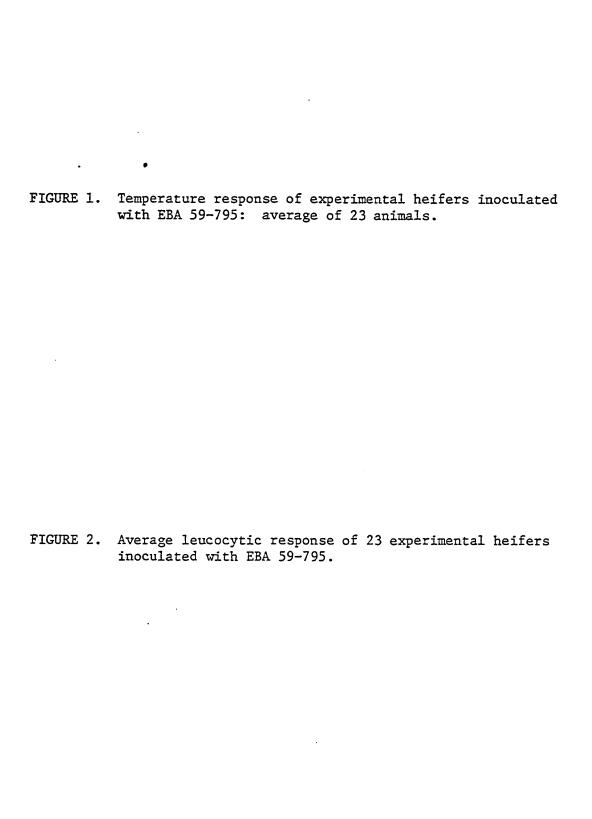
Table 11.—Multiway Comparison of Species-Specific CF Titers: EBA and
    LW-679 Sera vs. EBA and LW-679 antigens

	Antigen	
	EBA	LW-679
EBA serum (rabbit 1)	1:128	1:2
LW-679 serum (rabbit 5)	1:2	1:16
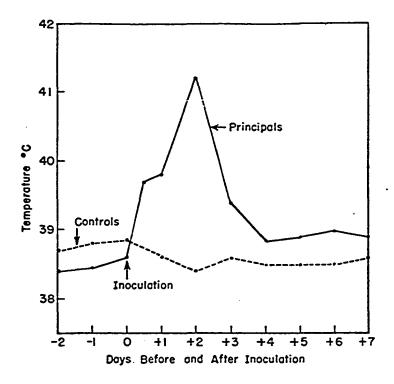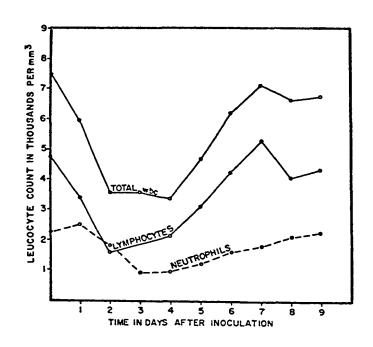
Table 12.—Multiway Comparison of Species-Specific CF Titers: EBA,
    LW-679, I-7666, and EBA Reisolate Antigens and Antisera
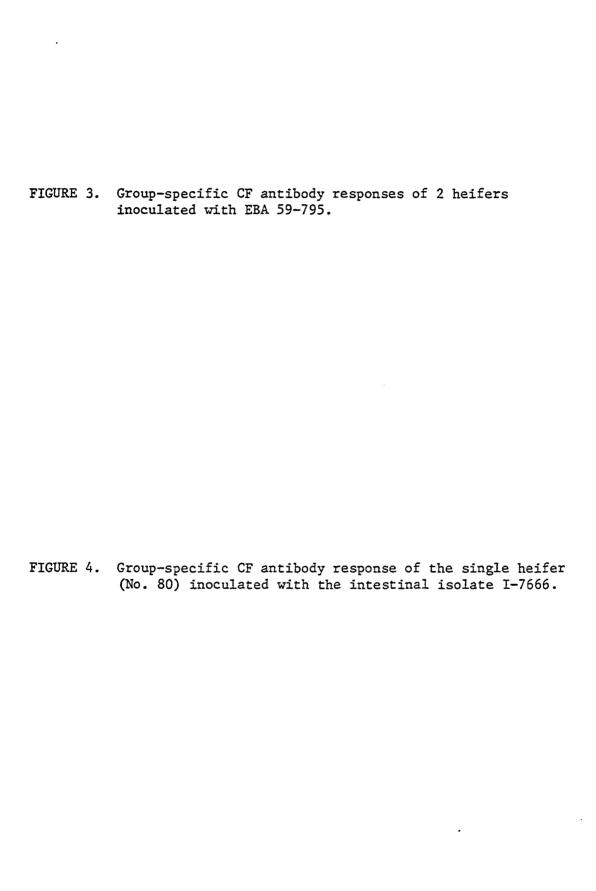
Antiserum	Antigen			
	EBA	LW-679	I-7666	EBA reisolate
EBA serum (rabbit 1)	1:128	1:2	1:256	1:64
(rabbit 2)	1:32	1:2	1:32	1:16
LW-679 serum (rabbit 5)	1:2	1:32	1:2	1:2
(rabbit 6)	1:8	1:64	1:2	1:2
I-7666 serum (rabbit 7)	1:128	1:2	1:128	1:128
(rabbit 8)	1:64	1:2	1:32	1:32
EBA reisolate (rabbit 9)	1:32	1:2	1:16	1:32
(rabbit 10)	1:64	1:32	1:64	1:64

rabbit 10 (EBA reisolate) vs. LW-679 antigen. Confirmation of the complete cross reaction with LW-679 antigen was attempted by testing all sera taken earlier in the immunization of rabbit 10. The results of these CF tests and the comparison of their homologous and heterologous species-specific titers are given in Figure 30.
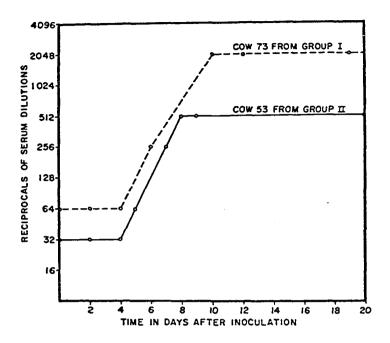
FIGURE 1. Temperature response of experimental heifers inoculated
          with EBA 59-795: average of 23 animals.

FIGURE 2. Average leucocytic response of 23 experimental heifers
          inoculated with EBA 59-795.

FIGURE 3. Group-specific CF antibody responses of 2 heifers
          inoculated with EBA 59-795.

FIGURE 4. Group-specific CF antibody response of the single heifer
          (No. 80) inoculated with the intestinal isolate I-7666.
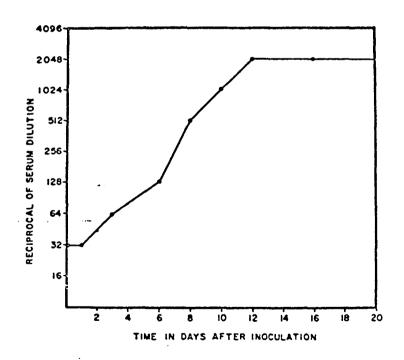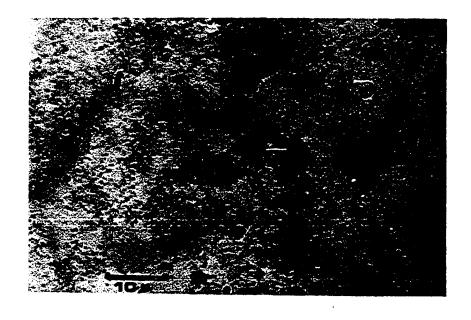
FIGURE 5. Gimenez stained smear of purified, yolk sac propagated,
          EBA 59-795. Elementary bodies are seen as numerous
          scattered black dots. Some less darkly stained debris
          is also present. X 1,600.

FIGURE 6. Electron micrograph of EBA 59-795 purified from unfrozen
          yolk sac material. Intact elementary bodies (EB) and
          initial bodies (IB) can be seen as well as some ruptured
          elementary bodies (R). Chromium shadowed preparation.
          X 11,400.
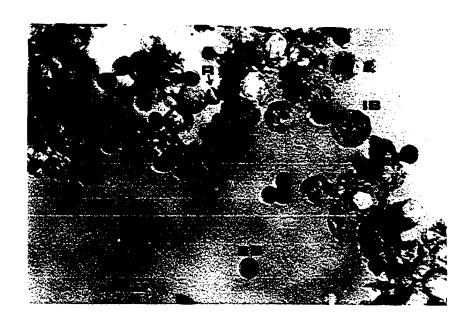
FIGURE 7. Electron micrograph of EBA 59-795 purified from unfrozen yolk sac material. The preparation was dispersed by mild sonification. Elementary bodies (EB), initial bodies (IB) and scattered debris (D) can be seen. The debris may have resulted from initial body disintegration. Chromium shadowed preparation. X 11,400.

FIGURE 8. Electron micrograph of EBA 59-795 purified from frozen yolk sac material. Intact elementary bodies (EB) and ruptured elementary bodies (R) are present along with a considerable amount of debris (D). There are no intact initial bodies. Chromium shadowed preparation. X 11,400.

FIGURE 9.   Electron micrograph of purified elementary bodies after
            5 minutes of high power sonification. Both intact
            elementary bodies (EB) and ruptured elementary bodies
            (R) are present. Chromium shadowed preparation. X 16,000.

FIGURE 10. Electron micrograph of purified elementary bodies after
            sonification and 4 hours of extraction with sodium deoxy-
            cholate (SDOC). No intact elementary bodies are present.
            Chromium shadowed preparation. X 16,000.
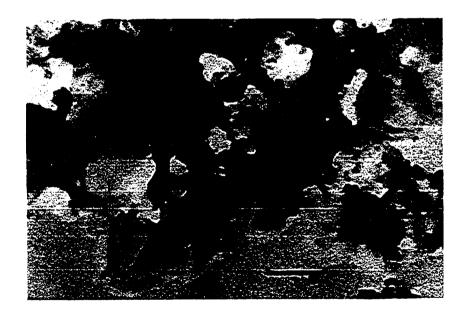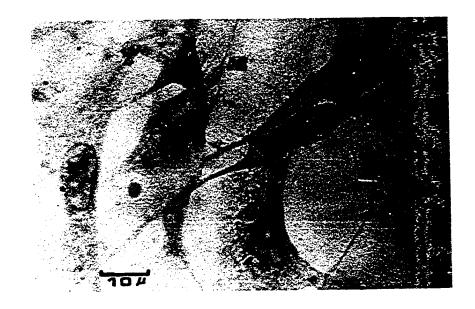
FIGURE 11. Photomicrograph of uninfected lamb thyroid cell·culture
            (LT-35). Gimenez stain. X 1,300.

FIGURE 12. Photomicrograph of LT-35 cell culture 60 hours after inocu-
            lation with EBA 59-795. Two mature inclusions of darkly
            stained elementary bodies are present in the cell at
            the center of the photomicrograph. The cell at the right
            edge contains a more loosely packed inclusion of
            elementary bodies. Gimenez Stain. X 1,300.
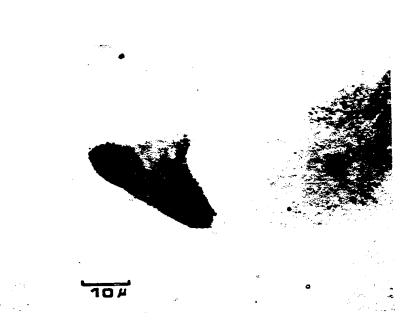
FIGURE 13. Photomicrograph of an LT-35 cell culture 36 hours after
            infection with EBA 59-795. Both immature (I) and
            mature (M) inclusions can be seen. Particles in the
            immature inclusion are larger and less darkly stained
            than those in the mature inclusion. The lightly stained
            particles are probably initial bodies and the darkly
            stained particles elementary bodies. Gimenez stain.
            X 1,300.

FIGURE 14. Electron micrograph: thin section through a portion
            of the cytoplasm of a lamb spleen cell 72 hours after
            infection with EAE B 577. A portion of an inclusion
            occupies the left 2/3 of the micrograph. In this in-
            clusion, elementary bodies (E), initial bodies (I) and
            intermediate bodies (R) can be found. The intermediate
            body (R) shows a discontinuous double membrane indicating
            breakage of cell wall and cell membrane. The remaining
            1/3 of the micrograph depicts host cell cytoplasm. The
            golgi (G) and mitochondria (M) appear unaffected by the
            infection. The black mark (A) in the cytoplasm is arti-
            fact of staining. Uranyl acetate and lead citrate stain.
            X 12,000.
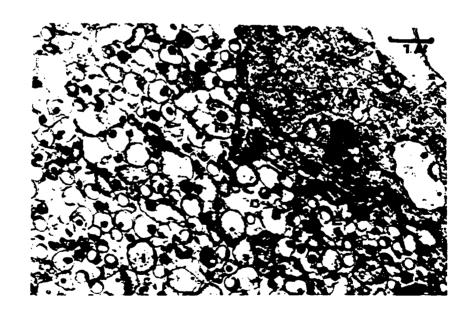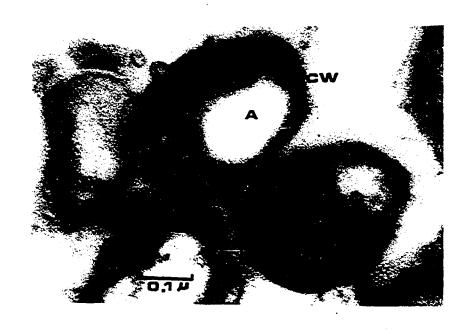
FIGURE 15. Electron micrograph: thin section of 2 EAE B 577 elemen-
tary bodies. Cell walls (CW) are visible as unit membranes
along the borders of the 2 elementary bodies. Electron
dense nucleoids (N) are seen in both elementary bodies.
The lighter portions in the elementary bodies are probably
artifact (A). Uranyl acetate and lead citrate stain.
X 142,000.

FIGURE 16. Electron micrograph: thin section of an EAE B 577 elemen-
tary body (EB) and portion of lamb spleen cell. The
elementary body contains numerous uniformly scattered ribo-
some-like granules. The lamb spleen cell appears to be in
the process of engulfing the elementary body. Mitochon-
dria (M) and polyribosomes (R) are visible in the host
cell. Uranyl acetate and lead citrate stain. X 82,000.
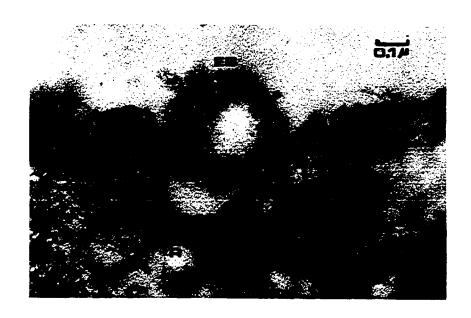
FIGURE 17. Electron micrograph: thin section of an EAE B 577 elemen-
           tary body. Both cell wall (CW) and cell membrane (CM) can
           be seen. The membranes are of the unit type and have
           dimensions of approximately 7.0 mu. Uranyl acetate and
           lead citrate stain. X 190,000.

FIGURE 18. Electron micrograph: thin section of an EAE B 577 inter-
           mediate body. The most prominent feature is the centrally
           located dense nucleoid with fine filaments radiating
           from it. Both cell wall and cell membrane can be
           resolved. Uranyl acetate and lead citrate stained.
           X 66,500.
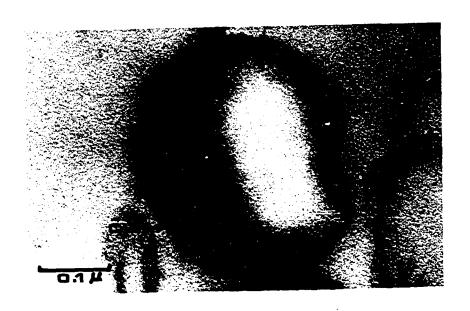
FIGURE 19. Electron micrograph: thin section of an EAE B 577 inter-
mediate body in the last stage of binary fission. Separa-
tion of the 2 daughter cells (arrow) is nearly complete.
The larger daughter cell has 2 nucleoids. Uranyl acetate
and lead citrate stain. X 82,000.

FIGURE 20. Electron micrograph: thin section of an unusual division
of an EAE B 577 initial body. Complete cell membrane (CM)
separation has occurred but cell wall (CW) separation has
not yet occurred. Numerous ribosome-like granules (R) are
present. Uranyl acetate and lead citrate stain. X 87,000.

64
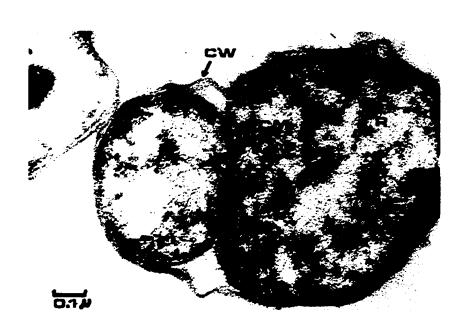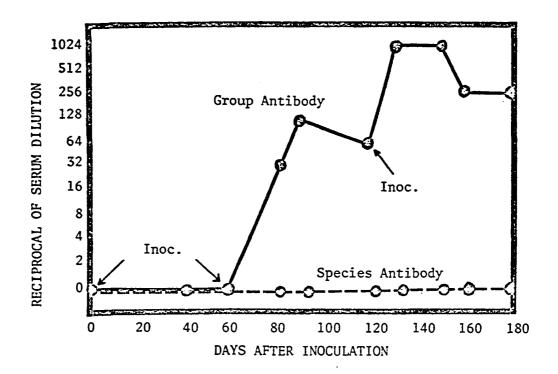
FIGURE 21. CF antibody response of rabbit No. 3 inoculated with
            group-specific (SDOC extracted) antigen prepared from
            EBA 59-795. The group-specific antibody response was
            determined by titration against group-specific CF
            antigen. The species-specific response was determined
            by titration against EBA 59-795 species-specific CF
            antigen. This rabbit had been inoculated intradermally
            at days 0 and 60 followed by an intravenous inoculation
            at 120 days.

FIGURE 22. CF antibody response of rabbit No. 1 inoculated with
            EBA 59-795 species-specific antigen. Titrations were
            performed against group-specific and homologous species-
            specific antigens. The rabbit had been inoculated intra-
            venously at days 0, 60 and 120.

FIGURE 23. CF antibody response of rabbit No. 2 inoculated with
            EBA 59-795 species-specific antigen. Titrations were
            performed against group-specific and homologous species-
            specific CF antigens. This rabbit was inoculated intra-
            venously at days 0, 60 and 120.

FIGURE 24. CF antibody response of rabbit No. 5 inoculated with
            species-specific LW-679 (sheep polyarthritis) antigen.
            Titrations were performed against group-specific and
            homologous species-specific CF antigens. This rabbit
            had been inoculated intravenously on days 0 and 24.

FIGURE 25. CF antibody response of rabbit No. 6 inoculated with
species-specific LW-679 (sheep polyarthritis) antigen.
Titrations were performed against group-specific and
homologous species-specific antigens. This rabbit
had been inoculated intravenously on days 0 and 24.

FIGURE 26. CF antibody response of rabbit No. 7 inoculated with
species-specific I-7666 (intestinal chlamydia) antigen.
Titrations were performed against group-specific and
homologous species-specific antigens. The rabbit had
been inoculated intravenously on days 0 and 14.
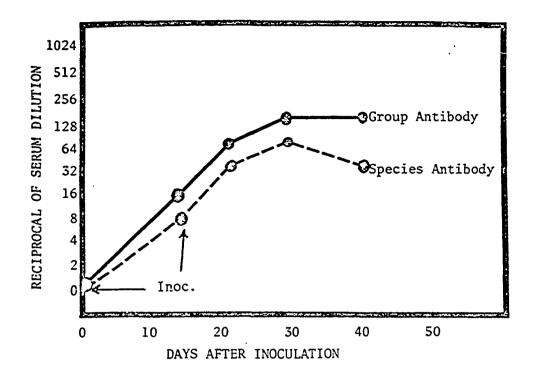
FIGURE 27. CF antibody response of rabbit No. 8 inoculated with
            species-specific I-7666 (intestinal chlamydia) antigen.
            Serum titrations were performed against group-specific
            and homologous species-specific CF antigens. The rabbit
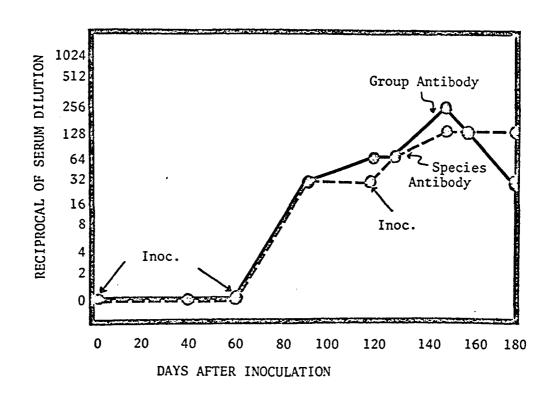   had been inoculated intravenously on days 0 and 14.

FIGURE 28. CF antibody response of rabbit No. 9 inoculated with
            species-specific antigen prepared from the EBA agent
            reisolated from the fetal spleen of cow No. 58.
            Titrations were performed against group-specific and
            homologous species-specific CF antigens. This rabbit
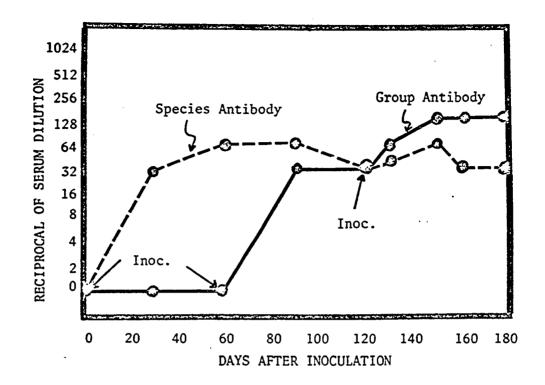   had been inoculated intravenously on days 0 and 14.
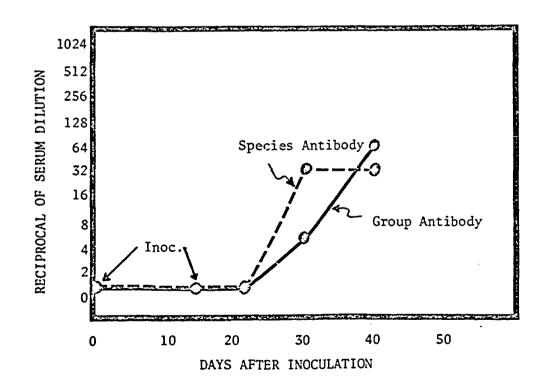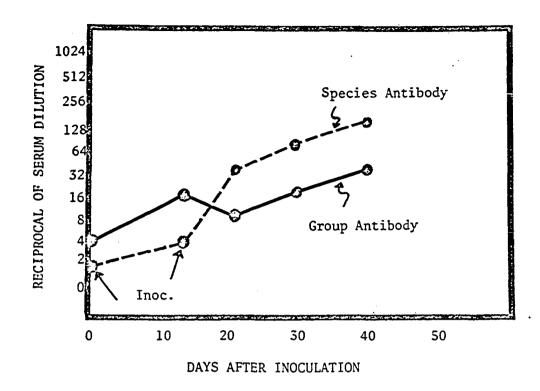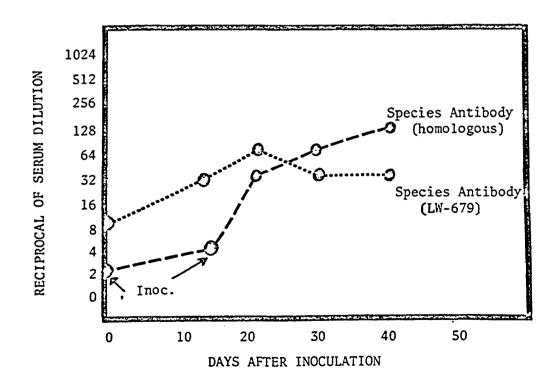
FIGURE 29. CF antibody response of rabbit No. 10 inoculated with
            species-specific antigen prepared from the EBA agent
            reisolated from the fetal spleen of cow No. 58.
            Titrations were performed against group-specific and
            homologous species-specific CF antigens. The rabbit
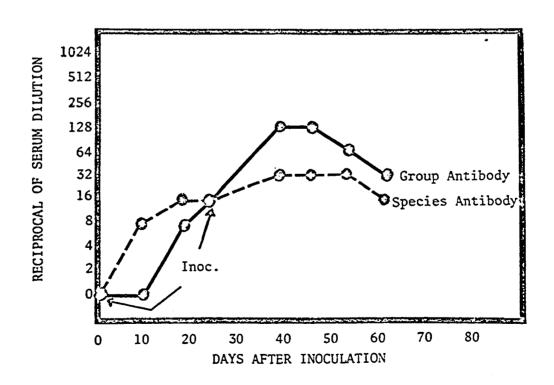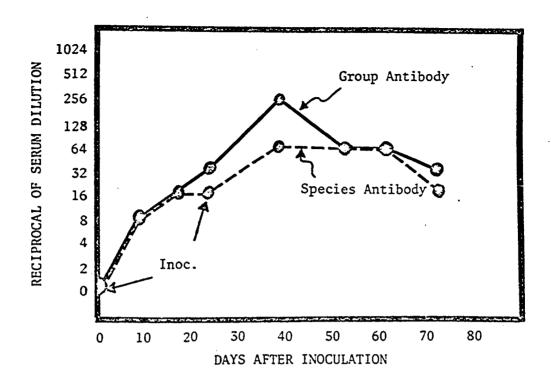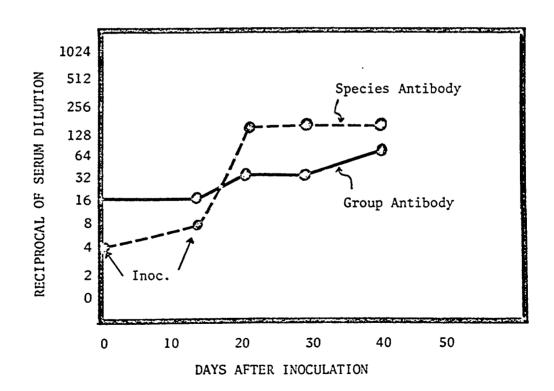   had been inoculated intravenously on days 0 and 14.

FIGURE 30. CF antibody response of rabbit No. 10 inoculated with
            species-specific antigen prepared from the EBA agent
            reisolated from the fetal spleen of cow No. 58.
            Titrations were performed against the homologous
            species-specific antigen and the heterologous LW-679
            species-specific antigen. The greatest increase in
            CF titer is shown by the homologous test system.

CHAPTER V

DISCUSSION

Clinical Response of Inoculated Cattle

Inoculation of the 23 pregnant heifers with the EBA agent resulted in marked febrile responses. Eleven of the 23 showed biphasic temperature responses similar to those reported by Studdert and McKercher (1968) in sheep inoculated with the EBA agent. The biphasic nature and height of the temperature response were evidently not functions of the inoculum titers as no correlations between them could be seen.

Twelve of the 23 infected heifers aborted or showed signs of impending abortion before the scheduled date of euthanasia. Attempts to correlate abortion with inoculum titer, maximum temperature response or biphasic temperature response were unsuccessful. Three infected heifers (No. 56, 57, and 69) aborted within 12 days after inoculation while the remaining 9 aborted from 27 to 49 days after inoculation.

The 3 early abortions may possibly have been the result of a toxic response of the dam. This hypothesis is supported by the findings of Studdert and McKercher (1968) who demonstrated the presence of a mouse lethal toxin in their preparations of EBA 59-795. Their studies of ovine abortion with the EBA agent also showed an early abortion syndrome which they concluded was the result of the toxin in their EBA preparation. It is probably significant that all early abortions in the present experiment occurred within one inoculation group. The 4 animals receiving this pooled inoculum were cows 56, 57, 69, and 65. Cow 65 might also have aborted early but was euthanatized 7 days after inoculation.

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One might expect that the toxin would also be responsible for the biphasic febrile response (in which case the first peak would be the result of the toxin and the second the results of the infection). The work of Studdert and McKercher (1968) supports this to a certain extent since their toxic preparations resulted in biphasic responses in many instances. In the present bovine abortion experiment, however, it was not possible to correlate early ("toxic") abortion with the biphasic temperature response.

Another explanation of biphasic response might be hypothesized on the basis of work by Storz, Marriot and Thornley (1968). These workers showed with EAE that two phases of blood infection were observed after intravenous inoculation. The first phase of bacteremia lasted only 2 hours after inoculation and the second phase began 24 to 30 hours after the initial clearance and lasted 2 to 3 days. Such detailed studies of bacteremia were not attempted in the present study but a relationship between biphasic bacteremia and biphasic fibrile response might exist.

Immunologic Response of Inoculated Cattle

All 23 heifers inoculated with the EBA agent showed increases in group-specific CF titers (Table 7). The rise in titer from time of inoculation to time of necropsy ranged between 4-fold for cattle killed 5 and 6 days post inoculation to as much as 128-fold for cattle necropsied later than 6 days after inoculation.

Analysis of the serologic results of preinoculation and control samples shows the group-specific CF titers of some uninfected animals varied as much as 8-fold over the 6 to 8 month period of testing. This variance could not be correlated with the presence of intestinal chlamydia found by Lincoln (1968) at the time of the preinoculation

serologic testing. The 6 cattle (Cow nos. 54, 61, 67, 75, 76, and 82) which Lincoln found to harbor intestinal chlamydia showed no pattern of CF titer rise. The variance in CF titers may therefore be non-specific and not a reflection of recent chlamydial infection.

All cattle tested had preinoculation titers of between 1:16 and 1:64 (2nd sampling) or 1:16 and 1:128 (1st sampling). This preinoculation titer evidently did not interfere with the susceptibility to experimental infection with EBA since essentially all heifers inoculated appeared to be infected (as determined by abortion, pathologic lesions or reisolation of the etiologic agent). The only exception was one cow (No. 65) necropsied 7 days post inoculation. This cow showed a febrile response and an immune response but failed to show any specific lesions (Lincoln et al., 1969 and Kwapian et al., 1970) and yielded negative reisolation results from both the placenta and fetus (see Table 9).

The rapid and dramatic antibody response after inoculation with the EBA agent was contrary to the results of other workers (Storz and McKercher, 1970; Storz, 1969; Studdert and McKercher, 1968; Storz et al., 1967 and Bassan, 1966). These workers found either a slight response or a very much delayed response. Storz (1969) showed that in both sheep (EAE) and cattle (EBA) experimental infection produced only a 2 fold rise in CF antibody titer. Two weeks after abortion, however, these animals showed a marked rise in antibody titer. The author concluded that the abortion process resulted in reinfection of the dam thus giving rise to an anamnestic antibody response.

There were striking similarities between the antibody responses of the 23 principles in the present experiment and the post-abortion

(anamnestic) antibody responses reported by Storz (1969) and Storz and McKercher (1970). A conclusion that the antibody responses of the 23 principles were also anamnestic would be well supported by the facts that all cattle tested had preinoculation chlamydia titers (Table 7) and that preinoculation chlamydial infections were shown to exist in the intestines of several of the 23 cattle (Lincoln, 1968).

Observations on the Pathogenesis of Experimental EBA

Reisolation of chlamydia from the blood of infected heifers indicated that inoculation of EBA produced a blood infection lasting up to 5 days after inoculation. This "chlamydemia" occurred with an infection of various non-placental organs of the dam for a period of 6 days post inoculation and resulted in a chronic infection of the placenta. Negative reisolation results from the placenta of cow 65 necropsied 7 days after inoculation (Table 9) indicated that the placental infection was quite low grade at such an early stage of incubation. The placental infection appeared to be chronic in nature as many heifers apparently carried the infection 30 to 49 days after inoculation.

Infection of the fetus was evident in over 50% of the infected heifers (12 out of 23). A tissue predilection or target organ was not seen in the fetuses (Table 10) as isolations were made from liver, spleen, kidney, lung and heart.

The results presented in Table 9 show a possible difference in the susceptability of the fetus in the second as opposed to the third trimester of gestation. Of the 12 heifers inoculated in the second trimester of pregnancy, infections were detected in 7 fetuses from days 5 to 49 post inoculation. No correlation between abortion and fetal infection was seen. Of the 11 heifers inoculated in third trimester,

however, all 5 fetuses obtained 29 days post inoculation were found to be infected while all of those obtained before 29 days of infection were not infected. Correlation between abortion and fetal infection was found to be nearly perfect.

There appear to be several possible explainations for the delay in infection of 3rd trimester fetuses:

1. The placentation in the 3rd trimester may be such that the EBA agent is unable to cross the placental barrier. The finding that fetal infections were established at the time of abortion or impending abortion is explainable as a break down of placental barriers by the abortion process and subsequent release of the EBA agent into the fetal circulation.

2. Interferon-like substances might play a role in delaying the infection in 3rd trimester fetuses. The differences seen between 2nd and 3rd trimester fetuses might represent differing abilities to pro-duce interferon.

3. It is also possible that the infection may have been present in 3rd trimester fetuses but at a level which was below detection by the methods used.

None of the above hypotheses to explain the delay in infection of 3rd trimester fetuses have been tested.

The placental pathology of experimental EBA was investigated by Kwapian (1968) and Kwapian et al. (1970). Using the same 23 heifers as in the present study, Kwapian showed that all heifers from day 12 to day 49 post inoculation had some degree of placentitis. The only heifers showing no placental lesions were No. 54, 62, and 65 necropsied 6 and 7 days after inoculation. Kwapian concluded that abortion,

except for the three early abortions, was the result of severe placentitis and not fetal infection. This finding is opposed to the findings of Kennedy et al. (1960) and Storz and McKercher (1962) who attributed abortion to the severity of the fetal infection. The possibility does exist that the severe placentitis seen in the present experiment was an artifact related to the high infection dosage and intravenous route of infection. The placentitis seen in the three early abortions was not thought to have caused the abortions.

The reisolation results from the placenta (Table 7) show that all those cattle with placentitis had infected placentas. The only negative isolation result was from a placenta (cow 65 necropsed 7 days post inoculation) with no gross or microscopic lesions visible. Those placentas which were not suitable for testing (contamination of the expelled membranes) were found (Kwapian et al., 1970) to contain numerous elementary bodies as demonstrated by Gimenez stained impression smears.

Chlamydial agents were recovered from the intestinal contents of 6 cattle tested before inoculation (Lincoln, 1968) and 3 cattle tested at necropsy. This would indicate a continuing intestinal infection in the herd. Preinoculation infections probably explain the presence of pre-inoculation CF titers. Our experimental infection of heifers with preinoculation titers confirms the report of Storz et al. (1967) who produced abortion in 2 cows with preinoculation EBA titers of 1:8 and 1:32. Later work by Storz et al (1968) showed that the rate of clearance of chlamydia (EAE agent) from the blood of sheep was evidently not influenced by the presence of humoral CF antibodies.

It is perhaps of significance that EBA antiserum obtained from an experimentally infected heifer showed a very high group-specific CF titer (1:1024) but essentially no serum neutralization titer (undiluted serum neutralized only 0.2 logs of EBA agent after 4 hours incubation) (Lincoln, 1968). With these results, one might propose two explanations for infection in the face of pre-existing antibody: first it is possible that a very large inoculum ($10^{4.9}$ to $10^{9.0}$ EID_{50}) would overwhelm any humoral immunity and second it is likely that CF antibody is of little significance in terms of immunity. The second explanation is favored if the absence of in vitro protection (serum neutralization) can be equated with the absence of in vivo immunity.

To summarize the findings related to pathogenesis, one might propose the following sequence of events in experimental EBA infection. Intravenous inoculation of the EBA agent produced a blood infection lasting up to 5 days and infection of various non-placental organs of the dam for a period of up to 6 days after inoculation. The placenta is usually infected by at least 5 days after inoculation and the infection persists until abortion. Abortion is probably the result of severe placentitis. The infection and abortion processes are apparently not altered by the presence of preinoculation CF antibodies or by the presence of a latent chlamydial infection in the intestine. After clearance of the infection from non-placental organs of the dam (6 days post inoculation), the only organs affected are the placenta and fetus and the internal iliac lymph node which drains the infected placenta.

Since chlamydia were isolated from the intestinal contents of control and experimental cattle before and after inoculation, no information could be gained on intestinal localization of the EBA agent.

Infection of the fetus may occur as early as at least 5 days after inoculation if the heifers are infected in the second trimester. Fetal infection probably does not occur until 29 days post inoculation if the heifer is inoculated in the third trimester of pregnancy. Time of abortion is extremely variable in the second trimester heifers but is remarkably constant (27 to 30 days) in third trimester heifers. Correlation between abortion and fetal isolations indicate that, in the third trimester, fetal infection may not occur until the abortion process has begun. This delay in fetal infection contrasts with the work of Storz et al. (1968) who demonstrated that ewes inoculated with the EAE agent developed a placental infection by 48 hours post inoculation and a fetal infection by 72 hours post inoculation.

Reisolation of the EBA agent from numerous different fetal tissues indicates that the fetus is probably infected hematogenously from the infected placenta. This agrees with the observation of Storz and McKercher (1962) that the fetus was infected hematogenously rather than through ingestion of infected amnionic fluid.

Infection of a pregnant heifer with the I-7666 Intestinal Agent

The isolation of chlamydial agents from the intestinal contents of 6 heifers before inoculation led to attempts to differentiate the intestinal agent from the experimental EBA agent. This was attempted first by inoculation of a pregnant heifer with the intestinal agent recovered from cow No. 76 before inoculation. The results of this experiment showed no differences in the disease picture grossly, histopathologically (Lincoln, 1968 and Kwapian, 1968) or immunologically. As with many EBA infected heifers, the agent could be recovered from the placenta and more than one fetal organ. These results are similar

to those of Storz (1963) who produced abortion and birth of live, infected sheep by inoculation of ovine intestinal agents into autologous sheep. Also Storz and McKercher (1962) inoculated a single pregnant heifer with a bovine intestinal agent isolated earlier by McKercher and Wada (1959). This isolate caused premature birth of a live but infected calf. The authors concluded that the EBA agent was similar to the intestinal agent except perhaps for a somewhat decreased pathogenicity of the intestinal agent. The I-7666 intestinal agent used in the present study showed no evidence of decreased pathogenicity.

Little new information was gained in this experiment regarding the epizootiologic significance of the latent intestinal infection. Of the six heifers found to be infected before inoculation, four were used as control animals. These four were necropsied, 11, 37, 41, and 48 days after placebo inoculation and no chlamydial agents were recovered from any organs or intestinal contents of the dams or fetuses. One control animal, however, which was found to be negative before inoculation showed the presence of intestinal chlamydia at necropsy (19 days after placebo inoculation). The validity of this finding of appearance and disappearance of intestinal chlamydial infections is prehaps questionable as the techniques used may not have been sensitive enough to consistently detect very low levels of infection. The absence of any abortions, infected placentas or infected fetuses among the controls indicated that the latency of the intestinal chlamydial infection was not broken. Conditions for breaking the latency of the intestinal infection must be determined before a role of intestinal chlamydia infections in EBA can definately be stated.

Production of Species-Specific and Group-Specific Antisera in Rabbits

All three rabbit-inoculation-schedules resulted in production of antisera of satisfactory CF titer and specificity. Because little difference was seen, the shortest schedule of immunization (2 injections spaced 14 days apart) appeared to be the most desirable. It is possible that three to four injections given at 10-14 day intervals would yield even higher antibody titers.

The maximum species-specific antibody responses seen in these rabbits ranged from 1:32 to 1:256 while the maximum group-specific response was 1:1024. These responses are similar to those responses obtained by other workers. Fraser and Berman (1965) showed in guinea pigs homologous species-specific responses of between 1:20 and 1:640 and Jenkin et al. (1961) found guinea pig serum titers of between 1:80 and 1:160. The results indicate that rabbits are probably as satisfactory as guinea pigs for production of antisera to chlamydial antigens.

Species-Specificity of Purified Preparations

The results presented in Fig. 22 - 29 show that cell wall antigens inoculated into rabbits produced not only species-specific responses but also group-specific CF antibody responses of approximately equal magnitude. Fig. 21, however, shows that inoculation of purified group-specific antigen (SDOC extract of purified elementary bodies) produced a group-specific response only. This can be taken as evidence that the procedure used for producing species-specific antigen did not remove all group-specific material. On the other hand, purified group-specific antigen evidently contained no species-specific antigen.

Complete cross reactions were found between EBA 59-795, the EBA reisolate and the I-7666 intestinal agent. With the exception of

rabbit number 10 antiserum, essentially no cross reaction was seen between the polyarthritis agent (LW-679) and the other 3 antigens. The high LW-679 titer in rabbit number 10 which had received the EBA reisolate species-specific antigen indicated probable previous exposure to an antigen similar to the sheep polyarthritis agent. This supposition was supported by the results illustrated in figure 30. Pre-inoculation titers to the homologous EBA and heterologous LW-679 were 1:2 and 1:8, respectively. At the final sampling, the titers were 1:128 and 1:32, respectively. This represented a 64-fold rise in EBA titer and a 4-fold rise in LW-679 titer. Thus, while the response was primarily to the EBA agent, the relatively high preinoculation LW-679 titer gave the appearance of no species-specificity.

Other small cross reactions of between 1:2 and 1:8 were seen between LW-679 and the other three strains. These, however, presented no difficulties in interpretation of the results. The slight cross reactions were probably the consequence of incomplete extraction of all the group antigen from the cell wall preparations.

The finding that the I-7666 intestinal isolate was antigenically identical to EBA conflicts with similar comparisons made by other workers. Fraser and Berman (1965), using the species-specific CF test, found the bovine intestinal isolate of York and Baker (1956) to be antigenically different from EBA. Wilson (1965) compared several cattle fecal strains to the EAE agent and found them to be unrelated. Wilson's test for antigenic relationship was based on serum neutralization instead of the species-specific CF test and therefore may not be completely comparable.

The work of Storz (1968) showed that more than one intestinal strain can be found in sheep. In investigating separate epizootics of ovine polyarthritis and ovine abortion, Storz found that the intestinal strains isolated from an EAE flock were serologically identical (serum neutralization test) to EAE and different from ovine polyarthritis. Those intestinal agents recovered during an outbreak of polyarthritis, on the other hand, were identical to the polyarthritis agent and different from the EAE agent. With precedent found in sheep, it may be speculated that several different strains of bovine intestinal chlamydia exist.

The identity found between the bovine intestinal strain and the EBA agent in the present work strengthens the case for an intestinal chlamydial role in EBA epizootiology.

Ultrastructural Morphology of Chlamydia

The ultrastructural studies of the purified EBA agent showed it to be identical to other members of the genus Chlamydia (Higashi, 1964; Manire, 1966). The degree of purity obtained appeared to be quite high except that the procedure used did not separate the larger, immature, bodies from the elementary bodies. The results of freezing and sonification during the purification procedure suggest that the larger immature particles are easily disrupted by these methods. Sonification in the presence of sodium deoxycholate resulted in complete disruption of the small, or elementary, bodies.

The ultrastructural studies of the EAE agent in lamb spleen cell culture showed the morphologic forms of initial, intermediate and elementary bodies essentially as described by other authors (Higashi, 1964; Armstrong and Reed, 1964 and 1967; Anderson et al, 1965; and

Armstrong, 1968). The structure of the elementary body, however, may be somewhat more uniformly compact than has been previously reported. It appeared to exist as a dense nucleoid uniformly surrounded by a cytoplasm which was evenly packed with ribosomes. This was tightly enclosed by the 2 unit membranes of cell wall and cell membrane. The observation that cell wall and cell membrane were morphologically identical is in agreement with the work of Armstrong (1968).

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