MYCOPLASMA MELEAGRIDIS INFECTION:
DEVELOPMENT OF LESIONS AND
DISTRIBUTION OF INFECTION IN
TURKEY EMBRYOS

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Received 29 March 1971

SUMMARY

Turkey embryos from eggs naturally and experimentally infected with Mycoplasma meleagridis were examined by immunofluorescence and light microscopy for distribution of infection and development of lesions. The time at which organisms could be demonstrated and their distribution suggested that the embryos became infected following ingestion or inhalation of amniotic fluid which contained M. meleagridis. Specific fluorescence was observed along the epithelial surfaces of respiratory and anterior digestive tracts of embryos after 3 weeks' incubation or more. The only inflammatory lesions observed were exudative airsacculitis and pneumonia, which occurred during late embryonic development. A hepatic perivascular granulocytopenic response also was evident near the time of hatching. The occurrence of lesions only during late embryonic development was believed to be related to maturation of inflammatory cells.

In ovo transmission of Mycoplasma meleagridis from mature turkeys to progeny is important in perpetuating this widely distributed infection of turkeys. The organism has been isolated from yolk sacs of embryonated eggs, dead-germ eggs, and infertile eggs laid by infected hens (3,7,10,11,14). It has also been isolated from both yolk and albumen of eggs recovered from the uterine portion of infected reproductive tracts (10).

Embryos experimentally infected with M. meleagridis have been examined by both immunofluorescence microscopy and cul-

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tural techniques to determine the distribution of infection (13). Organisms were found attached extracellularly to yolk-sac membranes; in the lumina of tracheae, small bronchi, and alveoli; and on the surfaces of air sacs. They were also found in the contents of the lumen and along the villi of lower intestines and in bursas of Fabricius.

Lesions, primarily exudative airsacculitis, are often seen in poults hatched from infected eggs, though little information is available on the pathologic response of embryos to *M. meleagridis* infection. This report describes a study of lesion development and distribution of infection in turkey embryos as determined by light and immunofluorescence microscopy.

**MATERIALS AND METHODS**

*Embryos.* Embryos from both naturally and experimentally infected turkey eggs, and counterpart control embryos from uninfected eggs were studied. The naturally infected eggs were from hens experimentally infected by intravaginal inoculation with *M. meleagridis*. Eggs from these hens and from similar but uninfected hens were incubated for 7, 14, 21, or 28 days, examined for fertility and viability, and cultured for mycoplasmas. Details of this portion of the study were given in a previous report (15).

Embryos viable at examination were killed by exsanguination and preserved for possible later study. Approximately half were fixed in 10% formalin; the others were frozen and stored at -65 C. Following examination for *M. meleagridis*, embryos from eggs found to be infected and a similar number of uninfected control embryos were sectioned, stained, and observed histologically. Tissue sections from the formalin-fixed embryos were examined for lesions, while sections from the frozen embryos were examined by immunofluorescence microscopy to determine the distribution of organisms. Sections of control embryo tissues were examined for comparative purposes.

Turkey embryos from the National Animal Disease Laboratory mycoplasma-free breeding flock were used to determine the lesions resulting from experimentally induced *M. meleagridis* infection. They were inoculated via the yolk sac with either a broth culture of *M. meleagridis* or sterile broth. Those inoculated with sterile broth served as unexposed controls.

Table 1 gives the schedule for inoculation and examination of these embryos, and the number examined.
At the appropriate time, the embryos were killed by exsanguination, allantoic fluid and yolk material were cultured for mycoplasmas, and the embryos were fixed in 10% formalin and processed for microscopic examination.

Distribution of infection was compared in experimentally infected embryos and naturally infected embryos. Five embryos, incubated for 7 days, were inoculated via the yolk sac with *M. meleagridis*. When the embryos had been incubated for 21 days, they were killed by exsanguination, cultured for mycoplasmas, and frozen at -65 C. Frozen sections from those embryos were examined by immunofluorescence microscopy. Equal numbers of unexposed embryos were examined in the same manner for comparative purposes.

**Inoculum and media.** *Mycoplasma meleagridis*, isolate 8M92, was obtained from Dr. M. L. Frey (Veterinary Medical Research Institute, Iowa State University, Ames, Iowa). Each experimentally exposed embryo received 0.1 ml of a 24-hour broth culture (approximately $1.3 \times 10^6$ organisms) by way of the yolk sac. The agar and broth media used were prepared as previously described (15).

**Isolation and identification techniques.** Yolk and allantoic fluids from each embryo were examined for mycoplasmas by cultural techniques described previously (15). Representative isolates from the embryos were identified by either tube agglutination or immunofluorescence tests. The tube agglutination test was conducted as described previously (14). In the immunofluorescence test, impression smears of colonies were fixed and stained with fluorescent antibody as described under histologic techniques.

**Histologic techniques.** Formalin-fixed embryos were trimmed transversely into 5 or 6 segments, which were dehydrated, infiltrated, embedded, and sectioned by conventional techniques. All sections were stained with hematoxylin and eosin.

The frozen embryos were also trimmed into 5 or 6 segments, embedded in albumen, and sectioned in a cryostat at -20 C. The embedding technique was similar to one reported previously (12). The frozen sections were picked up from the cryostat microtome blade with warm slides, allowed to thaw, and dried for a few minutes at 37 C. Those to be stained with fluorescent antibody (FA) were fixed in acetone for 2 minutes, allowed to dry, and stored at 5 C until used. Sections from the same block of tissue were fixed in either 10% formalin or absolute alcohol and were stained with
Lesions from Mycoplasma meleagridis in turkey embryos

Lesions from Mycoplasma meleagridis in turkey embryos were studied. Following FA staining and examination, some sections were restained with hematoxylin and eosin. These stained, frozen sections were used to verify the anatomic location of fluorescence.

To verify specificity of the FA reaction, 3 control measures were used: fluorescein-conjugated normal turkey gamma-globulin was used to stain selected tissue sections and smears; tissue sections from unexposed embryos were examined with FA against M. meleagridis; and immunofluorescence was blocked (6) with unlabeled specific antiserum but was not affected by unlabeled normal serum. Previous studies (5,14) using immunofluorescence techniques indicated that M. meleagridis was distinct from other avian Mycoplasma.

Tissue sections were stained with FA by flooding the tissue with an appropriate concentration of FA in phosphate-buffered saline (PBS) at pH 7.2. The FA-flooded tissue was incubated in a moist chamber at 37°C for 1 hour, washed 3 times in PBS, washed once in distilled water, and air-dried at 37°C. Cover slips were applied, with a solution of 50% glycerin in PBS used as mounting medium. Sections were examined by conventional immunofluorescence microscopy techniques.

Serum gamma-globulins were fractioned and the globulins subsequently conjugated with fluorescein isothiocyanate as described previously (8,9). Pooled serums from 4 turkeys with high antibody titers against M. meleagridis were used to prepare FA against M. meleagridis. Serum from an apparently normal turkey with no evidence of circulating antibody against M. meleagridis was used to prepare fluorescein-conjugated normal turkey gamma-globulin.

RESULTS

Isolation and identification of mycoplasmas. Mycoplasma were isolated from extra-embryonic fluids of each of the experimentally exposed embryos. Embryos from infected hens were selected for the present study as a result of isolation of mycoplasmas from extra-embryonic fluids. Representative isolates from 28 embryos were examined for identification purposes, and all were M. meleagridis. Mycoplasmas were not isolated from any of the control embryos.

Immunofluorescence examination. Examination of FA-stained sections from 2 embryos from naturally infected eggs incubated for 7 days did not reveal fluorescence.

Examination of 2 similar embryos after 14 days of incubation
revealed fluorescence only on the skin and developing feathers. The fluorescence was observed in only a few areas, appearing as small particles on surfaces.

Fluorescence was widely distributed in the 5 naturally infected embryos examined after 21 days of incubation. Specific
fluorescence was observed in the esophagus, trachea, bronchi, lungs, and air sacs of each embryo. Skin and feathers were involved as described above. Fluorescence was also observed in each crop and nasal cavity examined. Sections from 3 embryos included crop tissue, while those from 2 embryos included the nasal cavity.

Examination of a single naturally infected embryo incubated for 28 days revealed fluorescence in the same areas specified above, in the lumen of the intestine, and in intra-abdominal yolk.

The fluorescence observed within the embryos was found almost invariably along epithelial surfaces. Figs. 1, 2, 3, and 4 indicate the location of the fluorescence within the trachea, esophagus, air sac, and lung. Figs. 5 and 6 show fluorescent particles on developing feathers and skin. Fluorescent particles, not closely associated with epithelial surfaces, were observed in the lumina of crop and intestine and within yolk material.

Distribution of infection, as demonstrated by immunofluorescence microscopy, in experimentally infected 21-day-old embryos was similar to that in naturally infected 21-day-old embryos. Nasal cavities, trachea, bronchi, lungs, air sacs, esophagi, and crops had fluorescence distributed along epithelial surfaces. These fluorescent areas included more of the epithelial surface of involved tissue and were generally thicker than those of the naturally infected embryos. Fluorescent particles were found in the lumina of gizzards and proventriculi of several embryos. In 3 embryos, a few small fluorescent particles were observed in a small segment of intestine. The particles were found both in material in the lumen and adjacent

Fig. 1. Section of trachea from a naturally infected embryo after 21 days of incubation. Fluorescing areas indicate the presence of *Mycoplasma meleagridis* organisms along epithelial cells. ×200.

Fig. 2. Section of esophagus from a naturally infected 21-day-old embryo. Fluorescence indicates the location of *Mycoplasma meleagridis* organisms along epithelium. ×100.

Fig. 3. Section of tissue from a naturally infected 21-day-old embryo. Fluorescing areas indicate the location of *Mycoplasma meleagridis* organisms along air-sac epithelium (A) adjacent to liver (B). ×100.

Fig. 4. Section of lung from naturally infected embryo incubated for 21 days. Fluorescence indicates the location of *Mycoplasma meleagridis* along the epithelium of a secondary bronchus. ×100.

Fig. 5. Tissue section from a naturally infected 14-day-old embryo. Fluorescence indicates the presence of *Mycoplasma meleagridis* along the surface of a developing feather (A). ×100.

Fig. 6. Section of skin from a naturally infected 21-day-old embryo. Fluorescent areas indicate the presence of *Mycoplasma meleagridis* along the surface. ×200.
Fig. 7-12. Sections of air-sac tissue from *Mycoplasma-meleagrisidis*-infected (7, 8, 11, 12) and uninfected (9, 10) 28-day-old turkey embryos. Figs. 8, 10, and 12 are higher magnifications of portions of 7, 9, and 11. Exudate (arrows) is evident in air-sac lumina (A) of only infected embryos. ×40 and ×450.
Lesions from Mycoplasma meleagridis in turkey embryos

Table 1. Schedule of inoculation and examination of turkey embryos.

<table>
<thead>
<tr>
<th>No. of embryos</th>
<th>Inoculum</th>
<th>Age (wk) when inoculated</th>
<th>Age (wk) when examined</th>
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<tbody>
<tr>
<td>5</td>
<td>M. meleagridis</td>
<td>1</td>
<td>3</td>
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<tr>
<td>5</td>
<td>M. meleagridis</td>
<td>1</td>
<td>4</td>
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<td>5</td>
<td>M. meleagridis</td>
<td>2</td>
<td>4</td>
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<td>5</td>
<td>Sterile broth</td>
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<td>5</td>
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Lesions were observed in epithelial cells. In all 3, the involved segment was adjacent to pancreatic tissue, suggesting that it was duodenum, and consequently the anterior part of the intestinal tract.

The control measures used indicated that the fluorescence described above was specific. Examination of an equal number of uninfected embryos at the same stage of development revealed no fluorescence, and the use of conjugated normal turkey gamma-globulin did not result in fluorescence in infected embryo tissues. The one-step blocking technique also indicated specific fluorescence. Unlabeled turkey antiserum against M. meleagridis blocked fluorescence, while unlabeled normal turkey serum did not affect fluorescence.

**Microscopic lesions.** Examination of 7 naturally infected and 5 experimentally infected 21-day-old embryos revealed no lesions. Only 1 naturally infected embryo at the 28-day stage of development was available for examination. Lesions were observed in air sacs of this embryo, but other tissues were normal.

Examination of 10 experimentally infected 28-day-old embryos, 5 exposed after 7 days of incubation and 5 exposed after 14 days of incubation, revealed that all had air-sac lesions similar to

Table 2. Distribution of lesions in 28-day-old embryos experimentally infected with Mycoplasma meleagridis.

<table>
<thead>
<tr>
<th>Embryos</th>
<th>Air sacs</th>
<th>Lung</th>
<th>Liver</th>
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<tr>
<td>Exposed at 7 days</td>
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<td>Exposed at 14 days</td>
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*a*Lesions present.  
*b*Not examined.  
*c*No lesions observed.
those observed in the naturally infected 28-day-old embryo. Observed in many in addition to air-sac lesions were lung and liver lesions. Table 2 indicates the distribution of lesions among these embryos.

Air-sac lesions included both exudative and infiltrative inflammatory reactions. The predominant lesion was highly cellular
Lesions from Mycoplasma meleagridis in turkey embryos

exudate in air-sac lumina (Figs. 7, 8, 9, 10, 11, 12). Most of the cells were heterophils, although mononuclear cells were also present. Other constituents of the exudate were varying amounts of fibrin and cell debris. The most severely affected air sacs had large areas of epithelial necrosis.

Subepithelial heterophilic and mononuclear cell infiltration occurred often. Air-sac lesions usually affected all air sacs, but were most extensive in the aggregate air sacs. Sometimes the entire air sac was involved, but in most cases only small areas were affected.

The predominant lung lesion in the embryos was exudative pneumonia, involving air capillaries, atria, and bronchi (Figs. 13, 14). When the extent of the pneumonia was slight, the air tubules and atria were more often affected than bronchi, and lung tissue adjacent to air sacs was more severely affected than other areas. The exudate was similar to that in air sacs but contained more mononuclear cells and less fibrin. The mononuclear cells appeared to be either macrophages or epithelial cells. In addition to the exudative response, there were increased numbers of mononuclear cells in interstitial tissue, especially around the air tubules.

Liver lesions observed in the embryos consisted of perivascular aggregations of predominantly immature heterophils (Figs. 15, 16). A few cells of the same type were found perivascularly in liver sections of uninfected embryos (Figs. 17, 18), but livers of infected embryos were involved much more extensively. These cellular aggregates were considered to be sites of granulocytopoiesis, not an infiltrative inflammatory reaction.

Fig. 13. Exudative pneumonia in a 28-day-old turkey embryo experimentally infected with Mycoplasma meleagridis. An arrow indicates the location of an exudate-filled tertiary bronchus. ×40.

Fig. 14. Higher magnification of a portion of the tertiary bronchus indicated in Fig. 13. Cells in the exudate (A) are primarily heterophils. ×450.

Fig. 15. Section of liver tissue from a 28-day-old turkey embryo experimentally infected with Mycoplasma meleagridis. Arrows indicate the perivascular location of heterophils. ×125.

Fig. 16. Higher magnification of Fig. 15, showing immature heterophils (A) around blood vessel (B). ×450.

Fig. 17. Section of liver tissue from an uninfected 28-day-old turkey embryo. ×125.

Fig. 18. Higher magnification of portion of Fig. 17 which includes a blood vessel. ×450.
DISCUSSION

When 7-day-old embryos from naturally infected eggs were examined using immunofluorescence microscopy, *M. meleagris* could not be demonstrated. The organism could be demonstrated, however, on the skin and feathers of 14-day-old embryos. These findings suggest that between 7 and 14 days of incubation, organisms occurred in amniotic fluid surrounding the embryos. Organisms in amniotic fluid at this stage of incubation could result from contact with albumen containing *M. meleagris*. In chicken embryos after incubation for approximately 10 days, and presumably at about this time in turkey embryos, the seroamniotic connection which separates amniotic fluid and albumen ruptures and allows albumen to enter the amnion (16).

The presence of *M. meleagris* in respiratory and upper digestive tracts following the appearance of these organisms in amniotic fluid suggests ingestion or inhalation as a route of infection. At a stage of incubation following the addition of albumen to amniotic fluid there is a decrease in the volume of this fluid which is believed to be almost entirely due to ingestion by the embryo (16). In the chicken embryo, this begins at approximately 14 days (16). Material injected into amniotic fluid has been found later in the stomach, intestines, trachea, and primary bronchi (16). This distribution is similar to the distribution of *M. meleagris* in 21-day-old turkey embryos, and is additional evidence that infected amniotic fluid was responsible for infecting the embryos. A previously described study also indicated that ingestion and inhalation of infected amniotic fluid were involved in infecting embryo respiratory and digestive tracts (13).

Lesions were found only in 28-day-old embryos even though infection was widely distributed after 21 days of incubation.

The similarity of lesions in embryos exposed after either 7 or 14 days of incubation suggests that lesion formation was related to the stage of development of the embryo rather than to the duration of infection. Inflammatory cells in the lesions were primarily heterophils. There are few circulating leukocytes in avian embryos until near the time of hatching, and at that time most are heterophils (16). The development of lesions late in the incubation period may be related to the occurrence of inflammatory cells in the circulation.

Studies of respiratory-tract lesions in *M.-gallisepticum*-infected avian embryos indicated that they also developed late in
the incubation period (1,4,17). Lesions described in these reports and in an additional report (2) vary considerably, and consequently it is difficult to compare lesions resulting from infection with this organism with those of *M. meleagris* infection. In general, the lung and air-sac lesions described in 2 of these reports (4,17) are similar to those found in this study.

A previous study of *M.-gallisepticum*-infected chicken embryos found a widely distributed heterophilic perivasculitis (1). This perivasculitis was most evident in hepatic tissue. The heterophils involved were immature when first observed in 13-day-old embryos, and increased in number and maturity with continued incubation. In the present study, immature heterophils were also found surrounding hepatic blood vessels. They were much more numerous in infected than in uninfected 28-day-old embryos. Because of their immaturity and adventitial location, however, their occurrence was considered to indicate granulocytopenesis rather than perivasculitis. Granulocytopenesis in hepatic perivascular connective tissue of chicken embryos reaches a maximum after approximately 14 days of incubation and then gradually declines (16). The marked response in these turkey embryos near the time of hatching was probably the result of an increased demand for heterophils brought about by inflammatory responses to infection.

REFERENCES


ACKNOWLEDGMENTS

The technical assistance of Mr. E. L. Hall, Mr. J. E. Gallagher, and Dr. R. D. Cooper is greatly appreciated, as are the contributions made by Drs. M. S. Hofstad, M. L. Frey, F. K. Ramsey, and A. C. Pier, and Mr. K. L. Heddleston.