

# Growth and Cytopathology of *Mycoplasma synoviae* in Chicken Embryo Cell Cultures

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*Mycoplasma synoviae* was tested for its ability to grow and induce cytopathogenic changes in chicken embryo cell cultures. *M. synoviae* grew to high titers by day 5 in the presence of chick cells, but showed no growth in the tissue culture medium alone even though it was enriched with nicotinamide adenine dinucleotide and swine serum. Infected chick cell cultures showed a progressive cytoplasmic degeneration on successive days of examination. Early changes involved cytoplasmic granularity and mild vacuolation. On the last day of examination the cytoplasm of most cells was completely degenerated and some showed nuclear degeneration. *M. synoviae* was shown to be cytophilic for the chick cell membranes where the mycoplasmas reproduced and formed microcolonies which, on successive days, increased in size. The attachment site on the chick cell membrane was shown to be neuraminidase sensitive.

*Mycoplasma synoviae*, a fastidious mycoplasma with an absolute requirement for oxidized nicotinamide adenine dinucleotide (NAD), causes natural outbreaks of infectious synovitis in chickens (6, 10, 11). Experimental infection of chickens, either intratracheally or through the footpad, with *M. synoviae* produces an acute to chronic condition characterized by lesion production predominantly in the joints and heart. The joints show a marked thickening of the synovial membrane which may contain a cell-free exudate. The heart tissue also appears hyperplastic with scattered areas of necrosis and inflammation. Why lesions localize these in areas as opposed to other organs is not known, and what role *M. synoviae* plays in inducing these lesions is also unknown. In view of this, the following in vitro study was undertaken to determine (i) if *M. synoviae* would grow in tissue culture; (ii) what effect *M. synoviae* growth had on the tissue culture cells; and (iii) if other important cytopathogenic parameters could be recognized.

## MATERIALS AND METHODS

**Organism.** *M. synoviae* (ATCC 25204) was purchased from the American Type Culture Collection (Rockville, Md.) and was used in all the following studies. This organism was originally isolated from the hock of a chicken with infectious synovitis (9). Growth was obtained in an enriched mycoplasma medium (MS broth) containing the following (parts/

liter): mycoplasma medium base (Charles Pfizer and Co., New York), 22.5 g; swine serum (inactivated at 56 C for 30 min) (Microbiological Associates, Inc., Bethesda, Md.), 120 ml; Eagle vitamin supplement ( $\times 100$  concentration) (Microbiological Associates, Inc., Bethesda, Md.), 0.25 ml; NAD (Sigma Chemical Co., St. Louis, Mo.), 0.1 g; cysteine hydrochloride, 0.1 g; dextrose, 1.5 g; penicillin G, 100,000 U; thallium acetate, 0.1 g; phenol red, 2.5 ml of a 1% solution. MS broth was adjusted to pH 7.7 with 1 N NaOH and filter sterilized through 0.45- and 0.22- $\mu$ m membrane filters (Millipore Corp.) A solid medium for *M. synoviae* growth was prepared by adding 10 ml of a sterile 10% solution of purified agar to 90 ml of prewarmed MS broth (minus phenol red). The medium was allowed to mix for 15 to 30 min at 55 C and then dispensed into plastic petri dishes (15 by 60 mm) in 5-ml portions. All media were tested for sterility before use.

Batches of *M. synoviae* were grown for 3 to 5 days at 37 C until the phenol red indicator turned yellow. The medium was shaken to distribute the mycoplasmas evenly and 5-ml portions were frozen to -70 C. Each batch was titered by preparing triplicate serial 10-fold dilutions of a sample from each batch in MS broth (up to  $10^{-10}$ ). All dilutions were incubated at 37 C for 5 days and the end point was determined. The highest dilution which showed a yellow color (acid production) after incubation was considered to contain 1 color change unit per ml of original culture and the titer was the reciprocal of that dilution.

**Tissue culture media.** The chicken embryo cells used in this study were grown in Eagle minimal essential medium (MEM) with Hanks salts and L-glutamine (GIBCO, Grand Island, N. Y.). All tissue culture media were supplemented with 10% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.) and penicillin (100 U/ml)-streptomycin (100

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$\mu\text{g/ml}$ ) (GIBCO, Grand Island, N. Y.). For *M. synoviae*-infected cultures, the above MEM was modified by eliminating the penicillin-streptomycin and adding 2% inactivated swine serum and 0.01% NAD. All tissue culture media were adjusted to pH 7.2 to 7.4 and filter sterilized through a 0.45- and a 0.22- $\mu\text{m}$  membrane (filter Millipore Corp.). All media were tested for contamination before use.

**Preparation of chicken embryo cell suspension.** Ten-day-old fertile chicken eggs were thoroughly washed with 70% ethanol and then swabbed with 2% mercurochrome. The shell covering the air sac was removed, the chorioallantoic membrane was opened, and the embryo was aseptically removed and placed in a sterile petri dish. The head, appendages, and viscera were removed and the remainder of the embryo was washed in Hanks balanced salts solution (HBSS). The embryo was then minced and passed through a 10-ml syringe into a sterile Erlenmeyer flask. Sterile 0.25% trypsin (50 to 100 ml) was added to the flask and the mixture was stirred for 30 to 45 min at 37 C. The cell suspension was poured through four thicknesses of sterile cheesecloth contained in a Seitz filter. The cells were then spun down at  $500 \times g$  for 10 min and washed three times in HBSS. The cells were then suspended in MEM and a viability count was made by the erythrosin B exclusion technique (8). A 0.1-ml portion of the cell suspension was tested for bacterial and mycoplasma contamination.

**M. synoviae infection and titration in cell culture.** For titration of *M. synoviae* in tissue culture,  $10^6$  chicken embryo cells were seeded in 25-cm<sup>2</sup> Falcon flasks with addition of MEM with fetal calf serum and penicillin-streptomycin. The flasks were incubated 12 to 18 h to facilitate attachment, washed three times with HBSS, and infected with  $10^6$  color change units of *M. synoviae*. Control flasks were inoculated with an equal volume of sterile MS broth. The MEM was then modified to contain only fetal calf serum, swine serum, and NAD as described above. All flasks were incubated at 37 C and titered daily for 5 days by preparing serial 10-fold dilutions in MS broth as described above. The pH was adjusted, when needed, to 7.2 to 7.4 with sterile 0.7%  $\text{NaHCO}_3$ . In addition, an MS agar plate was inoculated at the time of titration and incubation to observe typical mycoplasma colonies.

For microscopy of *M. synoviae*-infected cultures, 10 growth Flaskettes (Lab Tek Products, Naperville, Ill.) were seeded with  $10^6$  chicken embryo cells followed by addition of 2.9 ml of MEM with fetal calf serum and penicillin-streptomycin. The Flaskettes were incubated at 37 C for 12 to 18 h to facilitate attachment and then washed three times with HBSS. Subsequently, each Flaskette received an equal volume of MEM with fetal calf serum, swine serum, and NAD. Five Flaskettes were inoculated with  $10^6$  color change units of *M. synoviae* while the other five (controls) received an equal volume of sterile MS broth. All Flaskettes were incubated at 37 C and the pH was adjusted to 7.2 to 7.4, when needed, with sterile 0.7%  $\text{NaHCO}_3$ . On each of the subsequent 5 days, one infected and one control Flaskette were washed three times with HBSS, fixed 3 min in absolute methanol, and stained in a 1:20 dilution of Giemsa stain (Allied

Chemical, Morristown, N. J.). The slides were then rinsed in HBSS and allowed to air dry. All slides were observed with bright-field illumination on a Leitz Ortholux microscope.

**Enzymatic characterization of the *M. synoviae* receptor site on chick cell membranes.** The method used was modified from that of Taylor-Robinson and Manchee (13). Plates of MS agar were inoculated with various dilutions of *M. synoviae* and incubated aerobically at 37 C for 3 to 5 days until distinct mycoplasma colonies appeared. Only plates containing 50 to 100 colonies were used. Chick cells that had been grown to confluency in 16-ounce (ca. 480 ml) medicine bottles were gently harvested by scraping with a sterile rubber policeman, washed three times in HBSS, and adjusted to  $10^6$  cells/ml. Portions (2 ml) of the cell suspension were then pipetted onto *M. synoviae* colony-containing plates. The plates were incubated at 37 C for 30 min. The cell suspension was then poured off and the colonies were gently washed three times with HBSS. The plates were then observed for cell attachment to *M. synoviae* colonies at  $\times 120$  magnification with a Leitz Ortholux microscope. Cytoadsorption was considered strong if 10 or more cells were attached to a colony after discarding the cell suspension and were still present after washing.

For enzymatic treatment of cells, 2-ml aliquots of the above cell suspension were mixed separately with an equal volume of trypsin (0.25%) (Difco Laboratories, Detroit, Mich.), viokase (0.25%) (GIBCO, Grand Island, N. Y.), and neuraminidase (50 U/ml) (Calbiochem, San Diego, Calif.). The mixtures were incubated at 37 C for 30 min and then centrifuged at  $500 \times g$  for 10 min, washed three times with HBSS, and suspended in 2 ml of HBSS. The treated cells were then pipetted onto *M. synoviae*-containing plates and tested as above for cytoadsorption.

## RESULTS

**Growth of *M. synoviae* in chick fibroblast cultures.** A steady increase in the number of *M. synoviae* organisms on each of the 5 days titrated is indicated in Fig. 1. On 4 of the 5 days an approximate 10-fold increase occurred. By

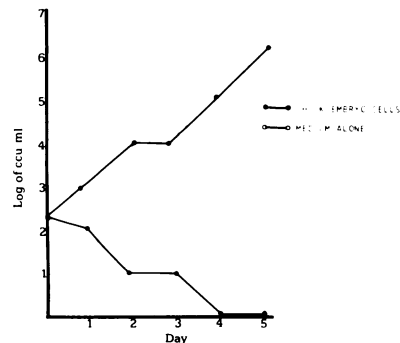


FIG. 1. Growth titration of *M. synoviae* in chicken embryo fibroblast cultures and medium alone.

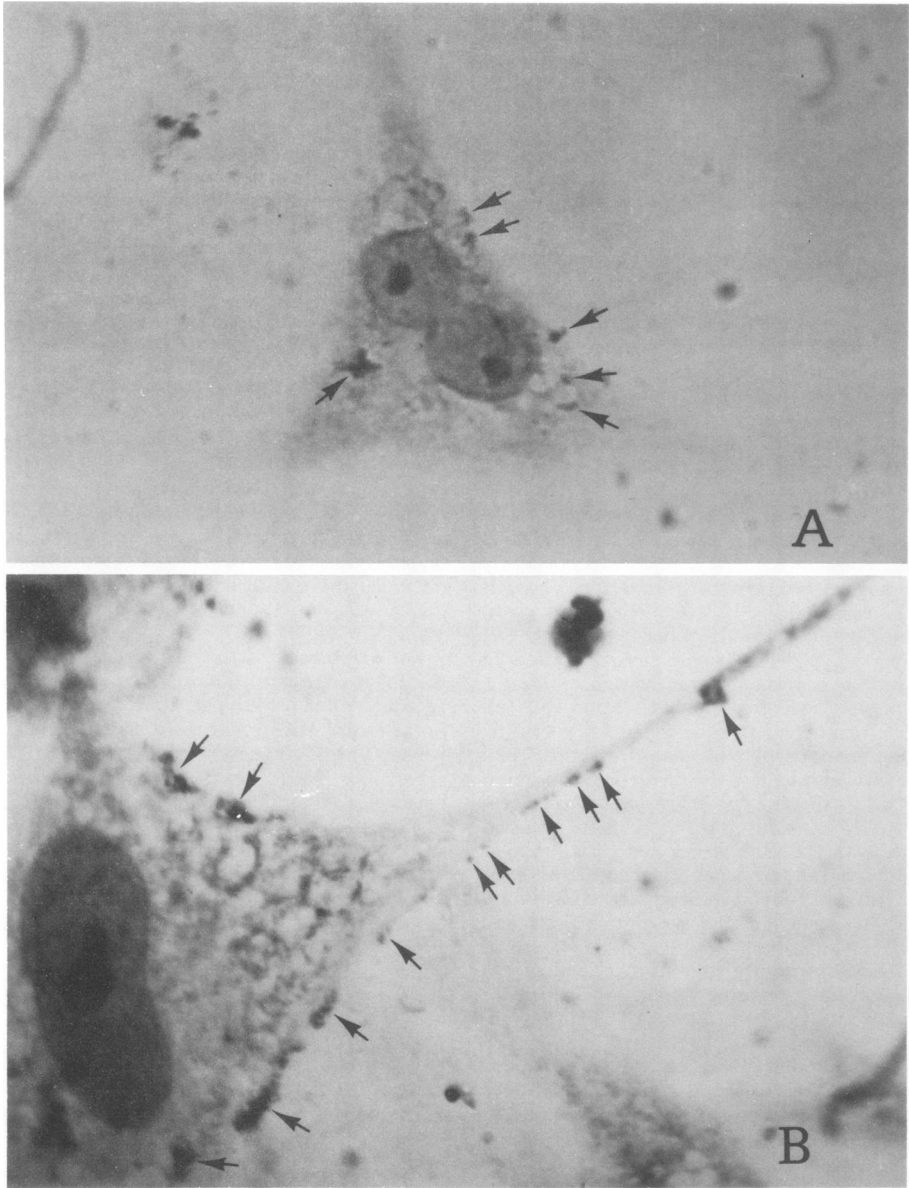


FIG. 2. *M. synoviae*-infected and control chicken embryo fibroblasts. (A) Day 2 postinfection; (B) day 3 postinfection; (C) day 4 postinfection; (D) and (E) day 5 postinfection; (F) day 5 uninfected control. In the infected cultures note the progressive deterioration of the cytoplasm beginning with mild vacuolation and granularity to almost complete dissolution of the cytoplasm by day 5. Also note the nuclear degeneration in infected cells (E). *M. synoviae* microcolonies are indicated by arrows (†). Note that the microcolonies begin as distinct colonies (A) and progressively spread over the surface of the cells (C, D, and E).  $\times 6750$ .

comparison, no increase in the number of mycoplasmas was found in the MEM containing 10% fetal calf serum, 2% swine serum, and 0.01% NAD. The mycoplasmas did survive in decreasing numbers until day 4, when none could be detected.

**Cytopathology induced by *M. synoviae*.** The Giemsa-stained preparations of chicken embryo cells infected with *M. synoviae* showed a general increase in pathological features on successive days along with evidence of overwhelming infection. Figures 2A through 2E

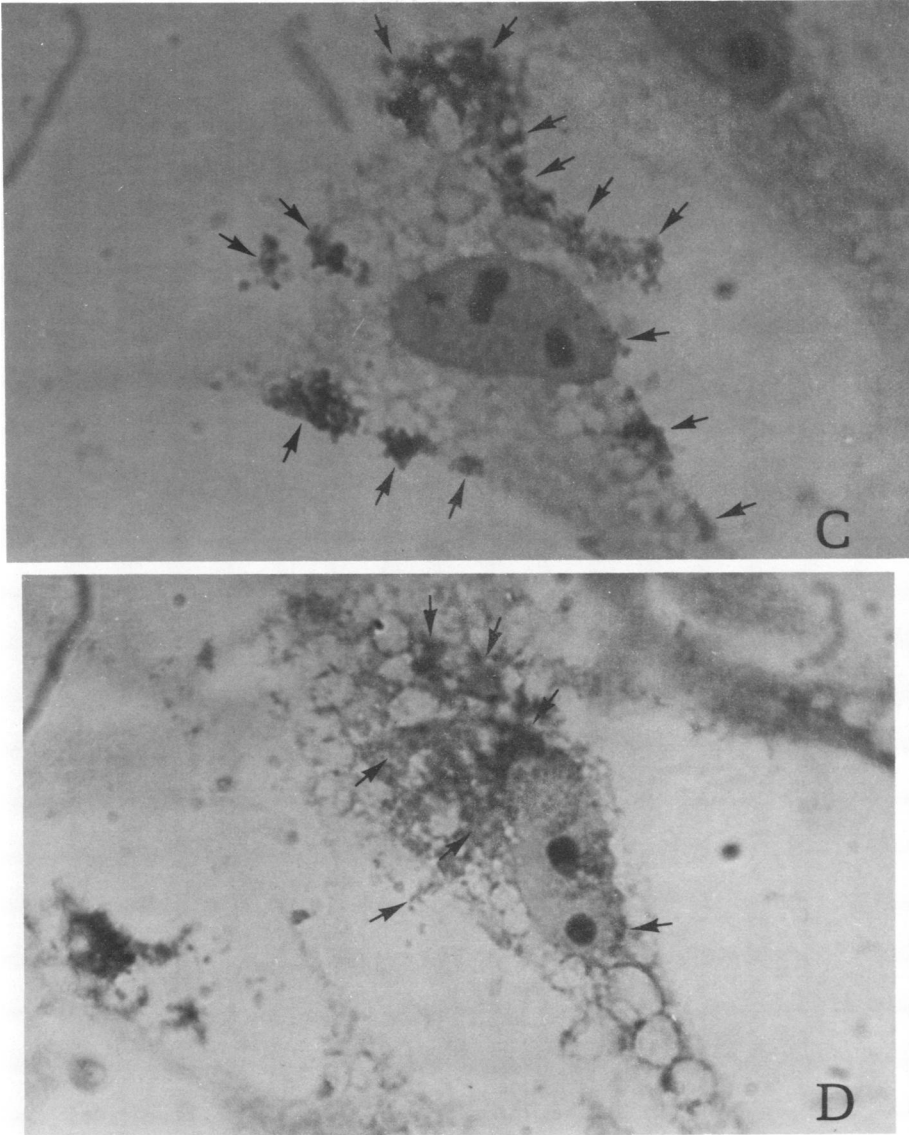


FIG. 2 C-D

show typical cells from infected cultures on days 2 to 5 while Fig. 2F shows a cell from a 5-day control (uninfected) culture. Also, during this study only those MS agar plates inoculated from infected cultures yielded positive results showing typical fried-egg colonies characteristic of *M. synoviae*.

On day 1 the cells appeared normal with some mycoplasmas attached to the membranes. No apparent cytoplasmic change had occurred even though the *M. synoviae* titer had risen approximately 10-fold. Day 2 yielded cells which were beginning to show cytoplasmic changes in the

form of mild vacuolation and granularity. Microcolonies of *M. synoviae* appeared at distinct sites along the membrane surface. Some cells contained only one or two such colonies whereas other cells contained between 10 and 20. On day 3 the vacuolation was more extensive and certain cells showed granular and vacuolated nuclei. The mycoplasma microcolonies were more widespread and larger in area. They covered large areas of the cell surface and portions appeared to extend out into the medium. At day 3 many of the cells appeared dead and a much slower rate of pH drop occurred

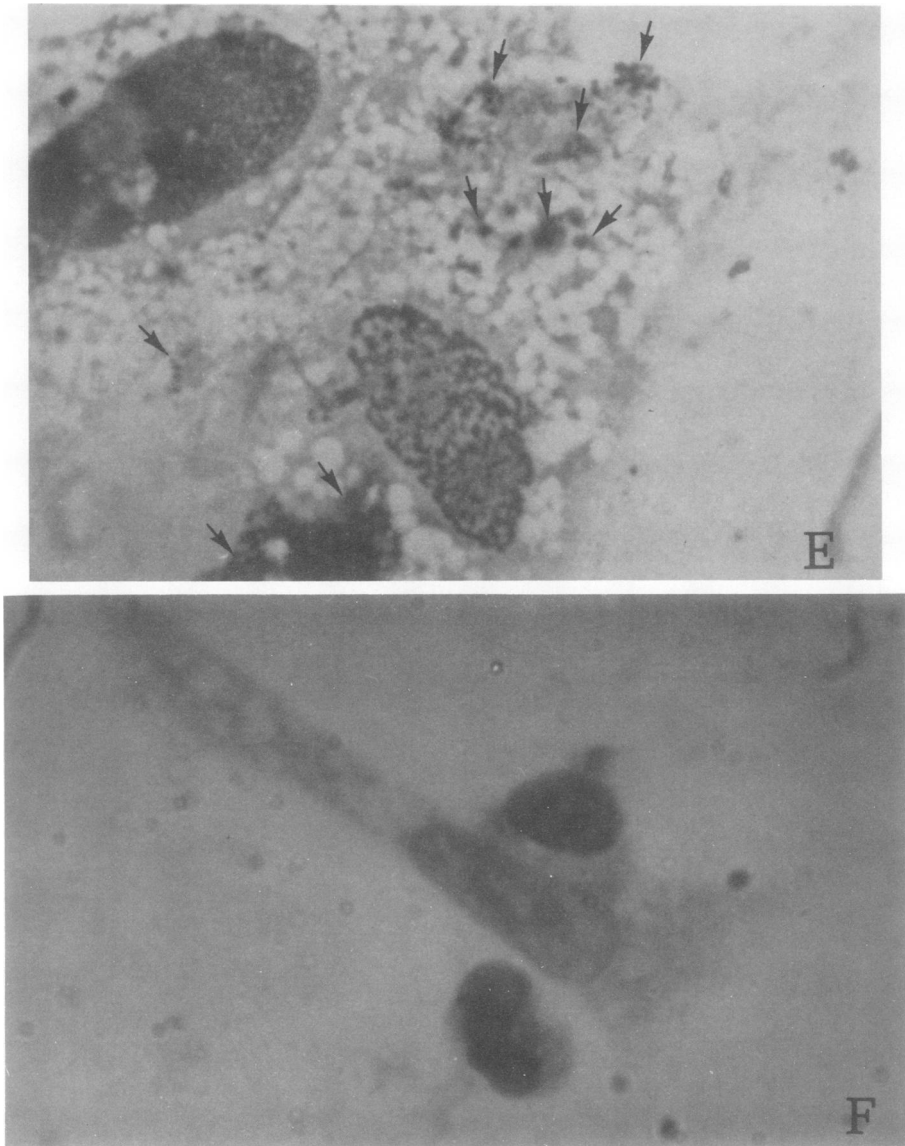


FIG. 2E-F

thereafter in infected cultures. On day 4 complete vacuolation of the cytoplasm had occurred as well as more cells with nuclear granularity and much less staining intensity. The *M. synoviae* microcolonies were large and many cells were dead and degenerating. Little was left of the cytoplasm and many cells had fallen off the glass slide. The remaining cells appeared covered with mycoplasmas.

Control (uninfected) cultures showed little or no pathology compared to the infected cultures. Even though occasional acid conditions existed

in the controls and were corrected with  $\text{NaHCO}_3$ , no extensive vacuolation occurred and confluent monolayers formed by day 5.

**Receptor site nature.** After observing microcolonies at the chick cell membrane, it was of interest to know the nature of this attachment site. As shown in Table 1, untreated (control) chicken embryo cells were attached to the *M. synoviae* colonies before and after washing. Only neuraminidase abolished the ability of the chick cells to attach to the mycoplasma colonies, whereas trypsin and viokase had no

TABLE 1. Effect of enzyme treatment on the attachment of chicken embryo cells to *M. synoviae* colonies

Control	Attachment after enzyme treatment		
	Neuraminidase	Trypsin	Viokase
(untreated) +	(50 U/ml) -	(0.25%) +	(0.25%) +

effect. Thus it is indicated that an essential part of the attachment or receptor site on the chick cell membrane is sialic acid. Interestingly, flooding plates, which had chick cells attached to *M. synoviae* colonies with neuraminidase, did not release the cells after 15 min of incubation at 37 C. Therefore, the sialic acid receptor appears to be protected from the enzyme after attachment to the mycoplasma colony.

## DISCUSSION

The growth of *M. synoviae* in the tissue culture medium of chicken embryo fibroblasts is probably not a true reflection of the total number of mycoplasmas in the culture because of the formation of microcolonies at the membrane level. Even so, such a steady increase should be regarded as an increase in the total number of organisms in the culture because the size of the microcolonies, as well as the number of organisms in the culture fluid, increased daily. Whether the increase in the number of organisms in the culture medium is due to shedding of organisms into the medium from the microcolonies or whether the organisms multiply in the medium alone is not known. It is probable that both occurs.

Interestingly, *M. synoviae* did not multiply in the tissue culture medium alone even though it was enriched with swine serum and NAD. Although *M. synoviae* is one of the most nutritionally exacting of the mycoplasmas, it would appear that all the necessary ingredients were present for *M. synoviae* growth in the tissue culture medium. Whatever was missing from the medium for *M. synoviae* growth was provided by the chicken embryo cells as evidenced by the increase in mycoplasmas.

The formation of microcolonies at the membrane level appears to result from the specific attachment of mycoplasmas to specific receptors. As the infection persisted, the microcolonies grew in size with concomitant increased chick cell cytopathology until the chick cells were dead and destroyed. Progressive chick cell destruction probably released the growth factor

necessary for *M. synoviae* to reach such high titers after 5 days of infection. The mechanism of growth factor release or induction of cytopathological effects by microcolonies is not known but may be due to hydrogen peroxide production as found in previous reports of mycoplasma pathology (1-3). Alternatively, membrane damage could occur from simple attachment of the mycoplasmas with loss of essential materials from the internal milieu of the cytoplasm and a pathological state resulting. This has been suggested by Collier (4). Stanbridge et al. (12) reported they were unable to grow *Acholeplasma laidlawii*, a nutritionally less exacting mycoplasma, in either fresh or conditioned BME tissue culture media. They further suggest that the close relationship between mycoplasmas and tissue cell membranes is more than casual and such an attachment leads to leaching nutrients from the tissue cell for their growth. The results in the present report readily support this suggestion not only in a quantitation of *M. synoviae* growth, but by their specific attachment to tissue cells via receptors.

Taylor-Robinson and Manchee (7, 13) have previously reported the presence of neuraminidase-sensitive receptors on tissue cells involved in attachment to mycoplasma colonies. They also indicated that the receptor counterpart on the mycoplasma membrane is not neuraminidase sensitive and is probably protein in nature. Gesner and Thomas (5) have also reported that neuraminidase-sensitive receptors participate in hemadsorption by mycoplasmas. In this study the occurrence of such receptors appeared to be unevenly distributed among the cell population examined. The number of microcolonies found in infected cultures varied from cells with none or a few to cells with 20 to 25 microcolonies each. This probably reflects an uneven distribution of receptor sites on the tissue cells since the higher titer of *M. synoviae* excludes inadequate contact of cell receptors with mycoplasmas. Since the chick cell suspension used was a conglomerate of disaggregated embryos, it is probable that most of the cells were in different stages in differentiation which could be reflected in the difference of receptors on the membrane.

In answering the questions proposed earlier, (i) *M. synoviae* did grow in chicken embryo cultures and reached high titers; (ii) *M. synoviae* infection induced a progressive cytoplasmic and nuclear degeneration of the chick cells; and (iii) *M. synoviae* attached to the chick cell membranes via neuraminidase-sensitive receptors and formed microcolonies which caused

release of a growth factor needed by the mycoplasmas for growth.

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