INFECTION AND IMMUNITY, May 1985, p. 576–578
0019-9567/85/050576-03$02.00/0

Vol. 48, No. 2

Isolation of a Cytopathic Factor from *Mycoplasma hyopneumoniae*

STEVEN J. GEARY†* AND EDWARD M. WALCZAK‡
Schering Animal Health, Omaha, Nebraska 68103

Received 5 November 1984/Accepted 17 January 1985

A cytopathic factor was isolated from *M. hyopneumoniae* VPP11 membranes. This factor had an isoelectric point of 6.2 and was capable of inducing cytopathic effect in MRC-5 human lung fibroblast culture at a concentration of 250 ng of protein per ml. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this cytopathic factor showed a major band with a molecular weight of approximately 54,000.

Mycoplasma hyopneumoniae is the etiologic agent of enzootic pneumonia of swine (8). A previous study demonstrated that a cytopathic factor (CF) is present in the membrane of *M. hyopneumoniae* (6). The CF is a protein which is capable of inducing cytopathic effect (CPE) in MRC-5 human lung fibroblast culture. The CPE induced in the MRC-5 culture monolayer is characterized by cleared areas of dead cells. The purpose of this study was to isolate the CF from the *M. hyopneumoniae* membrane.

M. hyopneumoniae VPP11 membranes were prepared by centrifugation of a culture (in stationary phase) containing ca. 4×10^7 organisms per ml at 12,500 × *g* for 15 min at 4°C. The organisms were washed three times in TEAN buffer (pH 7.4) (0.05 M Tris, 0.001 M EDTA-disodium salt, 0.003 M NaN_3, 0.2 M NaCl). They were suspended in TEAN buffer at 100 times the original concentration and lysed by subjecting them to 20 cycles of freezing (ethanol-dry ice) and thawing (37°C) (4). Unlysed organisms were pelleted by centrifugation at 4,000 × *g* for 15 min at 4°C. The supernatant was collected and centrifuged at 41,000 × *g* for 45 min at 4°C. The cytoplasmic fraction was collected and saved. The pelleted membranes were then washed three times in TEAN buffer and finally suspended in TEAN buffer at 1,000 times the original concentration. The membrane preparations were examined for the presence of viable mycoplasmas and bacterial contaminants. The protein concentration of the membrane preparation was determined by the technique of Bradford (1). The membrane preparation (2.6 mg) was solubilized in 1% Triton X-100 for 30 min at 25°C with constant mixing. The solubilized preparation was centrifuged at 41,000 × *g* for 45 min at 4°C and then dialyzed against distilled water. The sample was then separated by isoelectric focusing in a 2% ampholyte (Bio-Lyte) gel matrix (pH 3 to 10) (Bio-Rad Laboratories, Richmond, Calif.) at 5 W for 18 h at 4°C. The pH of each fraction was measured with a surface electrode. The fractions were collected individually and eluted from the gel in 1 ml of TEAN buffer over glass wool-packed Pasteur pipettes. Each fraction was assayed for the ability to cause CPE on MRC-5 human lung fibroblasts (cells were culture negative for mycoplasma contamination). The MRC-5 fibroblasts were maintained in Eagle minimum essential medium with Earle salts (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal bovine serum and an additional 1% nonessential amino acids (GIBCO). Cells were cultured in Tissue Culture Cluster 24 (Costar, Cambridge, Mass.) plates in 5% CO_2 at 37°C. A portion (10 μl) of each fraction was added to individual wells, followed by 1 ml of tissue culture medium each. Cells were evaluated at 24 h postinoculation. *M. hyopneumoniae* membranes served as a positive control, and TEAN buffer and tissue culture medium served as negative controls. The CF was mixed 1:1 with either preimmune or immune rabbit serum described previously (6), incubated at 37°C for 30 min with constant mixing, and then assayed on MRC-5 cells. The molecular weight of the CF was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed in a 10% gel by a modified procedure of Laemmli (7). The sample was run unheated and nonreduced. A sample of the *M. hyopneumoniae* membrane preparation was also run on a 10% SDS-PAGE gel. The proteins in the gels were stained with 0.2% Coomassie brilliant blue R-250 (Bio-Rad Laboratories). The cytoplasm of *M. hyopneumoniae* does not cause any CPE when assayed on MRC-5 cells (6). The membrane preparation was shown previously to be free of intact cells (6). *M. hyopneumoniae* membranes at a concentration of 2 μg/ml (2 μg per well) were capable of causing CPE, whereas CF was capable of causing CPE at 250 ng/ml (250 ng per well) (Fig. 1). The TEAN buffer control did not result in CPE. The CPE caused by *M. hyopneumoniae* membranes or CF was identical to that caused by viable *M. hyopneumoniae*. The cell monolayer showed cleared patches of dead cells which may be round and floating or lysed. Preimmune serum did not inhibit CF, whereas immune serum did inhibit CF from causing CPE. The CF had an isoelectric point of 6.2 and showed a major band with a molecular weight of approximately 54,000 on SDS-PAGE (Fig. 2). The membrane preparation contained approximately 23 protein bands which could be seen after staining with Coomassie brilliant blue R-250 (Fig. 3).

The demonstration of the cytopathic nature of the membranes of *M. hyopneumoniae* (6) is consistent with findings with other mycoplasma membranes. Membranes of *Mycoplasma pneumoniae* at a level of 25 μg of protein per ml induced a cytotoxic reaction in adult hamster trachea organ cultures (2). Membranes from a fresh isolate of *Mycoplasma fermentans* were found to contain a toxic factor which was lethal to mice when injected intraperitoneally (3). An inflammatory toxin was isolated from *Mycoplasma bovis* membranes which was shown to induce an eosinophilic mastitis in bovines (5). The isolation of this CF from the membrane of *M. hyopneumoniae* will now enable us to evaluate its role in the pathogenesis of the natural disease in swine.

* Corresponding author.
† Present address: Bionique Laboratories, Inc., Saranac Lake, NY 12983.
‡ Present address: Intervet USA, Inc., Millsboro, DE 19966.

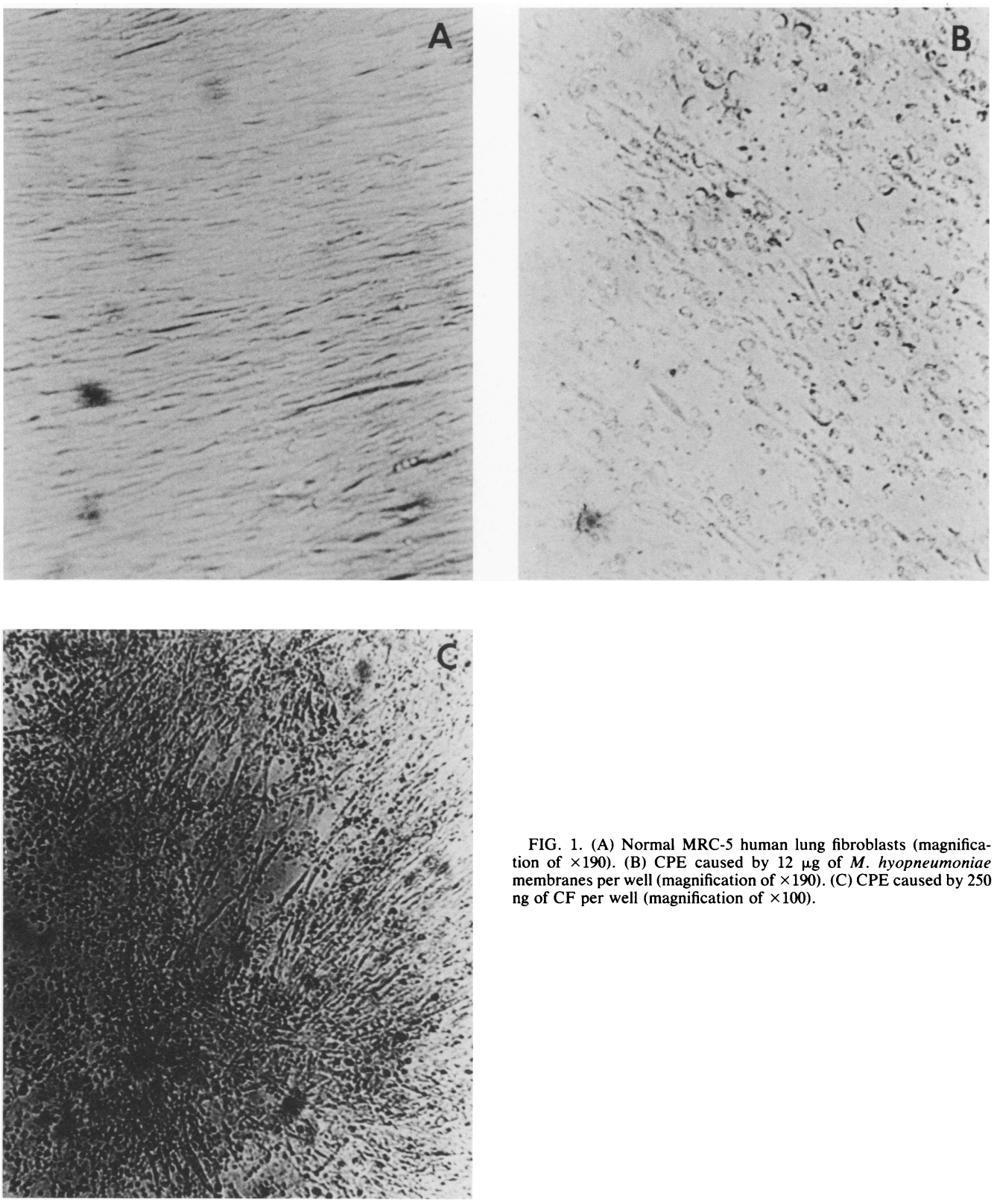

FIG. 1. (A) Normal MRC-5 human lung fibroblasts (magnification of ×190). (B) CPE caused by 12 μg of *M. hyopneumoniae* membranes per well (magnification of ×190). (C) CPE caused by 250 ng of CF per well (magnification of ×100).

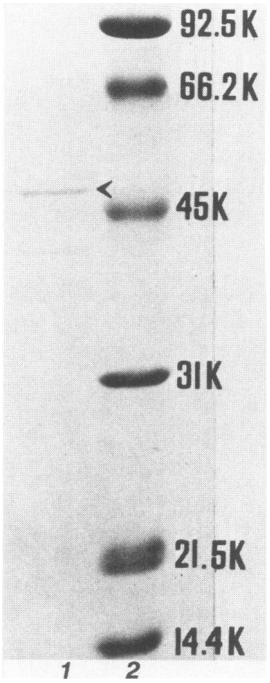

FIG. 2. SDS-PAGE of CF. Lane 1, CF (3 μg); lane 2, SDS-PAGE low-molecular-weight Bio-Rad standards as follows: phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

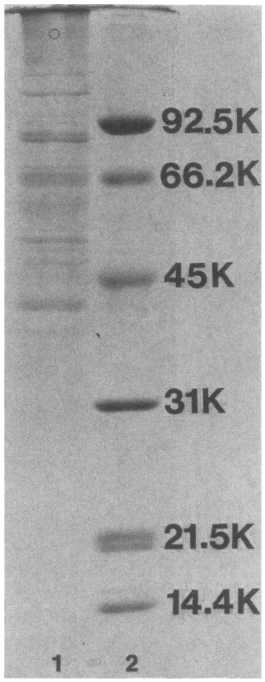

FIG. 3. SDS-PAGE of proteins from *M. hyopneumoniae* membranes. Lane 1, *M. hyopneumoniae* membranes (9 μg); lane 2, SDS-PAGE low-molecular-weight Bio-Rad standards as follows: phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

We thank Dawn Stickney for the typing of this manuscript.

LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Gabridge, M. G., C. K. Johnson, and A. M. Cameron.** 1974. Cytotoxicity of *Mycoplasma pneumoniae* membranes. Infect. Immun. **10**:1127–1134.
3. **Gabridge, M. G., and W. H. Murphy.** 1971. Toxic membrane fractions from *Mycoplasma fermentans*. Infect. Immun. **4**:678–682.
4. **Gabridge, M. G., and R. B. Polisky.** 1976. Quantitative reduction of 2,3,4-triphenyl tetrazolium chloride by hamster trachea organ cultures: effects of *Mycoplasma pneumoniae* cells and membranes. Infect. Immun. **13**:84–91.
5. **Geary, S. J., M. E. Tourtellotte, and J. A. Cameron.** 1981. Inflammatory toxin from *Mycoplasma bovis*: isolation and characterization. Science. **212**:1032–1033.
6. **Geary, S. J., and E. M. Walczak.** 1983. Cytopathic effect of whole cells and purified membranes of *Mycoplasma hyopneumoniae*. Infect. Immun. **41**:132–136.
7. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
8. **Ross, R. F.** 1973. Pathogenicity of swine mycoplasmas. Ann. N.Y. Acad. Sci. **225**:347–368.