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Ciliostasis and Loss of Cilia Induced by *Mycoplasma hyopneumoniae* in Porcine Tracheal Organ Cultures

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In vivo- and in vitro-grown *Mycoplasma hyopneumoniae* organisms were inoculated onto newborn piglet tracheal organ cultures to provide a model for interaction of this organism with ciliated respiratory epithelium. Ciliostasis and loss of cilia in tracheal rings were induced by *M. hyopneumoniae* grown in vivo and with low-passage cultures when grown in vitro. Levels of calmodulin or dehydrogenase enzymes in tracheal ring epithelium were not altered even though ciliostasis and loss of cilia induced by *M. hyopneumoniae* were extensive. The capacity for inducing epithelial damage diminished with in vitro passage of the organism. Attempts to induce higher-passage cultures to attach to cilia, cause ciliostasis, or cause ciliary damage by supplementation of mycoplasmal medium with porcine lung extract failed. Epithelial damage induced by *M. hyopneumoniae* in tracheal rings was averted by using porcine immune serum or by separating the organisms from ciliated epithelium with a 0.1-μm-pore-size membrane. Attachment, or at least close association, of *M. hyopneumoniae* to ciliated epithelium appeared to be necessary to induce ciliostasis and loss of cilia in this model.

Mycoplasma hyopneumoniae is a fastidious bacterium that causes enzootic pneumonia in swine. The organism apparently induces pneumonia by a variety of mechanisms, including damage to porcine ciliated epithelium. Microscopic examination of histologic sections of infected swine lungs revealed that *M. hyopneumoniae* induced clumping or loss of cilia in airways of swine with mycoplasmal pneumonia (11, 21, 31). Mycoplasmas were closely associated with ciliated epithelial cells of the trachea and bronchi in infected pigs (23). The precise mechanisms by which *M. hyopneumoniae* causes ciliary damage, and presumably ciliostasis, are not well understood.

Previous workers have investigated the interaction of *M. hyopneumoniae* and other mycoplasmas with mammalian cells or tissues in vitro in an effort to understand virulence mechanisms of these organisms. Changes in enzymatic activity of host cell proteins were used to quantitate epithelial damage induced by virulent mycoplasmas in tracheal organ cultures. *Mycoplasma pneumoniae*, the cause of atypical pneumonia in humans, was shown to reduce dehydrogenase enzyme levels in hamster tracheal rings (15). In other work, it was shown that *Mycoplasma equigenitalium*, a mycoplasma isolated from equine genital tracts, reduced calmodulin content in infected equine oviductal ring cultures (6). Tracheal ring explants were used to assess induction of ciliostasis and loss of ciliated epithelium after inoculation with *M. hyopneumoniae* (7, 10, 25, 26, 32). Progressive ciliostasis and epithelial exfoliation in porcine tracheal rings cocultivated with fetal porcine lung fibroblasts and *M. hyopneumoniae* were reported by Williams and Gallagher (32). However, most workers observed that *M. hyopneumoniae* caused no ciliostasis or loss of cilia in vitro (7, 10, 25, 26).

The reasons for conflicting results after inoculation of porcine tracheal rings with *M. hyopneumoniae* were not clear. However, information about virulence for pigs and number of passages in vitro was not always available for the *M. hyopneumoniae* strains used in tracheal ring studies. Inoculation of pigs indicated that virulence of *M. hyopneumoniae* was decreased by cultivation in artificial medium. In vitro growth for as few as six passages led to loss of pathogenicity of *M. hyopneumoniae* for pigs (29). Zielinski and Ross (33) reported that *M. hyopneumoniae* J (ATCC 25934) in the 60th passage failed to induce pneumonia in pigs, while strains 11, 232, and 144L, used as 19th- to 27th-passage cultures, caused lesions. On the other hand, a component of nonviable *M. hyopneumoniae* membranes which induced rounding and detachment of fibroblasts in vitro was not attenuated or lost after numerous broth passages (16, 17). Therefore, the role of this component in virulence is not clear.

In this study, we used porcine tracheal organ cultures to provide an in vitro model for detection of *M. hyopneumoniae*-induced damage to ciliated respiratory epithelium. Damage to tracheal ring epithelium caused by *M. hyopneumoniae* diminished with in vitro passage and was averted by the addition of convalescent-phase swine serum (CSS) containing polyclonal antibody specific for *M. hyopneumoniae*. Close association of *M. hyopneumoniae* with porcine tracheal organ cultures was necessary for induction of damage to ciliated respiratory epithelium.

MATERIALS AND METHODS

Media. Friis mycoplasmal medium was used for propagation of *M. hyopneumoniae* (14). Tracheal organ cultures were maintained in Friis mycoplasmal medium prepared without thallium acetate. Lung homogenates were suspended in Friis mycoplasmal medium prepared without any antibacterial agents.

M. hyopneumoniae. Lung homogenate containing *M. hyopneumoniae* was obtained from a specific-pathogen-free pig infected with *M. hyopneumoniae* 232, which was recovered from a pig inoculated with strain 11 (4, 22). The homogenate contained 10% (wt/vol) lung tissue in Friis mycoplasmal medium without any antibacterial agents and served as a source of in vivo-grown *M. hyopneumoniae* (termed *M. hyopneumoniae* grown in vivo). The *M. hyopneumoniae* grown in vivo was examined for mycoplasmas or other bacteria by culturing in mycoplasmal medium (27) and on blood agar and for viruses

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by transmission electron microscopy (TEM) and immune TEM. *M. hyopneumoniae* was the only organism isolated from *M. hyopneumoniae* grown in vivo. No viral agents were detected by TEM or immune TEM, and mycoplasmas were the only bacteria present. The *M. hyopneumoniae* grown in vivo was diluted 10-fold in Friis mycoplasmal medium, incubated, and passaged sufficiently in Friis mycoplasmal medium to provide 2nd-, 3rd-, 4th-, 5th-, 10th-, and 20th-passage *M. hyopneumoniae*; cultures of each passage were stored at −70°C. Strain 232 was cloned (clone 2A3; designated 232C), grown to passage 40 in Friis mycoplasmal broth, and frozen at −70°C. At passage 36, 232C induced pneumonia in pigs inoculated intratracheally (4). Supernatant of *M. hyopneumoniae* grown in vivo was subjected to 180,000 rads (termed irradiated *M. hyopneumoniae*). Supernatant of the lung homogenate was prepared by heating (100°C, 2 min) and centrifugation (740 × *g*, 15 min). The heated supernatant was passed through a filter retaining molecules with molecular weights greater than approximately 10,000 (Centricon-10 microconcentrator; Amicon, Division of W. R. Grace and Co., Danvers, Mass.) to obtain filtrate and retentate of the heated supernatant. Lung tissue collected from a normal specific-pathogen-free pig, free of infection with *M. hyopneumoniae* and other known swine respiratory pathogens, was prepared as a 10% (wt/vol) suspension in Friis mycoplasmal medium to obtain lung homogenate from a noninfected pig (termed normal lung homogenate). Aliquots of lung preparations were stored at −70°C until used. No mycoplasma or pathogenic bacteria were recovered from irradiated *M. hyopneumoniae* or normal lung homogenate.

For inoculation onto tracheal rings, lung homogenate preparations and frozen broth cultures of *M. hyopneumoniae* were thawed and diluted 1:10 in Friis mycoplasmal medium. The *M. hyopneumoniae* grown in vivo was diluted 1:10, 1:100, 1:1,000, and 1:100,000 in Friis mycoplasmal medium. The numbers of organisms inoculated onto tracheal rings were estimated by the tube dilution method for determination of color-changing units (CCU) per ml for every experiment. One CCU represented the highest dilution of culture changing the color of a tube of Friis mycoplasmal medium from red to yellow. CFU were not determined because *M. hyopneumoniae* produces low numbers of colonies on agar.

Tracheal rings. Crossbred Hampshire and Yorkshire piglets were obtained from sows from commercial swine herds by caesarean section or by manual removal of the piglet from the birth canal during normal parturition. Newborn piglets were placed immediately into a sterile transport container. Within 1 to 5 h of birth, each piglet was deeply anesthetized with approximately 65 mg of sodium pentobarbital given intraperitoneally and the trachea was aseptically removed at the level of the right bronchus as described previously for hamsters (8). The excised trachea was placed into approximately 5.0 ml of Friis mycoplasmal medium and cut into 20 to 25 rings. Appropriate control or mycoplasmal preparation in 0.3 ml of Friis mycoplasmal medium was randomly assigned to each well of a 24-well tissue culture plate (Tissue Culture Cluster[24] 3424; Costar, Cambridge, Mass.). Each ring was randomly assigned to a well. At least 10 tracheal rings were used for each control or mycoplasmal preparation. Rings were incubated with or without *M. hyopneumoniae* in Friis mycoplasmal medium at 35.5°C in 0.5% CO_2. Spent medium was removed daily, and 0.3 ml of fresh Friis mycoplasmal medium was added to each well until the ring was removed or the experiment was terminated. The medium from each well containing a ring was cultured for *M. hyopneumoniae* and other bacteria whenever a ring was removed.

Evaluation of damage to ciliated epithelium. The cytotoxicity of *M. hyopneumoniae* for tracheal ring epithelium, manifested as loss of cilia from epithelial cells and ciliostasis, was evaluated by using an inverted microscope (100× magnification) with slight modification of the methods described by Gabridge and Polisky (15). Loss of cilia was determined after estimating the percentage of epithelium retaining ciliated cells (0 to 100%). Ciliostasis, evaluated by observing the vigor of ciliary beating, was scored on a scale from 0 (no movement) to 3 (vigorous beating). Because loss of cilia and ciliostasis usually occurred simultaneously, ciliostatic data are not usually presented with ciliary loss data. For immunofluorescence, tracheal rings were frozen at −70°C in embedding medium (O.C.T. Compound; Miles Inc., Diagnostics Division, Elkhart, Ind.), sectioned at 5 μm, and stained with fluorescein-labeled porcine-origin antibody to *M. hyopneumoniae*. Sections were examined for the presence of immunofluorescence specific for *M. hyopneumoniae* (2) with an Olympus microscope with a reflected-light fluorescence attachment (model BHS with BH2-RFL; Olympus Corporation, Lake Success, N.Y.). For histopathology, selected tracheal rings were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 5 μm, processed, and stained with hematoxylin and eosin by standard methods. Selected tracheal rings were fixed at 4°C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), postfixed in 1% osmium tetroxide in sodium cacodylate buffer for 2 h at room temperature, washed with distilled water, dehydrated in a graded series of acetone of 50, 75, 95, and 100%, and embedded in Embed 812 resin (Electron Microscopy Sciences, Fort Washington, Pa.). Thin sections were cut at 70 to 90 nm with a diamond knife, stained with 2% uranyl acetate and Reynold's lead citrate, and examined in a Hitachi H500 TEM at 75 kV.

Effect of antibody on cytotoxicity induced by *M. hyopneumoniae*. CSS with antibodies specific for *M. hyopneumoniae* and normal swine serum (i.e., negative swine serum [NSS]) were obtained as described previously (4). On day 0, rings received an initial inoculum of *M. hyopneumoniae* grown in vivo (1:10) with or without NSS or CSS (1:2) in Friis mycoplasmal medium. On subsequent days, the wells were replenished with Friis mycoplasmal medium with the appropriate serum (1:4).

Evaluation of dehydrogenase enzyme levels in porcine tracheal rings. Reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) by dehydrogenase enzymes of metabolically active cells of tracheal rings was measured as described previously (15) with modifications. Rings with and without *M. hyopneumoniae* were evaluated. After incubation with TTC, each ring was placed in a vial containing 1.0 ml of methanol for 30 min to extract formazan dye resulting from reduction of TTC. Triplicate 200-μl aliquots of methanol from each vial were placed in wells in a 96-well plate, and the optical density was determined with an enzyme-linked immunosorbent assay plate reader with a 490-nm filter. Each tracheal ring was dried in a dessicator, the dry weight was determined, and the optical density per milligram of dry weight was calculated.

Evaluation of calmodulin levels in tracheal ring epithelium. Two or 5 days after inoculation, epithelium from each tracheal ring was dissected from the cartilage, weighed, and homogenized. Tissue homogenates were heated (100°C, 2 min) and centrifuged at 740 and 105,000 × *g*, and the supernatants obtained at each speed were assessed for protein content and total (740 × *g*) or unbound (105,000 × *g*) intracellular calmodulin. The volume of spent medium removed daily from each well was recorded and pooled by well for measurement of extracellular calmodulin content. Pooled medium from each well was cultured for *M. hyopneumoniae* and bacteria, heated

(100°C, 2 min), centrifuged (105,000 × *g*), and evaluated for calmodulin and protein content.

Calmodulin levels of triplicate samples were assessed indirectly by measuring phosphate release from AMP in a calmodulin-activated enzyme cascade (28) with modifications (5, 6). Cellular calmodulin content was calculated with reference to protein content (bicinchoninic acid protein assay; Pierce Chemical Co., Rockford, Ill.) and explant tissue weight. Extracellular calmodulin content was calculated with reference to protein content and total volume of medium.

Effect of in vitro passage and cocultivation with lung preparations on cytotoxicity induced by *M. hyopneumoniae*. To evaluate the effect of in vitro passage on cytotoxicity, *M. hyopneumoniae* organisms in the 2nd, 3rd, 4th, 5th, 10th, and 20th passages were assessed for the ability to induce ciliary loss. Lung homogenate preparations with or without *M. hyopneumoniae* were diluted 1:10 in Friis mycoplasmal medium for inoculation onto tracheal rings. To evaluate the effect of cocultivation with lung preparations on cytotoxicity, *M. hyopneumoniae* was propagated in Friis mycoplasmal medium from passage 2 to passage 10 with or without 10% lung homogenate from a noninfected pig or supernatant of heated lung homogenate from a pig infected with *M. hyopneumoniae* (see "*M. hyopneumoniae*" in Materials and Methods).

Assessment for diffusibility of cytotoxic substance(s) produced by *M. hyopneumoniae*. For assessment of the diffusibility of a cytotoxic substance, tracheal rings were incubated with *M. hyopneumoniae* in a dual-chamber Millicell system. Sterile Millicell inserts containing a membrane with a 0.1-μm pore size (Millicell, custom order no. SD2P111E1; Millipore Corp., Bedford, Mass.) and designed for 24-well plates were placed in each well of a plate. Tracheal rings were placed above the membrane in the upper chamber of the inserts. Spent medium under the membrane in each well was removed daily, and fresh Friis mycoplasmal medium was added until the experiment was terminated. Control rings were inoculated with sterile Friis mycoplasmal broth on top of and below the Millicell insert membrane. Positive control rings were inoculated with *M. hyopneumoniae* on top of (direct exposure) and below the Millicell membrane to ensure direct contact of ciliated epithelium with viable mycoplasmal organisms. Test rings were inoculated with Friis mycoplasmal medium on top of the Millicell membrane, and *M. hyopneumoniae* was placed below the membrane (indirect exposure). All tracheal rings receiving *M. hyopneumoniae* either by direct or indirect exposure were inoculated with the same total number of organisms, approximately 10^6 CCU of *M. hyopneumoniae*. The medium from above and below the Millicell membrane in each well containing a ring was cultured for *M. hyopneumoniae* and other bacteria when the experiment was terminated. Colonization of tracheal rings by *M. hyopneumoniae* was evaluated by immunofluorescence.

Statistical analysis. The effects of *M. hyopneumoniae* on tracheal rings were determined by analysis of variance using an F-statistic ($P < 0.05$) and the Student *t* test to locate significant differences. The relationship between presence of *M. hyopneumoniae* and calmodulin level was determined with Pearson correlation coefficients ($r > 0.8$).

RESULTS

***M. hyopneumoniae* with tracheal rings.** In vivo-grown *M. hyopneumoniae* and passage 2 of *M. hyopneumoniae* (*M. hyopneumoniae* passaged twice in vitro) caused significant ciliostasis and loss of cilia ($P < 0.04$) (Fig. 1 and 2) when compared with controls 4 and 5 days after inoculation of tracheal rings.

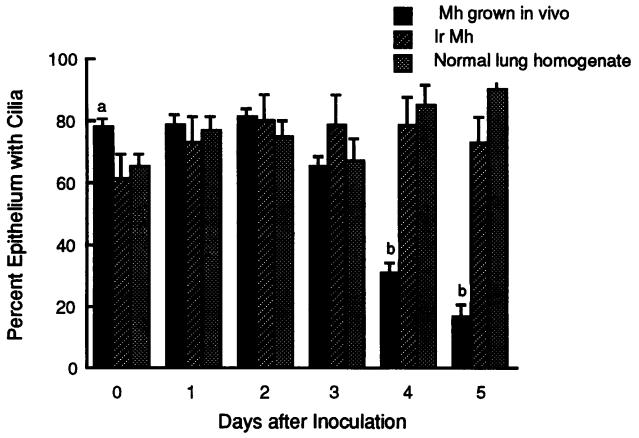

FIG. 1. Loss of cilia from porcine tracheal ring epithelium after inoculation with in vivo-grown *M. hyopneumoniae* (Mh grown in vivo) from the lung of a pig infected with *M. hyopneumoniae*, irradiated lung homogenate from a pig infected with *M. hyopneumoniae* (Ir Mh), or lung homogenate from a noninfected pig (Normal lung homogenate). On the day of inoculation, tracheal rings inoculated with *M. hyopneumoniae* grown in vivo possessed more epithelium with cilia than rings inoculated with irradiated *M. hyopneumoniae* or normal lung homogenate (bar a; $P < 0.04$). There was no difference in the percentages of epithelium with cilia 1, 2, and 3 days after inoculation. Four and 5 days after inoculation, tracheal rings inoculated with *M. hyopneumoniae* grown in vivo exhibited marked loss of cilia when compared with irradiated *M. hyopneumoniae* and normal lung homogenate (bars b; $P < 0.001$). Results are means ± standard errors of the means of three experiments.

Inocula containing *M. hyopneumoniae* grown in vivo (1:10) or *M. hyopneumoniae* broth cultures possessed 10^5 to 10^8 CCU of *M. hyopneumoniae*. Medium bathing rings inoculated with *M. hyopneumoniae* grown in vivo contained 10^6 CCU of *M. hyopneumoniae* 1 day postinoculation (p.i.) and approximately 10^8 CCU thereafter to 7 days p.i. The CCU of *M. hyopneumoniae* in spent medium at the termination of experiments was usually 10^8.

Immunofluorescence specific for *M. hyopneumoniae* was evident in inoculated rings from 1 to 8 days p.i. Specific immunofluorescence was not observed on a few rings exhibiting severe loss of cilia, e.g., retaining less than 20% ciliated epithelium on the luminal surface (data not shown).

Microcolonies of organisms closely associated with ciliated epithelium were detected by using TEM 2 days after inoculation. Extensive colonization of almost all intact ciliated epithelium was evident 5 days after inoculation with 10^5 CCU or more of *M. hyopneumoniae* grown in vivo (Fig. 3) or passage 2 of *M. hyopneumoniae*.

Examination of tracheal ring sections by using light microscopy and TEM 5 days after inoculation with *M. hyopneumoniae* grown in vivo or passaged twice in vitro revealed that ciliated epithelium was replaced by a single layer of low cuboidal to flat epithelial cells where the luminal epithelial surface had been damaged. Cilia from control rings usually appeared straight and separated from one another when viewed by light microscopy. In contrast, cilia of inoculated rings viewed by light microscopy were attached to one another at the tips, forming ciliary clumps up to 30 μm wide. Basophilic, spherical organisms (mycoplasmas) approximately 0.3 μm in diameter and adherent to cilia were visible in areas of ciliary clumping.

Time required for induction of epithelial damage by in vivo-grown *M. hyopneumoniae* was delayed by dilution of the

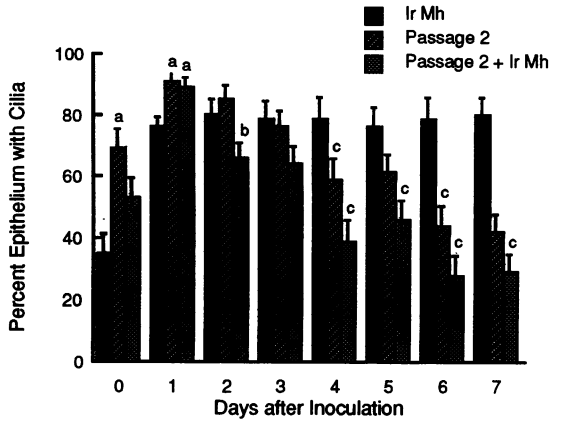

FIG. 2. Loss of cilia from porcine tracheal ring epithelium after inoculation with *M. hyopneumoniae* passaged twice in broth with (Passage 2 + Ir Mh) and without (Passage 2) irradiated lung homogenate from a pig infected with *M. hyopneumoniae*. On the day of and the day after inoculation, tracheal rings inoculated with Passage 2 possessed more epithelium with cilia than rings inoculated with irradiated *M. hyopneumoniae* (Ir Mh) only (bars a; $P < 0.005$). Two days after inoculation, tracheal rings inoculated with Passage 2 plus irradiated *M. hyopneumoniae* exhibited significantly greater loss of cilia than rings inoculated with Passage 2 or irradiated *M. hyopneumoniae* (bar b; $P < 0.05$). Loss of cilia from rings inoculated with Passage 2 or Passage 2 plus irradiated *M. hyopneumoniae* was not significantly different for the remainder of the experiment (bars c; significantly less than irradiated *M. hyopneumoniae*; $P < 0.04$). Results are means ± standard errors of the means of one experiment ($n = 14$ for each datum point). Ciliary loss after inoculation with Passage 2 or Passage 2 plus irradiated *M. hyopneumoniae* and inability of irradiated *M. hyopneumoniae* to induce epithelial damage in tracheal rings were independently confirmed in three or more experiments.

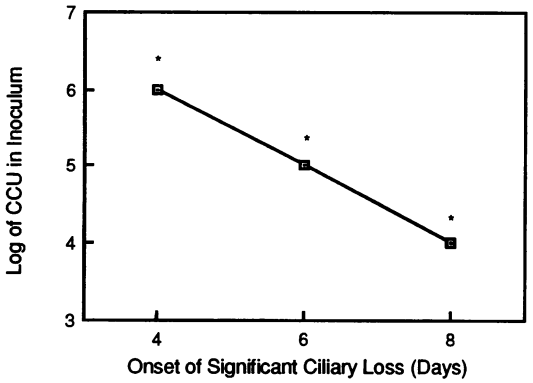

FIG. 4. Tracheal rings were inoculated with 10-fold dilutions of *M. hyopneumoniae*, and the percentage of epithelium retaining cilia was recorded daily. The effect of dose of inoculum on time of onset of significant ciliary loss after inoculation is illustrated. *, $P < 0.05$ when compared with other dilutions. Results are means of three experiments.

mycoplasma inoculum and was dose related (Fig. 4). Colonization of tracheal ring epithelium was evidenced by positive immunofluorescence of almost all rings inoculated with dilutions of *M. hyopneumoniae* grown in vivo upon termination of the experiment.

Effect of antibody on cytotoxicity of *M. hyopneumoniae*. The CSS significantly mitigated *M. hyopneumoniae*-induced damage of ciliated cells at 3 to 5 days p.i. ($P < 0.03$) (Fig. 5). The number of CCU of *M. hyopneumoniae* recovered from medium containing CSS was 1,000 times less than the number of CCU from medium containing NSS. Intense colonization of tracheal epithelium of NSS- and CSS-treated rings was evident 5 days p.i. when examined by immunofluorescence and TEM.

Effect of in vitro passage on cytotoxicity of *M. hyopneumoniae*. Passage-2 *M. hyopneumoniae* consistently induced more ciliary loss in piglet tracheal rings than *M. hyopneumoniae* of higher in vitro passages. Ciliostasis was closely associated with loss of cilia. Data from a single experiment with cultures of *M. hyopneumoniae* 232 at the 2nd to 20th passage are shown in Fig. 6; in this experiment, *M. hyopneumoniae* at passage 20 induced significant ciliary loss and ciliostasis in comparison with uninoculated controls ($P < 0.01$). However, induction of significant epithelial damage by passage 20 of *M. hyopneumoniae* was not consistent in other experiments. *M. hyopneumoniae* 232C at the 40th passage was consistently noncytotoxic (mean percentages of intact ciliated tracheal ring

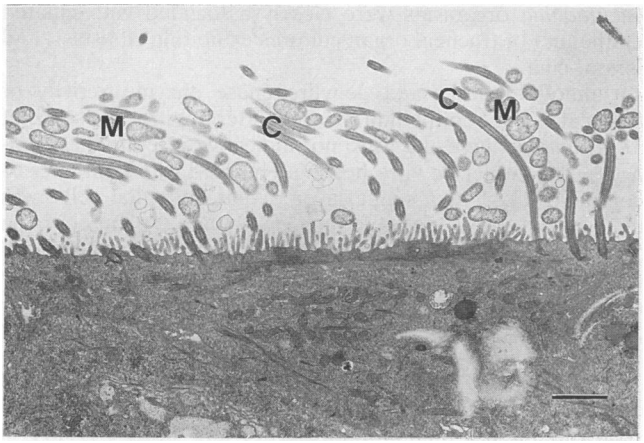

FIG. 3. Colonization of tracheal ring epithelium by *M. hyopneumoniae* (M) 5 days after inoculation. Mycoplasma cells are closely associated with cilia (C). Bar, 0.83 μm.

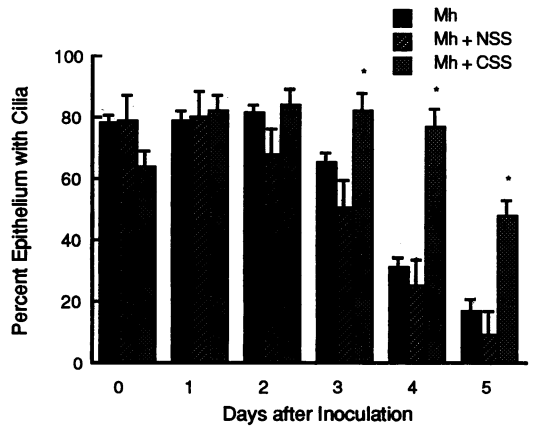

FIG. 5. Loss of cilia from porcine tracheal ring epithelium after inoculation with *M. hyopneumoniae* (Mh) with or without NSS or CSS. Three, 4, and 5 days after inoculation, tracheal rings inoculated with *M. hyopneumoniae* plus CSS containing antibody specific for *M. hyopneumoniae* possessed more epithelium with cilia than rings inoculated with *M. hyopneumoniae* or *M. hyopneumoniae* plus NSS ($P < 0.02$). Results are means ± standard errors of the means for two experiments.

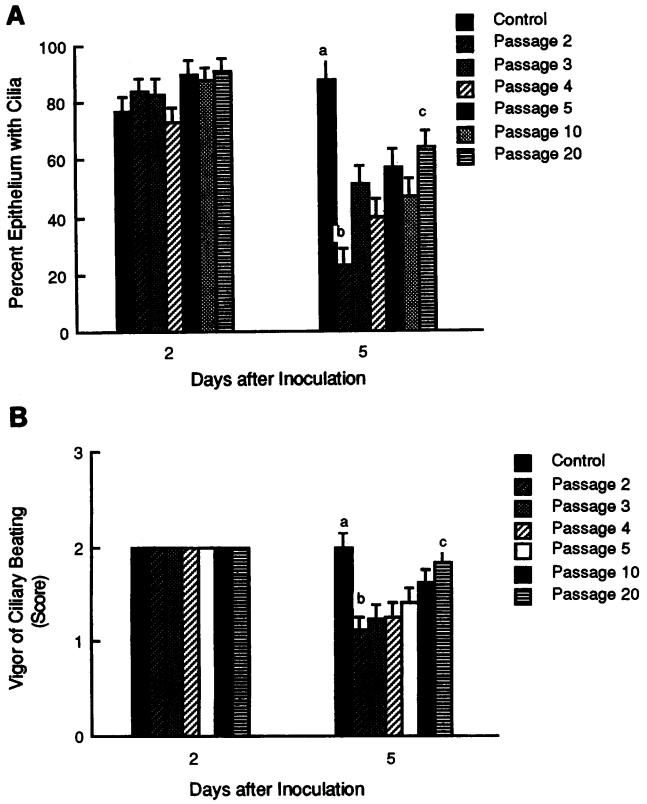

FIG. 6. Loss of cilia from (A) and vigor of ciliary beating on (B) tracheal ring epithelium 2 and 5 days after inoculation with *M. hyopneumoniae* passaged in vitro. There was no difference in values for *M. hyopneumoniae* passages or the control 2 days after inoculation. The ability to induce ciliostasis in ciliated epithelium 5 days after inoculation decreased with in vitro passage. Five days after inoculation, tracheal rings inoculated with sterile mycoplasma medium (Control) retained significantly more ciliated epithelium than all passages (panel A, bar a; $P < 0.009$) and significantly more ciliary activity than passages 2 to 5 (panel B, bar a; $P < 0.007$). Passage 2 induced significantly more ciliary loss and ciliostasis than all other passages except passage 4 (panels A and B, bars b; $P < 0.02$). Passages 2 and 4 were not significantly different. Passage 20 was significantly less cytotoxic than passages 2, 4, and 10 (panel A, bar c; $P < 0.05$) and induced significantly less ciliostasis than all other passages except passage 10 (panel B, bar c; $P < 0.04$). Results are means ± standard errors of the means of one experiment which included all passages of *M. hyopneumoniae* (n = 12 to 16 tracheal rings for each datum point). Loss of cytotoxic and ciliostatic potential with in vitro passage was confirmed by using passages 2, 10, and 20 in another experiment.

epithelium after 5 days, 60 [control] and 72% [strain 232C]). Rings inoculated with strain 232C had only rare focal areas of specific immunofluorescence on the epithelium.

Effect of lung preparations on cytotoxicity of *M. hyopneumoniae*. Clear evidence of a potentiating effect of lung preparations, added either during in vitro passage of *M. hyopneumoniae* or at the time of inoculation to tracheal rings, was not found (data not shown).

Diffusibility of substances inducing ciliary loss. Separation of *M. hyopneumoniae* from ciliated epithelium by a 0.1-μm-pore-size membrane prevented loss of cilia ($P < 0.01$) (Fig. 7). At termination of the experiment 5 days p.i., 10^7 to 10^9 CCU of *M. hyopneumoniae* were recovered from media below the Millicell membrane in wells inoculated with the organism. Mycoplasmas were not usually recovered from above the

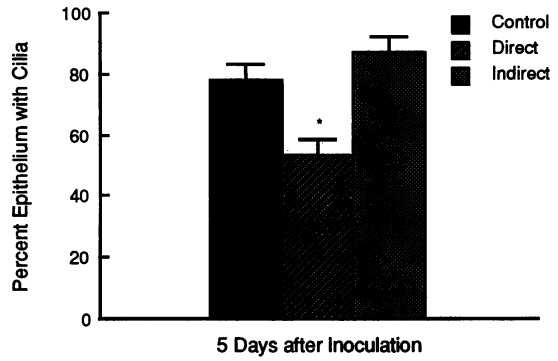

FIG. 7. Loss of cilia after direct inoculation of porcine tracheal rings with *M. hyopneumoniae* (Direct) but not after indirect exposure to mycoplasmas through a membrane with a 0.1-μm pore size (Indirect). Each tracheal ring was placed on top of the membrane of a well insert. *M. hyopneumoniae* was inoculated above and below (Direct) or only below (Indirect) the membrane. The loss of cilia from tracheal rings separated from *M. hyopneumoniae* by the membrane and that from rings inoculated with sterile mycoplasma medium (Control) were not significantly different. Tracheal rings directly inoculated with *M. hyopneumoniae* exhibited significant loss of cilia (*, $P < 0.002$). Results are means ± standard errors of the means for two experiments.

Millicell membrane of rings indirectly exposed to *M. hyopneumoniae*. When recovered, the number of CCU of mycoplasma in medium above the Millicell membrane (10^5) was 1,000 times less than the number of CCU recovered below the membrane (10^8) in the same well. No mycoplasmas were recovered from negative control wells.

Metabolic assays. Intracellular and extracellular calmodulin levels of rings inoculated with *M. hyopneumoniae* were not significantly different from those of controls at any time (data not shown). There was no relationship between calmodulin level and loss of ciliated epithelium or ciliary activity. Mean dehydrogenase enzyme levels were not significantly affected by *M. hyopneumoniae* (data not shown).

DISCUSSION

In vivo-grown or passage-2 *M. hyopneumoniae* consistently induced marked loss of cilia and reduction in the ciliary activity of porcine tracheal rings. Positive immunofluorescence and TEM visualization provided evidence that numerous *M. hyopneumoniae* organisms were closely associated with ciliated epithelium of tracheal organ cultures exhibiting ciliostasis and loss of cilia.

Calmodulin level and dehydrogenase enzyme activity of tracheal explant epithelium were not altered by the presence of *M. hyopneumoniae* and were not indicative of epithelial damage in this model. In vitro healing, observed with light microscopy and TEM as a single layer of flat epithelial cells, was evident in tissue sections of damaged porcine explant epithelia 5 days after inoculation with *M. hyopneumoniae*. Epithelial cell damage caused by *M. hyopneumoniae* may not be reflected by calmodulin or dehydrogenase enzyme levels because the enzyme activity of metabolically active cells participating in the healing response along with that of dying cells is measured.

Although CSS reduced ciliary loss and ciliostasis, tracheal ring cultures treated with CSS and *M. hyopneumoniae* had relatively intense immunofluorescence and TEM evidence of colonization with *M. hyopneumoniae* 5 days after inoculation. The high numbers of organisms inoculated into and proliferating within the organ culture system may have overwhelmed

the antibody capable of blocking attachment to ciliated cells. Another explanation for colonization in the presence of CSS is that antibodies protecting against ciliated cell damage may not block attachment. Evidence for this hypothesis has been indirectly corroborated in experiences with commercial vaccines for *M. hyopneumoniae* currently in use. Lesion severity was reduced in vaccinated pigs, but colonization of airways by *M. hyopneumoniae* was not averted by vaccination (9, 24). Antibodies capable of metabolic inhibition may explain the reduced numbers of *M. hyopneumoniae* in medium containing CSS when compared with those in medium containing NSS.

Close association of *M. hyopneumoniae* with ciliated cells was essential for induction of epithelial damage in this model. Tracheal rings indirectly exposed to *M. hyopneumoniae* through a 0.1-μm-pore-size membrane failed to exhibit loss of cilia when compared with rings directly inoculated with the organism. Further evidence for the necessity of colonization is that noncytotoxic strains of *M. hyopneumoniae* exhibited little or no evidence of colonization by immunofluorescence or TEM, while cytotoxic strains exhibited strong immunofluorescence and clusters of organisms were associated with cilia on TEM. Because similar numbers of organisms were recovered after inoculation with in vitro passages of *M. hyopneumoniae*, it appears that differences in growth rate or cell numbers of the cultures did not account for differences in cytotoxic potential. Perturbation of cell function through the host cell membrane is likely to be an important mechanism in cytotoxicity caused by *M. hyopneumoniae*.

M. hyopneumoniae apparently does not secrete a diffusible substance cytotoxic for ciliated epithelium in vitro. However, a toxin may be produced and induce peribronchiolar lymphocytic infiltration (21, 31), changes in lectin affinity of bronchiolar epithelium (1), or histochemical alterations of mucus-secreting cells (11) in infected swine. The role of the membrane-associated cytopathic factor previously described (16, 17) in *M. hyopneumoniae*-induced cytotoxicity to tracheal ring cultures is unclear.

In vitro passage of *M. hyopneumoniae* significantly decreased the cytotoxic potential of the organism for newborn piglet tracheal organ cultures. Apparently, broth passage of *M. hyopneumoniae* causes reduction of virulence. Loss of cytotoxic potential during in vitro passage may be caused by loss of ability to attach to cilia or selective growth of noncytotoxic population subsets. The environment provided by Friis mycoplasmal medium and standard incubation conditions may cause down-regulation of a gene(s) coding for a protein(s) associated with cytotoxic potential. Growth conditions are known to alter the virulence of *Salmonella* species. *Salmonella* cells in the stationary phase of growth were unable to invade Madin-Darby kidney cells in vitro, and oxygen-limited conditions during growth enhanced adherence and invasiveness of the bacteria (20). Environmental factors including low temperature, $MgSO_4$, and nicotinic acid modulate expression of proteins associated with virulence of *Bordetella pertussis* through signal transduction of gene products of the *bvg* locus (3). Modification of growth medium and/or incubation conditions may be useful for maintenance of virulence of *M. hyopneumoniae* in vitro. Alternatively, spontaneous deletions such as those described for *Escherichia coli* (19) or antigenic variation may occur during growth, leading to loss of virulence. Variation in the V-1 surface antigen of *Mycoplasma pulmonis* occurs in vitro and in vivo (30). The presence of a similar protein in *M. hyopneumoniae* has not been reported.

Production of new proteins by *Salmonella choleraesuis* and *Salmonella typhimurium* was induced by contact of the organisms with host cell components (12, 13). Lung homogenate was evaluated as a source of components which might induce signal transduction, leading to enhanced cytotoxicity of *M. hyopneumoniae*. In early studies, it was shown that the use of a medium containing extract of boiled pig lung monolayer cultures facilitated growth of *M. hyopneumoniae* (18). Additionally, lung homogenate prepared from swine infected with *M. hyopneumoniae* has proven to be reliable for induction of experimental pneumonia (2). However, cytotoxicity of *M. hyopneumoniae* was not enhanced by the presence of lung preparations during growth or incubation with tracheal rings. The concentration of lung preparations or a factor in the lung preparations may have been too low to induce signal transduction. Another possibility is that the factors involved in in vivo enhancement of virulence may be short-lived and unable to withstand conditions used for heating of mycoplasmas or collection and preparation of the lung homogenates.

Several workers have reported that *M. hyopneumoniae* did not induce ciliostasis or loss of cilia in tracheal ring epithelium (7, 25, 26). *M. hyopneumoniae* strains used in previous studies were highly passaged in vitro, or the passage history and viability of the inoculum were not provided. For this report, we determined that 10^5 CCU of viable *M. hyopneumoniae* organisms were necessary to induce significant cytotoxicity in tracheal organ cultures by 6 days p.i.; furthermore, the inoculum consisted of nonpassaged infected lung homogenate or second-passage broth cultures. Ciliary loss was delayed when insufficient numbers of viable mycoplasmas were present in the inoculum. Additionally, irradiated *M. hyopneumoniae* was unable to induce damage to ciliated epithelium. The discrepancy in reported results of *M. hyopneumoniae* cytotoxicity studies among laboratories may be related to strain or passage history of *M. hyopneumoniae*.

Interaction of *M. hyopneumoniae* with porcine tracheal rings may imitate the relationship of pathogen to host cell in the infected pig. Our findings demonstrate that attachment of *M. hyopneumoniae* to ciliated epithelium is very likely necessary to induce ciliostasis and loss of cilia in this model. Evidence that attachment is necessary for induction of ciliary damage occurring during *M. hyopneumoniae* infection in vitro further demonstrates the importance of identifying the immunogens associated with attachment of *M. hyopneumoniae* in vivo for production of vaccines that prevent infection, as well as pneumonic lesions, in the pig.

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