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Immunofluorescence Studies on the Pathogenesis of Intestinal Chlamydial Infections[1]

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Newborn calves were exposed orally to the chlamydial agent of bovine polyarthritis. The chlamydial infection in the gastrointestinal tract was traced by reisolation of the agent and by fluorescent-antibody techniques. Absorption of the fluorescein isothiocyanate (FITC)-labeled antiserum with bovine fetal intestinal tissue powder eliminated effectively the nonspecific fluorescence of eosinophilic granules in intestinal tissue sections. Cells of the eosinophilic series were observed in great numbers in the gastrointestinal tract of inoculated and normal calves. Although chlamydial agents could be reisolated from mucosal scrapings of abomasum and duodenum for 5 days after inoculation, specific fluorescence was not observed in these gastrointestinal portions. As the chlamydial infection progressed, it localized in the mucosal epithelial cells of the jejunum and ileum. Fluorescing chlamydial inclusions were observed most consistently in the cytoplasm of mucosal epithelial cells on the tips of the jejunal and ileal villi. The inclusions were located between the nucleus and the free border of the epithelial cells. In the deeper parts of the villi, the inclusions in the epithelial cells were situated frequently between the nucleus and the basement membrane of the mucosal lamina propria. In calves examined 7 days after inoculation, fluorescing chlamydial inclusions were seen in the cells of the crypts and the mucosal lamina propria of the lower portions of the small intestine. Chlamydial infection of cells in the intestinal interstitium reflected a process of systemic invasion.

Fluorescent-antibody (FA) techniques have been applied successfully to the detection of chlamydial antigens in infected cultured cells, and in impression smears prepared from the human conjunctiva and infected yolk sacs (1, 7, 11, 12). The inherent advantages of immunofluorescence have not been applied to the study of pathogenetic events in chlamydial infections of man and animals.

In intestinal infections, FA techniques have been used with varying degrees of success to locate viral antigens in frozen sections of intestinal specimens. Pensaert and co-workers (13) found viral antigens in the cytoplasm of epithelial cells covering the villi of the small intestine of piglets affected with transmissible gastroenteritis. Colgrove and his associates (4) located viral antigens of African swine fever in the mucosal lamina propria and submucosa of the lower portions of the small intestine of pigs. In contrast, Fernelius and Lambert (5) were unable to identify by immunofluorescence the cell type or the tissue structures of the infected intestine of calves after exposure to the virus of bovine diarrhea. Immunofluorescence studies on the poliovirus infection of the intestinal tract in monkeys were inconclusive (8).

It is well established that chlamydial agents infect the intestinal tract of cattle (10, 18). In young calves, enteritis may be associated with the intestinal chlamydial infection (15, 19). Although the majority of cattle excreting chlamydiae in their feces are clinically normal, intestinal chlamydial infections may play an important role in the pathogenesis of chlamydia-induced diseases, such as polyarthritis, abortion, pneumonia, encephalomyelitis, or conjunctivitis. The present knowledge concerning intestinal chlamydial infections in calves is limited to the mere fact that these agents infect the intestinal tract and are excreted in the feces.

This investigation was undertaken (i) to study

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the sites and cell types of chlamydial multiplication in the gastrointestinal tract, (ii) to explore the dynamics of the intestinal infection after oral inoculation of calves with the chlamydial agent of polyarthritis, and (iii) to compare results of isolation, FA tracing, and other cytological techniques in detecting intestinal chlamydial infections.

MATERIALS AND METHODS

Experimental design. Six newborn and two 1-week-old calves were inoculated orally with chlamydial strain LW 613. This chlamydial strain was isolated originally from synovial fluids of a polyarthritic calf (15). Another calf was inoculated intra-articularly with the same strain. The inoculum per calf consisted of a suspension of infectious yolk sac material, containing 4×10^8 to $4 \times 10^{9.5}$ 50% chicken embryo lethal doses $(CELD)_{50}$. Other experimental details concerning these calves are listed in Table 1.

Chlamydial reisolation. Seven-day-old chicken embryos were inoculated by the yolk sac route with decimal dilutions of suspensions prepared from mucosal scrapings from different portions of the gastrointestinal tract. The procedures for decontamination and chicken embryo cultivation of intestinal specimens were described elsewhere (16).

Immunofluorescence studies. Hyperimmune serum against the LW 613 strain was prepared in rabbits by giving five intravenous injections of crude and partially purified inoculum at 15-day intervals. The serum of one rabbit with a complement-fixing group-specific chlamydial antibody titer of 1:2,048 was used throughout this study.

The globulin fraction of the antiserum was conjugated with 0.02 mg of fluorescein isothiocyanate (FITC) per mg of protein as described by Spendlove (14). The labeled globulin was fractionated by chromatography on diethylaminoethyl (DEAE)-Sephadex A-50. The fractions eluted with 1 M NaCl were used. For the examination of intestinal tissue sections, the labeled globulin was absorbed with acetone-dried intestinal tissue powder prepared from an 8-month-old bovine fetus (9). Merthiolate was added to the absorbed serum at a final concentration of 1:10,000.

Annular segments from duodenum, jejunum, and ileum, and pieces with a surface area of about 1 cm^2 from abomasum, cecum, colon, and rectum, were placed in plastic bags and quick-frozen in a dry ice-alcohol bath. Tissue sections with a thickness of 4 to 5 μm were cut in a refrigerated microtome, air-dried, and then fixed in acetone at -20 C for 15 min. The slides with the tissue sections were washed once in cold buffered saline and air-dried. The FITC-labeled rabbit LW 613 antiserum was placed on the tissue sections, incubated for 20 min at 37 C in a moist chamber, washed three times for 5 min in buffered saline, and mounted in buffered glycerol.

Cytological procedures. Parallel frozen tissue sections were heat-fixed and subsequently stained according to the method of Gimenez (6). Other frozen sections were fixed for 4 min in absolute methanol and stained in Giemsa solution.

In vitro studies. Mouse L-cells (NCTC 929) grown on cover slips were infected with a partially purified suspension of the yolk sac-propagated LW 613 agent. Cover slips were removed at certain intervals after inoculation. They were fixed in acetone and stained with either FITC-labeled LW 613 antiserum or Giemsa solution, or by the method of Gimenez (6).

Fluorescence microscopy and photography. A Leitz Orthoplan microscope with an illuminating system containing a built-in heat filter, a UG1 and BG12 filter, a barrier filter (no. K510), and an H B0 200-w mercury vapor lamp was used. Photomicrographs were taken with a Leitz Orthomat camera and with Kodak TRI-X-PAN film (ASA 400).

RESULTS

Conditions for FA staining. The direct immunofluorescence test gave brighter fluorescence and was more sensitive in detecting chlamydial antigens than the indirect test. The addition of unlabeled guinea pig complement in the direct test did not enhance the staining reaction. Optimal fluorescence was obtained with the labeled globulin eluted from the DEAE-Sephadex column with 1 M NaCl.

Impression smears from chlamydia-infected yolk sacs contained almost exclusively individual elementary bodies located extracellularly. Cytological evidence for the different stages of the chlamydial developmental cycle was not found. In addition, yolk sac impression smears revealed some nonspecific fluorescence inherent with the homologous system, since infected yolk sac suspensions were used as immunizing antigen for the rabbits. Impression smears of chlamydia-infected yolk sacs were, therefore, considered inadequate for evaluating the specificity and sensitivity of this test.

Different chlamydial developmental forms were present in mouse L-cells infected with strain LW 613 (Fig. 1). Cytological features of the chlamydial infection could be studied in situ, and the specificity of the test could be established through the following controls: (i) fluorescence was not detected in uninfected L-cell cultures after staining with labeled rabbit LW 613 antiserum (Fig. 1A); (ii) bright fluorescence was observed in the cytoplasm of chlamydia-infected L-cells when they were stained with labeled rabbit LW 613 antiserum; (iii) fluorescence in chlamydia-infected L-cells was abolished or diminished if the cells were exposed to unlabeled rabbit LW 613 antiserum prior to staining with the labeled antiserum; (iv) treatment of chlamydia-infected L-cells with unlabeled normal rabbit serum prior to staining with labeled antiserum did not influence the specific fluorescence; (v) the fluorescing inclusions always reflected characteristic features of chlamydia-infected cells. The inclusions of most

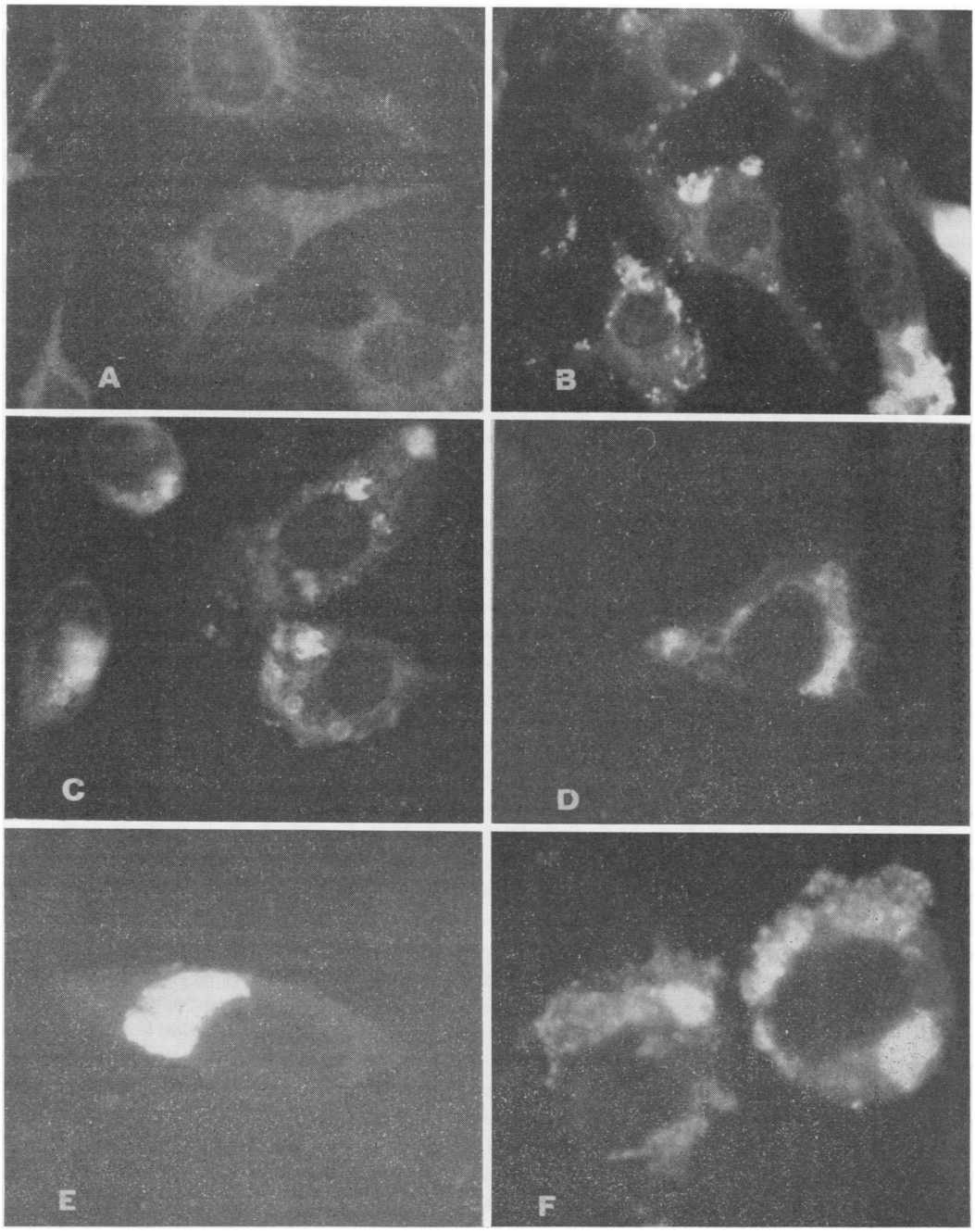

FIG. 1. *Fluorescing inclusions of the chlamydial developmental cycle in mouse L-cells infected with strain LW 613 (× 780). (A) Uninoculated L-cells. (B) L-cells 24 hr postinoculation. (C) Multiple inclusions. (D) Juxtanuclearly located inclusion. (E) Helmet-shaped inclusion hugging the nucleus. (F) Late inclusions.*

stages of the chlamydial developmental cycle could be relocated in Giemsa-stained preparations.

L-cells grown on cover slips were stained with labeled LW 613 rabbit antiserum at different time intervals after inoculation with the LW 613 strain. The purpose of this experiment was to study the immunofluorescent properties of different

chlamydial inclusions that arise during the developmental cycle. This knowledge facilitated the identification of different chlamydial inclusions in tissue sections of the gastrointestinal tract of calves.

The outstanding immunofluorescent characteristics observed in infected tissue cultures were as follows. In the early stage of infection, chlamydial antigen, in the form of small brightly fluorescing globules resembling elementary bodies from the inoculum, was present extracellularly or scattered throughout the cytoplasm (Fig. 1B). Subsequently, multiple inclusions were found singly or in cluster forms in the cytoplasm. The cluster forms had a reticulated appearance. The single forms stained more intensively at the periphery of the inclusion than in the center (Fig. 1C). This type of chlamydial inclusion was seen occasionally as a band or an arc next to the nucleus (Fig. 1D). In more advanced stages of the infection, dense, helmet-shaped inclusions hugging the nucleus were observed. These inclusions were often very bright (Fig. 1E). Inclusions occupying most of the cytoplasm were found in late stages of the infection. Cells containing such inclusions had nuclei which were dislocated to the margins of the cells. Particles of different sizes within the inclusion had brighter green color than the matrix (Fig. 1F). These types of inclusion were packed with elementary bodies in Giemsa- and Gimenez-stained cells.

The main difficulty involved in staining gastrointestinal sections with FITC-labeled LW 613 antiserum was the elimination of nonspecific fluorescence. This type of fluorescence had the characteristic apple-green color. Corresponding sections from normal newborn calves stained with Giemsa revealed that the fluorescing cells were of the eosinophilic series. These cells were found in all sections of the gastrointestinal tract, but they were seen most frequently in the small intestine and cecum, where they accumulated focally. Fluorescence due to these cells was largely limited to the villous interstitium and was also observed in the mucosal epithelium and between fibers of the muscularis (Fig. 2A and B). The granules of these cells had fluorescence of green color. Occasionally these fluorescing granules were spread over parts of tissue sections, giving the appearance of chlamydial elementary bodies.

Absorption of the labeled antiserum with bovine liver powder or counterstaining with rhodamine bovine albumin did not reduce this nonspecific fluorescence. However, it was eliminated completely by absorbing the antiserum with acetone-dried tissue powder prepared from the intestine and its contents of an 8-month-old bovine fetus. The absorbed serum retained its reactivity for specific chlamydial inclusions when tested in infected L-cells. In sections stained with this absorbed serum, the eosinophilic cells could still be recognized, but their granules no longer showed apple-green fluorescence. Instead, their color was orange and had a dull appearance.

Localization of chlamydial infection in the gastrointestinal tract by immunofluorescence. Specific fluorescence was found in the mucosal epithelial cells in calves inoculated orally with LW 613. Epithelial cells of the jejunum and ileum always revealed extensive fluorescence (Fig. 3A and B), whereas those of the cecum, colon, and rectum were involved less frequently. The whole cytoplasm of infected epithelial cells usually fluoresced. Granularity indicative of individual ele-

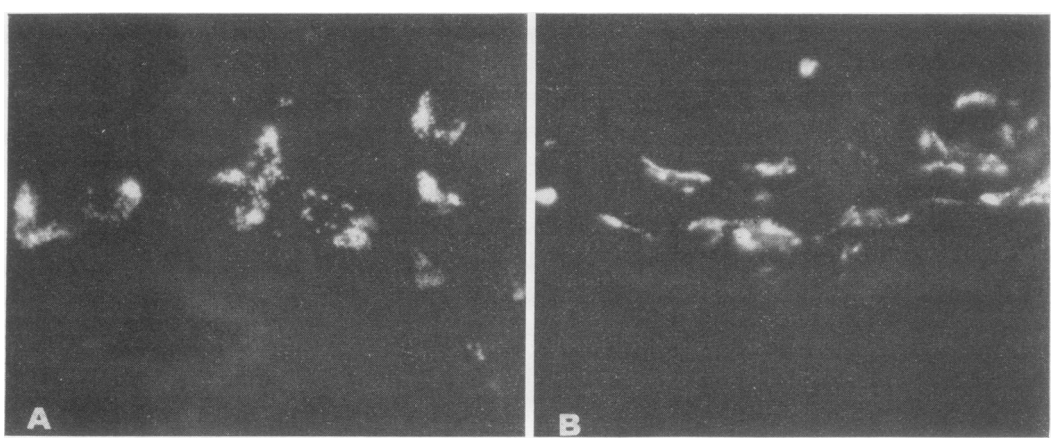

FIG. 2. *Frozen intestinal sections stained with unabsorbed FITC-labeled rabbit LW 613 antiserum* (× 780). *Nonspecific fluorescence of granules of eosinophils located in the mucosal lamina propria of the ileum* (*A*) *and jejunum* (*B*).

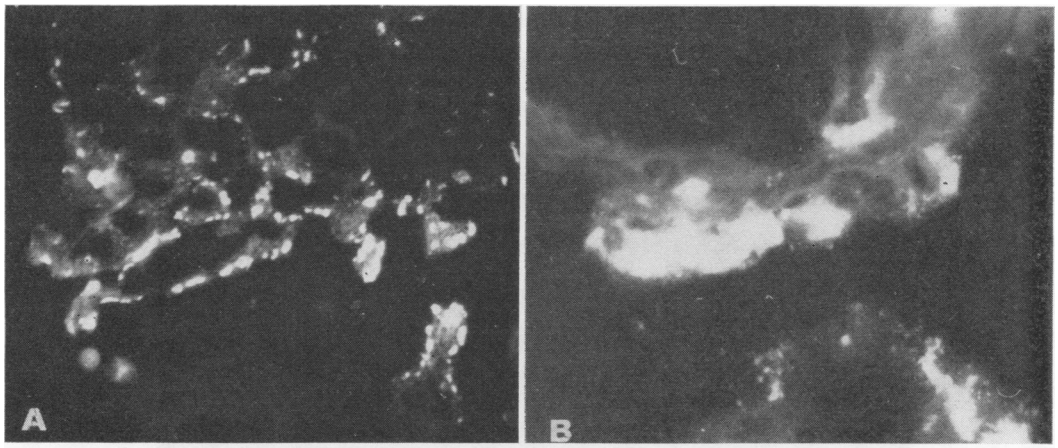

FIG. 3. *Frozen sections of jejunum stained with absorbed FITC-labeled LW 613 antiserum. (A) Heavily infected area (× 145). (B) Fluorescing inclusions in mucosal epithelial cells (× 780).*

mentary bodies was noticed within the fluorescing matrix of the inclusion. Desquamated fluorescing epithelial cells and free elementary bodies could be seen in the lumen of the intestine in heavily infected areas.

The tips of the intestinal villi were predominantly involved (Fig. 4A and B). The inclusions either filled the entire cytoplasm, or they occupied the space between the nucleus and the striated border of the cells. In deeper parts of the villi, the inclusions were located frequently between the nucleus and the supporting basement membrane of the mucosal lamina propria (Fig. 5A and B). Fluorescence in endothelial and interstitial cells of the intestinal villi was not observed, although petechial hemorrhages in the intestinal mucosa were a common feature in the younger calves. Partial denuding of the intestinal villi was observed only in heavily infected areas. In such areas, chlamydial antigen appeared to be present in the villous interstitium (Fig. 5C).

Fluorescence was not as extensive in older calves. The predominant feature was still the fluorescing epithelial cell at the tips of the villi in the lower portions of the small intestine, especially the ileum. However, epithelial cells in deeper parts of the villi and in the crypts also fluoresced. In addition, cells in the lamina propria and submucosa were fluorescing in calves 75 and 77, which were examined 11 and 25 days after inoculation (Fig. 5D).

Fluorescing inclusions in the gastrointestinal tract of calf 74, inoculated intra-articularly with strain LW 613, were limited to the mucosal epithelial cells of the ileum.

Epithelial cells with chlamydial elementary bodies located in the cytoplasm could be identified by the staining method of Gimenez (6), but not as readily as by immunofluorescence. The different forms of the developmental chlamydial cycle and chlamydial inclusions in the lamina propria could not be identified positively by this method. It was definitely inferior to isolation or immunofluorescence for the detection of chlamydial infection (Table 1). Microscopic examination of Gimenez-stained sections was rather tedious because entire sections had to be examined under highest magnification, whereas FA-stained sections could be screened quickly under low power to localize infected cells (Fig. 3A). Giemsa-stained sections of the intestine were not suitable for identifying chlamydial inclusions because of the abundance of eosinophilic cells and their granules.

Isolation of chlamydial agents in developing chicken embryos from intestinal sites appeared to be most sensitive for the detection of chlamydial infection (Table 1). A localization of the infection in the lower portions of the small intestine became more pronounced with passage of time between inoculation and necropsy.

DISCUSSION

Tissue-cultured cells were better suited than yolk sac impression smears for the evaluation of the specificity of FITC-labeled antiserum. Yolk sac smears contained almost exclusively individual elementary bodies located extracellularly, whereas all stages of the chlamydial developmental cycle were present in infected L-cells. Morphologically similar inclusions were found in chlamydia-infected L-cells and tissue sections from gastrointestinal tracts of calves orally inoculated with chlamydial agents. This finding reflected the speci-

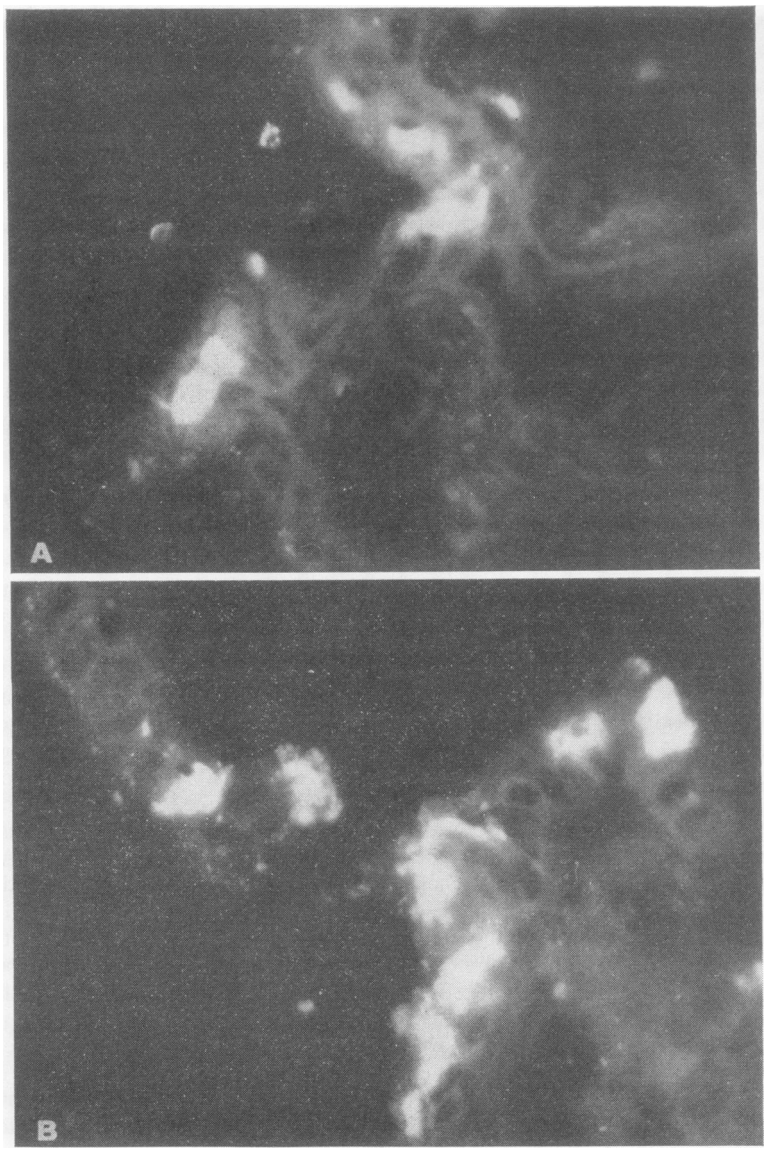

FIG. 4. *Frozen sections of the tips of the ileal villi stained with absorbed FITC-labeled rabbit LW 613 antiserum.* (× *780*). (*A*) *Fluorescing inclusions are located between nucleus and free border of the mucosal epithelial cell.* (*B*) *Inclusions occupy most of the cytoplasm of epithelial cells.*

ficity of the immunofluorescence test applied to gastrointestinal specimens. One might postulate that the cytological changes observed by immunofluorescence in intestinal infections with other obligate intracellular parasites should resemble those seen in correspondingly infected cultured cells. This postulate was not met in several studies on intestinal viral infections (5, 8).

One of the major problems encountered in staining intestinal sections from the calves with labeled antibodies was nonspecific fluorescence due to eosinophils. This nonspecific fluorescence was not reduced in this study by counterstaining with rhodamine bovine albumin. Also, absorption of the labeled rabbit serum with bovine liver powder was of little value. The nonspecific fluorescence was eliminated effectively by absorption of the labeled serum with fetal bovine intestinal powder. The cells associated with nonspecific fluorescence were identified as eosinophils and their immature forms. The cytoplasmic granules of these cells are known to cause nonspecific

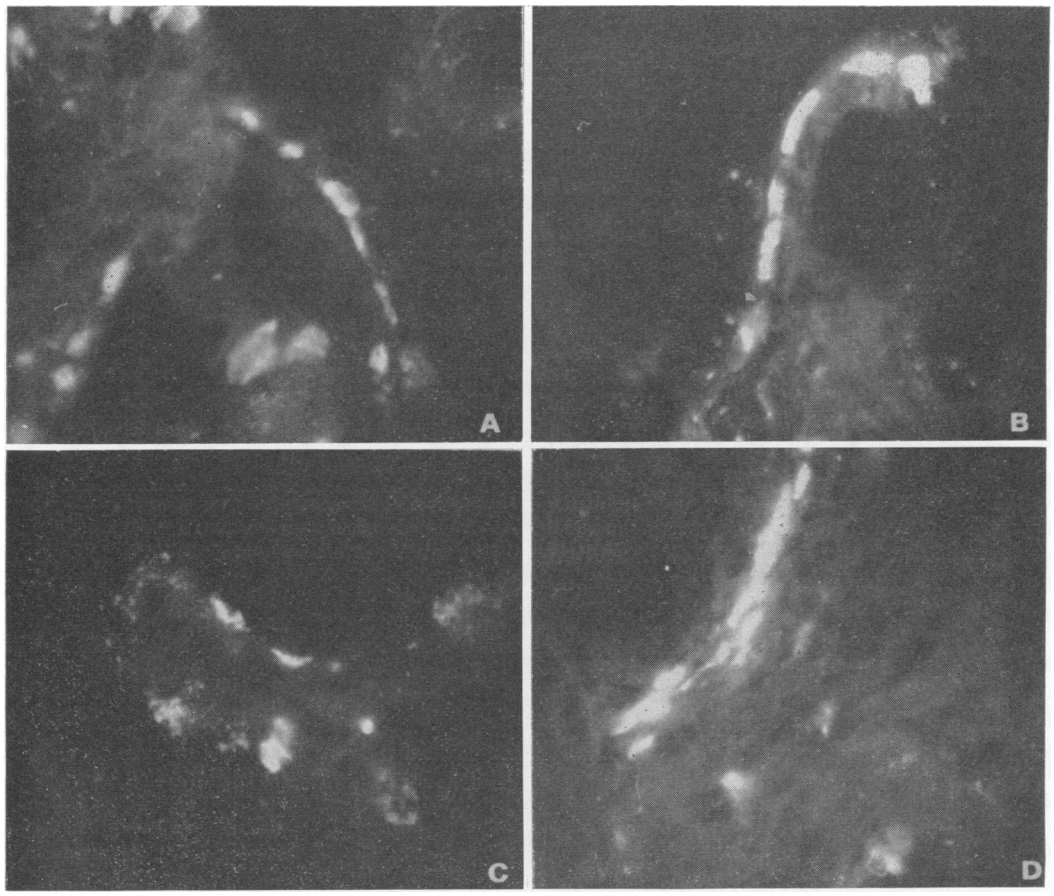

FIG. 5. *Frozen sections of ileum stained with FITC-labeled LW 613 antiserum. Deeper parts of the villi: (A, B) inclusions are located between nucleus and basement membrane. (C) Partially denuded villus. (D) Inclusions in cells of the mucosal lamina propria.*

TABLE 1. *Relative sensitivity of isolation, immunofluorescence, and Gimenez staining methods for the detection of chlamydiae in the gastrointestinal tract of calves*[a]

Calf no.	Age at inoculation (days)	Necropsy (days post-inoculation)	Colustrum administered	Abomasum I F G[b]	Duodenum I F G	Jejunum I F G	Ileum I F G	Cecum I F G	Colon I F G	Rectum I F G
11	0.25	4	No	+−−	+−−	+++	++−	+−−	+−−	++−
12	0.5	4	No	+−−	+−−	+++	++−	+−−	+−−	+−−
10	0.5	5	No	nt−−	+−−	++−	+++	++−	+−−	+−−
73	1	4	No	−−−	+−−	+++	++−	+−−	++−	++−
76	1	5	No	+−−	−−−	++−	+++	++−	++−	+−−
72	1	7	Yes	−−−	+−−	++−	+++	+−−	−−−	−−−
75	6	11	Yes	−−−	c−−	+−−	++−	+−−	−−−	−−−
77	7	25	Yes	−−−	−−−	−−−	++−	c−−	−−−	−−−
74x	16	5	Yes	c−−	+−−	c−−	++−	c−−	+−−	c−−

[a] All calves were inoculated orally with LW 613 agent, except calf 74x, which was inoculated intra-articularly.

[b] I = chlamydial agent isolated in developing chicken embryos; F = immunofluorescence of frozen tissue section; G = staining of frozen tissue section by the method of Gimenez (6); c = sample not culturable owing to heavy bacterial contamination; nt = not tested.

fluorescence (4). Chennekatu and co-workers (2) studied infection with the herpesvirus of infectious bovine rhinotracheitis and attributed fluorescence in eosinophilic cells of the spleen to viral antigens. This interpretation should be evaluated cautiously in view of the behavior of eosinophilic cells stained with unabsorbed labeled serum.

The high numbers of eosinophils seen in this study in the intestinal tract of newborn calves have not been reported in the literature. The presence of cells of the eosinophilic series in the villous interstitium and between mucosal epithelial cells of the gastrointestinal tract was not associated with the chlamydial infection, because normal newborn calves had similar eosinophilic reactions.

FA techniques have not been applied to chlamydial infections of animals. In these experiments, the mucosal epithelial cells of the tips of villi in lower portions of the small intestine were most frequently infected. The fluorescing inclusions here were located between the nucleus and the free border of the cell. In deeper parts of the villi, the inclusions were located between the nucleus and the basement membrane of the mucosal lamina propria. It is conceivable that under these circumstances a direct cell to cell transmission of the chlamydial infection could occur, in addition to the release of chlamydiae into the intestinal lumen and subsequent infection of neighboring epithelial cells via the striated borders. This type of transmission could be important in reducing the effectiveness of colostral antibodies. As the infection progressed, cells of the crypts and the mucosal lamina propria also contained inclusions reflecting the initial step leading to systemic chlamydiosis.

Desquamation of the infected cells and partial denuding of the villi were seldom observed. The continued regeneration of the epithelial cells most likely prevented denuding of the villi. Even the highly virulent virus of transmissible gastroenteritis does not denude the jejunal villi in piglets. Instead, the villi are shortened and the epithelial cells become atrophic (13).

The localization of the chlamydial infection in lower portions of the small intestine is comparable to intestinal infections with certain viruses (3, 8, 13, 17). The terminal parts of the small intestine are important sites for the preservation of the normal ecology of the intestinal bacterial flora. Changes in the bacterial flora were observed in calves exposed orally to chlamydial agents (Storz et al., Ann. N.Y. Acad. Sci., *in press*).

In a comparison of the results obtained by isolation of the chlamydial agent and immunofluorescence, isolation appeared to be superior to immunofluorescence for the detection of intestinal chlamydial infections. However, the following factors must be considered. (i) For culture, the mucosa was scraped from an area of 5 to 10 cm^2. The scrapings were cultured together with residual intestinal contents. For immunofluorescence, a section of about 0.5 to 1 cm^2 and 5 μm in thickness was examined, and serial sectioning was not employed. (ii) Intestinal chlamydial infections were found to be focal in nature. (iii) An infection located proximally in the gastrointestinal tract could have led to positive isolation in all distal portions owing to the mere passage of the agents along the intestinal tract. Immunofluorescence gave direct evidence for cellular infections as indicated by the presence of cytoplasmic chlamydial inclusions.

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