Immunofluorescence and Electron Microscopy of the Attachment of *Mycoplasma synoviae* to Chicken Embryo Fibroblasts

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The techniques of immunofluorescence and of transmission and scanning electron microscopy were used to demonstrate the attachment of *Mycoplasma synoviae* to chicken embryo fibroblasts. Although the organisms colonized the surface of many of the cells, there was marked variation in the number of organisms associated with individual cells.

As noted in the review by Stanbridge (10), the adsorption of mycoplasmas to fibroblastic, epithelial, or lymphocytic cells can produce a variety of cytotoxic effects sometimes leading to cell death. *Mycoplasma pneumoniae* has been shown to attach to the epithelial-lining cells of tracheal organ cultures, inhibit the normal ciliary movement, and induce necrosis (7, 8). Cohen and Somerson (4) have shown that *M. pneumoniae* produces large amounts of hydrogen peroxide which lyses erythrocytes and suggested that the hydrogen peroxide may induce extensive injury to the respiratory epithelium. Others (11) have suggested that the attachment itself or that the leaching out of nutrients from the cell by the attached mycoplasmas produces pathological changes in the eukaryotic host.

A previous report (2) showed that *M. synoviae*, the causative agent of chicken synovitis (9), attach to chicken embryo fibroblasts via a neuraminidase-sensitive receptor. The present study was undertaken to demonstrate this attachment by using immunofluorescence and transmission and scanning electron microscopy.

Two strains of *M. synoviae* were used throughout this study. The laboratory strain *M. synoviae* LS is a high in vitro passage strain, and its origin has been previously discussed (2). The second strain, designated *M. synoviae* 1331 p9, is a low passage strain and was used in this study in the ninth in vitro passage. The latter strain was originally isolated from an arthritic chicken and was kindly provided by T. H. Vardaman (South Central Poultry Research Laboratory, State College, Miss.). The culture medium and procedure for growing these organisms was as previously described (2). Colony-forming units per milliliter were quantitated by plating 0.01 ml amounts of serial 10-fold dilutions to *M. synoviae* agar.

The preparation of chicken embryo cells was performed as previously reported (2), except that RPMI 1640 tissue culture medium (Flow Laboratories, Rockville, Md.), supplemented only with 10% (vol/vol) inactivated fetal calf serum and 200 U of penicillin per ml, was used.

Morphological observations on infected fibroblasts were conducted on cells infected at a mycoplasma-cell ratio of 10:1. In all studies, three test groups were established, consisting of (i) cells infected with strain LS, (ii) cells infected with strain 1331 p9, and (iii) cells which received only medium and served as uninfected controls.

For indirect immunofluorescence, each group was grown in Flaskettes (Lab Teck Products, Naperville, Ill.). On days 1, 3, and 5 postinfection, one Flaskette from each group was washed three times with Hanks balanced salts solution (HBSS), and 1 ml of a 1:10 dilution (in HBSS) of chicken anti-*M. synoviae* antiserum (provided by T. H. Vardaman) was added. After incubation for 30 min at 37°C, the Flaskettes were washed three times with HBSS, and then each received 1 ml of a 1:10 dilution (in HBSS) of fluorescein-labeled anti-chicken globulin (Grand Island Biological Co., Grand Island, N.Y.). After a further incubation for 30 min at 37°C, all Flaskettes were washed three times with HBSS, a cover slip was mounted with glycerol, and the preparations were observed under UV light with a...
Zeiss Universal microscope using a BG12 and a K10 filter.

For transmission electron microscopy, each test group was set up in large prescription bottles (growth area, ca. 125 cm²). The bottles were incubated at 37°C, and after 3 days the cells were washed three times with HBSS and then gently teased off the glass with a sterile rubber policeman. The suspensions were centrifuged and washed three times with HBSS. Each cell pellet was then fixed 2 to 3 days in phosphate-buffered glutaraldehyde (5%), rinsed with phosphate buffer, and postfixed for 2 to 3 h in phosphate-buffered osmium tetroxide (1%). Each pellet was dehydrated through a graded series of ethanol and embedded in Epon. Sections were cut on a Porter-Blum MT2 ultramicrotome (Sorvall Instruments, San Mateo, Calif.) using a 75-0-nm thickness setting and were placed on copper grids and stained with uranyl acetate and lead citrate. The sections were then examined by transmission electron microscopy with an RCA EM U3H microscope (RCA Instruments, Camden, N.J.).

For scanning electron microscopy, cells were grown in Leighton tubes (Bellco Glass, Inc., Vineland, N.J.) containing cover slips (9 by 22 mm). All tubes were incubated at 37°C and, at 1, 3, and 5 days postinfection, one cover slip from each group was processed as follows. Each cover slip was washed three times with HBSS, fixed 20 min with phosphate-buffered glutaraldehyde (3%), washed three times with HBSS, and dehydrated through a graded series of ethanol. The cover slips were placed in 100% ethanol, perfused with liquid CO₂, and dried in a critical-point drying apparatus. The cover slips were then cut in half, mounted on metal stubs with silver paint, and gold coated. The specimens were examined on a Cambridge Mark IIA scanning electron microscope (Cambridge Instruments, Cambridge, England).

Immunofluorescent staining of cell cultures showed a progressive increase in the amount of fluorescence to day 3. Degeneration of the cells occurred after 3 days. Figure 1 shows the staining intensity of the LS strain of M. synoviae at day 3. The 1331 p9 strain gave similar results, although the LS strain consistently gave more intensive staining. Also, the LS strain appeared to produce larger microcolonies on the membranes than did the P9 strain. However, at day 3 both mycoplasma strains could be seen to completely cover the membrane of many of the cells. Other cells showed a few to several microcolonies, whereas some cells appeared to have no organisms attached at all. The uninfected controls showed no fluorescence.

Figure 2 illustrates the attachment of the 1331 p9 strain of M. synoviae to chicken embryo fibroblasts with transmission electron microscopy. Almost all of the mycoplasmas illustrated show direct contact or close association with chicken cell membranes. The majority of the mycoplasmas appear as round or coccoid forms. The LS strain gave almost identical results but

Fig. 1. Indirect immunofluorescence of M. synoviae-infected chicken fibroblast 3 days postinfection. M. synoviae LS-infected cell. ×2,000.
is not shown. No mycoplasmas were seen in uninfected controls.

Scanning electron microscopy very dramatically reveals the attachment of the \textit{M. synoviae} strains to chicken embryo fibroblasts (Fig. 3A to E). The distribution of mycoplasmas on different fibroblasts appeared to be quite uneven. Much of the cell surface in Fig. 3A is covered with attached organisms, whereas in Fig. 3B only a single microcolony can be seen. At higher magnification (3C and D), the individual components of the microcolonies can be seen. Figure 3E

\textbf{Fig. 2.} Transmission electron microscopy of \textit{M. synoviae}-infected chicken fibroblast 3 days postinfection. \textit{M}, Mycoplasma cells. \textit{M. synoviae} 1331 p9-infected cell. Approximately $\times27,000$. Note the close mycoplasma-fibroblast association.
shows the intimate mycoplasma-fibroblast relationship at higher magnification. Mycoplasma particles were not detected on any of the uninfected fibroblasts.

Collier (7), using transmission electron microscopy, observed that *M. pneumoniae* attached to tracheal culture epithelial cells via a specialized terminal structure located at one end of the elongated, cylindrical mycoplasma cells. Boatman et al. (3), by using both transmission and scanning electron microscopy, showed that the attachment of bovine mycoplasmas to HeLa
cells appeared similar to that reported here with *M. synoviae*.

The present and previous studies (2) substantiate that *M. synoviae* attaches to the surface of chicken embryo cells. The role played by this attachment in cytotoxicity in vitro and in cell damage in vivo remains to be determined. The reason for the uneven distribution of mycoplasmas to different fibroblast cells is not known but could reflect a difference in distribution of receptor sites. Additional studies should be conducted to elucidate this possibility, since it may have a bearing on the tropisms exhibited in many mycoplasma infections.

*M. synoviae* exhibits other effects on mammalian cell functions. We have shown that it is strongly mitogenic for mouse lymphocytes (5) and will induce cultured human lymphocytes to produce interferon (6). Further studies are required on the role of these mycoplasma-cell interactions in the pathogenesis of *M. synoviae*-induced disease.

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**LITERATURE CITED**