Pathogenicity of *Mycoplasma fermentans* and *Mycoplasma penetrans* in Experimentally Infected Chicken Embryos

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Cumulative mortality for chicken embryos infected via the yolk sac was 10 to 30% for *Mycoplasma fermentans* and 70 to 100% for *M. penetrans*. Morbidity included dwarfing, deformed limbs, and petechiae. Both mycoplasmas were reisolated from the allantoic fluid, yolk sac, liver, and brain. Histopathological changes in livers included a diffuse infiltration of lymphocytes after *M. fermentans* infection and an acute inflammatory response with periportal infiltration of granulocytes after *M. penetrans* infection. This model system may provide a means to study mycoplasma disease processes in vivo.

Mycoplasmas are bacteria which lack a cell wall. They cause a variety of acute and chronic debilitating diseases in animals (26). Fifteen species are currently known to colonize or infect humans, producing diseases such as pneumonia and urogenital tract infections (17). *Mycoplasma fermentans* and *Mycoplasma penetrans* infect patients with AIDS more frequently than they do human immunodeficiency virus (HIV)-negative low-risk individuals (18). These AIDS-associated mycoplasmas may be cofactors in the progression of AIDS (12, 18).

Infection by *M. fermentans* can be detected in the blood, oral, and urine samples of patients with AIDS by serology, PCR, and isolation (7, 8, 13, 15). *M. fermentans* has been identified in autopsy tissues from patients with AIDS and in placentas delivered by patients with AIDS (3, 18, 20). Infection by *M. fermentans* has also been associated with adult respiratory distress syndrome with or without systemic illness in HIV-negative patients (19, 23).

Mycoplasma penetrans is a new species of human mycoplasma recently isolated from the urogenital tracts of male homosexual patients with AIDS (22). *M. penetrans* has the unusual capability of penetrating into the cytoplasm of mammalian cells (2, 11, 21). In the United States, *M. penetrans* infects 40% of HIV-positive homosexual men and 10 to 20% of HIV-negative homosexual men but less than 0.5% of the general population and less than 1% of HIV-positive intravenous drug users, HIV-positive hemophiliac patients, and HIV-negative patients with immune dysfunctions and/or low leukocyte counts (32, 33). Seroepidemiological studies have revealed that infection by *M. penetrans* is associated with the development of Kaposi's sarcoma in HIV-infected homosexual men (33). However, the actual role, if any, of *M. penetrans* in the development of Kaposi's sarcoma is not yet understood.

Laboratory model systems are an important tool in infectious-disease research. There are very few animal models available for studying human mycoplasmas. Embryonated chicken eggs have been employed as a model system for demonstrating viral pathology (4, 31). Studies of mycoplasmas which affect poultry, for example, *Mycoplasma iowae*, *Mycoplasma gallisepticum*, and *Mycoplasma meleagridis*, have also utilized embryonated eggs as a model system (5, 25).

In the present study, we experimentally infected chicken

embryos with *M. fermentans* and *M. penetrans*, monitored mortality and morbidity, reisolated mycoplasmas from internal organs, and examined tissues for histopathological changes which occurred in infected embryos. This model system appears to be useful in assessing and comparing the pathogenicities of *M. fermentans* and *M. penetrans*, examining their abilities to invade different organ systems, and studying the tissue pathologies associated with infections by these mycoplasmas.

Embryonated chicken eggs. Specific pathogen-free embryonated chicken eggs (research quality) were purchased from SPAFAS Inc. (Rheinholt, Pa.).

Mycoplasma. *M. fermentans* (incognitus strain) (18, 20) and *M. penetrans* (GTU54-6A1 strain) (18, 22) were cultured by two methods. Mycoplasmas were grown in modified SP4 broth medium (0.1% glucose, 0.01% arginine, without antibiotics, thallium acetate, or amphotericin B) (22) aerobically at 37°C or in NIH 3T3 cells (cultured in RPMI 1640–10% fetal bovine serum without antibiotics or amphotericin B in a 5% CO₂ atmosphere). NIH 3T3 cells cultures were infected for at least 4 weeks (1 week per passage) before use as egg inocula. NIH 3T3 supernatants were centrifuged at 1,000 × g for 15 min and filtered (0.45-μm pore size) prior to egg inoculation. The mycoplasma titers in inocula were determined as previously described (14). Mycoplasma titers were expressed in color change units (CCU) per ml.

Experimental infections. Embryonated chicken eggs were inoculated via the yolk sac at 7 days of age (4). Eggs were divided into the following six groups: (i) *M. fermentans* from SP4 broth culture, (ii) *M. fermentans* from NIH 3T3 cell culture, (iii) *M. penetrans* from SP4 broth culture, (iv) *M. penetrans* from NIH 3T3 culture, (v) uninoculated SP4 broth, and (vi) supernatant from uninfected NIH 3T3 cells. Eggs were incubated at 37°C in a humidified incubator on a rocker platform set at slow speed. Eggs were candled daily. For subsequent passages, yolk sac fluid was collected aseptically and diluted with SP4 broth for use as inoculum.

Necropsy. Embryos which died within 48 h of inoculation were discarded. Embryos which died after 48 h were necropsied. The shell of the egg was disinfected and then opened aseptically. Allantoic fluid was collected with a sterile pipette, and yolk sac fluid was collected with a sterile syringe and needle. Then the yolk sac was removed, and the embryo was immersed in disinfectant solution and rinsed with sterile phosphate-buffered saline. The abdomen was opened by using sterile instruments. A second set of sterile instruments was used to

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FIG. 1. Mortality of chicken embryos infected by *M. fermentans* (A) and *M. penetrans* (B). (A) □, 5.0×10^7 CCU per egg from SP4 broth, first passage into embryos ($n = 5$); ■, 1.4×10^7 CCU per egg from NIH 3T3 cell culture, first passage into embryos ($n = 6$); ○, 1.4×10^6 CCU per egg from NIH 3T3 cell culture, second passage into embryos ($n = 10$); ●, 8.1×10^5 CCU per egg from NIH 3T3 cell culture, fifth passage into embryos ($n = 9$). (B) □, 1.5×10^8 CCU per egg from SP4 broth, first passage into embryos ($n = 6$); ■, 8.0×10^5 CCU per egg from NIH 3T3 cell culture, first passage into embryos ($n = 6$); ○, 8.0×10^5 CCU per egg from NIH 3T3 cell culture, second passage into embryos ($n = 10$); ●, 2.0×10^6 CCU per egg from NIH 3T3 cell culture, fifth passage into embryos ($n = 9$). No mortality was seen in the control embryos inoculated with either SP4 broth ($n = 8$) or supernatant of infected NIH 3T3 ($n = 8$) at 13 days postinoculation.

remove the liver. The skin covering the skull was removed by using a third set of sterile instruments. A fourth set of sterile instruments was used to open the skull and remove the brain.

Mycoplasma culture. Mycoplasmas were cultured in SP4

broth medium containing 100 U of penicillin per ml, 500 U of polymyxin B per ml, 0.25 μ g of amphotericin B per ml, and 100 μ g of thallium acetate per ml (22). Allantoic and yolk sac fluids were inoculated directly into SP4 broth medium. Liver and

FIG. 2. Morbidity of mycoplasma-infected chicken embryos. (A) Uninfected control embryo (SP4 medium inoculation) at 13 days postinoculation. (B) *M. fermentans*-infected (3.8×10^5 CCU per egg from SP4 broth, first passage in embryos) embryo at 13 days postinoculation. Note the deformities of the legs. (C) Uninfected control embryo (SP4 medium inoculation) at 10 days postinoculation. (D) *M. penetrans*-infected (2.7×10^3 CCU per egg from SP4 broth, second passage in embryos) embryo at 10 days postinoculation. Note dwarfing and the deformity of the right leg. Bar = 1 cm.

brain tissues were finely minced and inoculated into SP4 broth medium. Samples were diluted 10-fold (10^{-1} to 10^{-8}) and incubated aerobically at 37°C for 3 weeks before being discarded. Cultures which exhibited an acidic color change were subcultured to SP4 solid medium (1% agarose) in order to confirm mycoplasma growth by observation of mycoplasma colonies. Subcultures were incubated for 1 week. The identities of the mycoplasmas isolated from embryos were confirmed by immunoblot testing of SP4 broth supernatants for *M. fermentans* and *M. penetrans* antigens (16).

Histological procedures. Tissue samples from the brain, heart, kidney, liver, lung, spleen, and yolk sac were preserved in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, Brown and Brenn, Brown and Hopps, and Grocott's methanamine silver stains.

Mortality of embryos. The mortality of embryos infected by *M. fermentans* grown in either SP4 broth or NIH 3T3 cells was 20 to 30%. No increase in mortality was observed upon serial passage of *M. fermentans* from yolk sac fluid to new embryos. The mortality of embryos infected by *M. penetrans* grown in SP4 broth or NIH 3T3 cells was 70 to 80%. *M. penetrans* grown in NIH 3T3 cells appeared to cause more rapid mortality than did *M. penetrans* grown in SP4 broth. Mortality occurred more rapidly and increased to 100% upon further passage of *M. penetrans* (initially grown in NIH 3T3 cells) (Fig. 1). No mortality was observed in the control groups inoculated with SP4 medium or supernatant from NIH 3T3 cell culture.

Morbidity of embryos. The signs of morbidity included dwarfing, deformed limbs, petechiae, and a mottled liver. Dwarfing of embryos occurred in 80% of both *M. fermentans*-

and *M. penetrans*-infected embryos. Deformed limbs were evident in 20% of *M. fermentans*- and *M. penetrans*-infected embryos (Fig. 2). Petechiae were observed in 10% of embryos with both mycoplasmas. Livers from infected embryos were often grossly abnormal, with a mottled yellow appearance. There was no evidence of morbidity in the control groups inoculated with SP4 medium or supernatant from NIH 3T3 cell culture.

Reisolation of mycoplasma. Both *M. fermentans* and *M. penetrans* were reisolated from the yolk sac fluids of infected embryos (approximately 80%). The titers were usually very high for both mycoplasmas (average for *M. fermentans*, 4.6×10^7 CCU/ml; average for *M. penetrans*, 8.5×10^7 CCU/ml). The recovery of both mycoplasmas from the allantoic fluids of infected embryos was 60 to 70%. The average titer from allantoic fluid was 6.2×10^7 CCU/ml for *M. fermentans* and 6.1×10^5 CCU/ml for *M. penetrans*. The reisolation of mycoplasmas from the liver was more frequent in embryos infected by *M. penetrans* than it was in embryos infected by *M. fermentans* (55 and 20%, respectively). The mycoplasma titers for *M. penetrans* (average, 8.3×10^5 CCU/g of tissue) and *M. fermentans* (average, 3.5×10^6 CCU/g of tissue) were similar. *M. penetrans* was reisolated from the brain in approximately 25% of all passages. The reisolation of *M. penetrans* from the brain averaged approximately 1.5×10^3 CCU/g of tissue. The reisolation of *M. fermentans* from the brain was not successful until after several passages in embryos and ranged from 15 to 20%. The reisolation of *M. fermentans* was less than 100 CCU/g of tissue. Mycoplasmas were not isolated from the control groups inoc-

FIG. 3. Histopathology of livers from mycoplasma-infected embryos. (A) Liver from a control embryo (SP4 medium inoculation, first passage) at 9 days postinoculation. Magnification, $\times 49$. (B) Same as for panel A. Magnification, $\times 196$. (C) Liver from an *M. fermentans*-infected (1.6×10^6 CCU per egg, originating from SP4 broth, third passage) embryo at 9 days postinoculation. The *M. fermentans* reisolation titer was 1.4×10^6 CCU/g of tissue. Magnification, $\times 49$. (D) Same as for panel C. Magnification, $\times 196$. (E) Liver from an *M. penetrans*-infected (2.6×10^5 CCU per egg from SP4 broth, first passage) embryo at 13 days postinoculation. The *M. penetrans* reisolation titer was 7.8×10^2 CCU/g of tissue. Magnification, $\times 49$. (F) Same as for panel E. Magnification, $\times 196$.

ulated with SP4 medium or supernatant from NIH 3T3 cell culture.

Histopathology. Histopathological changes were found only in the liver tissues of infected embryos from which *M. fermentans* or *M. penetrans* was reisolated. Liver tissues from *M. fermentans*-infected embryos showed extensive mononuclear lymphoid cell infiltration in the periportal areas and in the distended vessels (Fig. 3C and D). In comparison, *M. penetrans*-infected livers had prominent infiltration of granulocytes in the periportal areas (Fig. 3E and F). Brain tissues from which mycoplasmas were reisolated did not show significant histological changes. No histopathological changes were seen in tissues from the control groups inoculated with SP4 medium or supernatant from NIH 3T3 cell culture (Fig. 3A and B). Brown and Brenn, Brown and Hopps, and Grocott's methanamine silver stains did not reveal evidence of bacterial, fungal, or viral infection in any experimental group.

There are advantages in using embryonated eggs as a model system for studying the pathogenesis of infectious agents. Because the cells of an embryo rapidly divide and undergo differentiation, the developing embryo is more sensitive to many infectious processes. Pathogenesis manifested as mortality and different forms of morbidity due to infection can be assessed in a short time.

Both *M. fermentans* and *M. penetrans* infections resulted in significant mortality in embryonated chicken eggs. The difference in embryo mortality (10 to 30% for *M. fermentans* and 70 to 100% for *M. penetrans*) indicates that *M. penetrans* is more pathogenic than *M. fermentans* in experimental infections of chicken embryos. The mortality produced by *M. penetrans* infection was comparable to those reported for experimental infections of embryonated eggs with pathogenic poultry mycoplasmas *M. iowae*, *M. gallisepticum*, and *M. meleagridis* (5, 25). In comparison, mortality has been reported to occur only occasionally in embryonated eggs infected by *Mycoplasma pneumoniae* (9).

From comparisons of the pathogenicities of mycoplasmas grown in SP4 broth and those grown in NIH 3T3 cell culture, two different observations can be made. First, there is no significant difference in pathogenicity between *M. fermentans* grown in SP4 broth and *M. fermentans* grown in NIH 3T3 cell culture. Second, *M. penetrans* grown in NIH 3T3 cell culture kills embryos earlier than does *M. penetrans* grown in SP4 broth. Mycoplasmas grown outside the natural host in artificial media are known to lose virulence after extended passage (29, 30). Both mycoplasmas have been passed a large number of times in artificial media (approximately 60 passages for *M. fermentans* and 20 for *M. penetrans*). Factors associated with virulence, such as capsular material, can be regained when mycoplasmas are passed in tissue culture (1, 28). This could account for the apparently increased virulence of *M. penetrans* after passage in NIH 3T3 cells.

The signs of morbidity (i.e., dwarfing, deformed limbs, and petechiae) in embryos experimentally infected by *M. penetrans* and *M. fermentans* were similar to those reported for *M. iowae* (5). In this context, the only reported indication of morbidity associated with *M. pneumoniae* infection was the occasional dwarfing of embryos (9).

The reisolation of *M. fermentans* and *M. penetrans* from the livers and brains of infected embryos indicates that these mycoplasmas caused systemic infections in embryos. In the case of *M. fermentans*, it has been well established that systemic infection occurs in humans (3, 13, 18–20, 23). In this respect, the embryo model is consistent with human infections. The recovery of *M. iowae*, *M. gallisepticum*, and *M. meleagridis* from some internal organs of experimentally infected embryos was reported, although the livers and brains of embryos were not examined (5, 25). For embryonated eggs infected by *M. pneumoniae*, the recovery of infectious organisms from livers but not from brains was reported (10).

The histopathological changes in the livers of embryos infected by *M. fermentans* and *M. penetrans* differed. A diffuse infiltration of lymphocytes was often noted in the liver, spleen, or lung of an embryo that died of *M. fermentans* infection. It was difficult to differentiate lymphoid cells from degenerated nucleated erythrocytes in the early-age embryos. An acute inflammatory response was identified, with many granulocytes infiltrating the portal areas of livers from *M. penetrans*-infected embryos. *M. penetrans* could often be reisolated from these livers. These findings suggest that there are different mechanisms which underlie the disease processes responsible for the acute illness and fatality produced by *M. fermentans* and *M. penetrans* infection in chicken embryos. Mycoplasmas are known to stimulate the production of immunoregulatory mediators, i.e., cytokines and prostaglandins (6, 24, 27). Mycoplasmas in the livers of infected embryos may have stimulated the production of mediators, resulting in the infiltration and/or proliferation of lymphocytic cells in the case of *M. fermentans* and of granulocytes in the case of *M. penetrans*. The differences in cell populations observed in embryos infected by *M. fermentans*

and *M. penetrans* may be due to different factors being produced in response to these different mycoplasma species.

Since presently there is no rapid and inexpensive system for studying the pathogenesis of human mycoplasmas, the chicken embryo model provides a valuable system for studying the in vivo disease processes of *M. fermentans* and *M. penetrans*. Studies of the interactions between mycoplasmas and various host target cells and of the host responses in tissues infected by each mycoplasma will shed light on the complex pathogenesis and histopathology associated with these mycoplasmal infections in humans.

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