5. J. B. Dougherty, H. Samanta, P. J. Farrell, P. Lengyel, *J. Biol. Chem.* **255**, 3813 (1980).
6. H. Samanta, J. B. Dougherty, P. Lengyel, *ibid.*, p. 9807.
7. C. Baglioni, M. A. Minks, P. A. Maroney, *Nature (London)* **273**, 684 (1978).
8. M. J. Clemens and B. R. G. Williams, *Cell* **13**, 565 (1978).
9. P. J. Farrell, G. C. Sen, M. F. Dubois, L. Ratner, E. Slattery, P. Lengyel, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5893 (1978); A. Schmidt, A. Zilberstein, L. Shulman, P. Federman, H. Berissi, M. Revel, *FEBS Lett.* **95**, 257 (1978); D. A. Eppstein and C. E. Samuel, *Virology* **89**, 240 (1978).
10. E. Slattery, N. Ghosh, H. Samanta, P. Lengyel, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4778 (1979).
11. G. Floyd-Smith, in preparation.
12. J. L. Oakley, R. E. Strothkamp, A. H. Sarris, J. E. Coleman, *Biochemistry* **18**, 328 (1979).
13. H. Osterman and J. E. Coleman, in preparation.
14. H. Donis-Keller, A. M. Maxam, W. Gilbert, *Nucleic Acids Res.* **4**, 2527 (1977).
15. S. Cory, J. M. Adams, P.-F. Spahr, *J. Mol. Biol.* **63**, 41 (1972).
16. D. Kowalski and M. Laskowski, in *Handbook of Biochemistry and Molecular Biology*, section B, vol. 2, *Nucleic Acids*, G. D. Fasman, Ed. (CRC Press, Cleveland, 1976), pp. 508–517.
17. L. Ratner *et al.*, *Eur. J. Biochem.* **79**, 565 (1977).
18. Some of these results were presented at the symposium on Frontiers in Molecular Biology, Nutley, N.J., September 1980. Studies on the cleavage specificity of ribonuclease L are also pursued in the laboratory of I. Kerr.
19. D. H. Wreschner, J. W. McCauley, J. J. Skehel, I. M. Kerr, *Nature (London)* **289**, 414 (1981).
20. C. L. Peebles, R. C. Ogden, G. Knapp, J. Abelson, *Cell* **18**, 27 (1979).
21. We thank H. Chao for help in preparing ribonuclease L and R17 RNA, Drs. H. Osterman and J. Coleman for the bacteriophage T7 Hpa II 140b segment and T7 RNA polymerase, D. Soll and S. Sharp for useful discussions, and T. Novicki for preparing cell extracts. This work was supported by NIH research grants AI-12320 and CA-16038 and a national research service award to G.F.S.
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Inflammatory Toxin from *Mycoplasma bovis*: Isolation and Characterization

Abstract. *An inflammatory toxin was extracted from* Mycoplasma bovis *with 75 percent aqueous ethanol. The toxin is a complex polysaccharide composed of glucose, glucosamine or galactosamine, and a heptose, is heat-stable, devoid of protein and lipid, and has a molecular weight of 73,000. The holotoxin in the cell membrane is a glycoprotein; however, it is the polysaccharide portion that is toxic. This inflammatory toxin increases vascular permeability and is capable of activating complement. Infusion of 0.9 milligram of toxin into the bovine udder resulted in the characteristic eosinophilic mastitis produced by* Mycoplasma bovis.

Most cases of bovine mycoplasmal mastitis are due to *Mycoplasma bovis* (*1*). In cattle with mastitis caused by this organism, the inflammation at the acute stage is characterized by exudation of mostly eosinophils in the alveoli. Later on an interstitial reaction with eosinophils and mononuclear cells, including plasma cells and lymphocytes, develops. The chronic stage is characterized by progressive fibroplasia around ductuli and alveoli, with hypertrophy of alveolar epithelium (*2*). Some animals may return to secretion of normal-appearing milk within a few weeks; others may not do so until the next lactation, if at all. In either situation the yield of milk is far below that prior to the disease (*3*).

Of the mycoplasmas that are known pathogens, only a few appear to produce a pathogenic toxin. Relatively little is known about these toxins.

A neuroactive exotoxin is produced in cultures of *Mycoplasma neurolyticum*, which is the causative agent of "rolling disease" in mice (*4, 5*). The toxin is a protein with a molecular weight greater than 200,000. It is thermolabile, being destroyed by heating at 45°C for 15 minutes, and is destroyed by treatment with trypsin (*6*). *Mycoplasma gallisepticum* also has a neurotoxic property; however, the organism does not produce an exotoxin; all of its neurotoxicity is associated with the living organism (*7*). *Mycoplasma fermentans* possesses a toxic factor that is associated with both viable and lysed cells (*8*). When injected intraperitoneally into mice, a lethal toxicity syndrome is induced which resembles shock and is similar to that caused by endotoxins of Gram-negative organisms (*9*). Viable cells and membrane preparations of *Mycoplasma pneumoniae* induce necrosis of organ cultures of adult hamster trachea. The lesions produced include ciliostasis, vacuolization, loss of ciliated respiratory epithelial cells, disorganization, and a loss of polarity (*10*). *Mycoplasma pulmonis* and *Mycoplasma arthritidis* are toxic for mice and rats. Intravenous injection of either organism is usually lethal within 2 to 3 days (*11*). A galactan was isolated from *Mycoplasma mycoides*, the causative agent of contagious bovine pleuropneumonia, which is composed of 90 percent D-galactose, about 4 percent lipid, less than 2 percent nitrogen, and less than 0.1 percent phosphorus (*12*). Intravenous injection of this galactan into cattle causes dramatic stress: the rate of respiration increases, the animals cough and salivate, and then they collapse. The animals will, however, return to normal within a few days (*13*). Lysed *Mycoplasma bovis* cells are also toxic. Infusion of lysed *Mycoplasma bovis* into the udders of cows resulted in severe, acute mastitis characterized by an eosinophilic response (*14*).

In this report we describe the isolation and purification of an inflammatory toxin from *Mycoplasma bovis*, and discuss its biochemical and biological characteristics.

Mycoplasma bovis was cultured in Bacto PPLO broth (Difco) supplemented with 20 percent sterile swine serum at 37°C for 48 hours. The cells were centrifuged for 20 minutes at 12,500*g*, washed twice in phosphate-buffered saline (PBS), resuspended at 1000 times the original concentration, lysed by freezing and thawing, and washed twice in PBS. Lipids were extracted from this membrane preparation with a mixture of chloroform and methanol (2:1 by volume) and then with 75 percent aqueous ethanol (by volume) at 56°C with constant stirring for 1 hour. This membrane residue was hydrolyzed with the broad spectrum proteolytic enzyme, Pronase B (1.0 mg per 4.5 mg of membranes) for 18 hours at 37°C, after which it was again extracted with 75 percent aqueous ethanol. The toxin, which was extracted into this aqueous ethanol, was run on a Bio-Gel A-5M column, with PBS as the eluant. The molecular weight of the toxin was determined to be 73,000 by comparison to a curve for standard dextrans that were also run on this column.

Protein determination by the method of Lowry *et al.* (*15*) showed that the toxin contained no detectable protein. Sugar analysis by gas-liquid chromatography of trimethylsilyl derivatives (*16*) of the toxin revealed the toxin to be composed of glucose, glucosamine or galactosamine, and a heptose. Fatty acid analysis (*17*) of the toxin by gas-liquid chromatography failed to demonstrate the presence of any fatty acids, and a test for 2-keto-3-deoxyoctonate was also negative (*18*).

Since extraction of the toxin required prior digestion of the protein, it was possible that the toxin was covalently bound to a protein embedded in the membrane. We therefore solubilized *Mycoplasma bovis* membranes with sodium dodecyl sulfate and then ran them on 7.5 percent polyacrylamide gels. Scans of these gels, stained with Coomassie blue for proteins and periodic acid–Schiff for carbohydrate, revealed 32 protein bands and one carbohydrate band which corresponded to one of the protein peaks (Fig. 1). The presence of glycoproteins in

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prokaryotes is rare (*19*), and to our knowledge this is the first report of a glycoprotein in the membrane of *Mycoplasma bovis*.

Infusion of 0.9, 9.0, and 18.0 mg of polysaccharide toxin into the udders of cows resulted in increased white cell counts in the milk of, respectively, 10, 100, and 175 times that of controls infused with PBS. Rectal temperature also increased. Physical examination of the inflamed quarters showed large, firm masses, and the milk obtained from these quarters was thick and ropy. Histologic examination of the inflamed tissue revealed many eosinophils and polymorphonuclear leukocytes in the alveoli, symptoms characteristic of mycoplasmal mastitis caused by *Mycoplasma bovis* (*14*).

Intradermal injection of 0.1 mg of the polysaccharide toxin into guinea pigs resulted in red swelling reactions measuring approximately 1.0 cm in diameter approximately 2 hours after inoculation. Maximum reactions were usually reached by 6 to 8 hours and subsided completely within 24 hours. Histologic examination of the reaction sites showed edema with large influxes of eosinophils.

Cytotoxicity (*20*) and mitogenicity tests (*21*) revealed this toxin to be neither directly cytotoxic for bovine kidney cells nor mitogenic for C57L/6 mouse lymphocytes. When the toxin was assayed by the *Limulus* amoebocyte lysate test (*22*) no significant degree of gelation was observed.

An increase in vascular permeability (*23*), which was dose-dependent, occurred when this toxin was inoculated intradermally into guinea pigs. In addition, the toxin activated complement (*24*) to an extent similar to both zymosan and *Salmonella typhimurium* lipopolysaccharide.

Mycoplasma bovis toxin thus differs from bacterial lipopolysaccharide both chemically and biologically. It does not contain 2-keto-3-deoxyoctonate or lipid A, does not cause gelation of *Limulus* amoebocyte lysate, and is not mitogenic for mouse lymphocytes. And, also unlike bacterial lipopolysaccharide, it occurs in the cell as a glycoprotein.

Eosinophilic responses are usually ascribed to parasitic infestation or certain allergic reactions (*25*); however, Cohen and Sapp (*26*) demonstrated that carbohydrates such as glycogen, inulin, and dextran are themselves eosinotactic.

It is perhaps significant that *Mycoplasma bovis* toxin, being a polysaccharide, also evokes an eosinophilic response that is not observed in bovine mastitis caused by *Staphylococcus*, *Streptococcus*, or other bacterial agents. Since some dextrans activate complement by the alternative pathway (*27*), the finding that *Mycoplasma bovis* toxin activates complement suggests a mechanism by which it may cause the inflammatory reaction. Activated complement is known to increase the vascular permeability of small blood vessels, which may account for the edema, and is chemotactic for leukocytes (*28*), which may cause the accumulation of eosinophils at the reaction site. The increase in vascular permeability caused by this toxin may also play a role in clinical cases of bovine mastitis by allowing for the widespread dissemination of the organism throughout the mammary gland.

Recently, a glycoprotein (molecular weight of 60,000) was extracted with lithium diiodosalicylate from the cell membrane of *Mycoplasma pneumoniae* (*29*), and a glycoprotein was also isolated from *M. gallisepticum* (*30*). However, only the glycoprotein from *Mycoplasma bovis* has been demonstrated to be toxic.

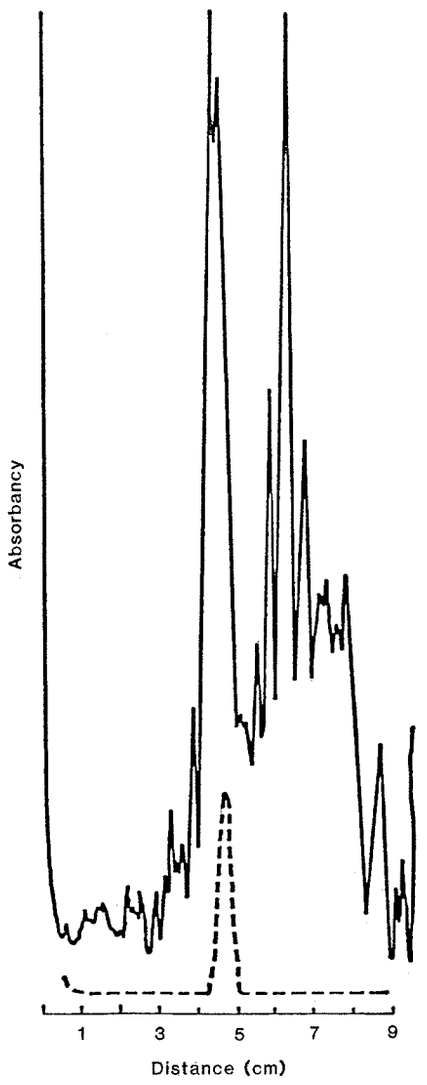

Fig. 1. Spectrophotometric scan of *M. bovis* membranes separated on polyacrylamide gels and stained with Coomassie blue for proteins (540 nm, solid line) and periodic acid–Schiff for glycoproteins (520 nm, dashed line).

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References and Notes

1. D. E. Jasper, *J. Am. Vet. Med. Assoc.* **170**, 1072 (1979).
2. E. J. van der Molen and G. Grootenhuis, *Vet. Q.* **1**, 126 (1979).
3. E. Boughton and C. D. Wilson, *Vet. Rec.* **103**, 70 (1978).
4. G. M. Findlay, E. E. Kleineberger, F. F. O. MacCallum, R. D. MacKenzie, *Lancet* **235**, 1511 (1938).
5. A. B. Sabin, *Science* **88**, 575 (1938).
6. L. Thomas, *Ann. N.Y. Acad. Sci.* **143**, 218 (1967).
7. L. Thomas, M. Davidson, R. T. McCluskey, *J. Exp. Med.* **123**, 897 (1966).
8. M. G. Gabridge and W. H. Murphy, *Infect. Immun.* **4**, 678 (1971).
9. M. G. Gabridge, G. D. Abrams, W. H. Murphy, *J. Infect. Dis.* **125**, 153 (1972).
10. M. G. Gabridge, C. K. Johnson, A. M. Cameron, *Infect. Immun.* **10**, 1127 (1974).
11. E. Kaklamanis and L. Thomas, in *Microbial Toxins*, vol. 3, *Bacterial Protein Toxins*, T. C. Montie, S. Kadis, S. Ajl, Eds. (Academic Press, New York, 1970), p. 493.
12. P. Plackett and S. H. Buttery, *Nature (London)* **182**, 1236 (1958).
13. J. R. Hudson, S. H. Buttery, G. S. Cottew, *J. Pathol. Bacteriol.* **94**, 257 (1967).
14. A. H. Mosher, W. N. Plastridge, M. E. Tourtellotte, C. F. Helmboldt, *Am. J. Vet. Res.* **29**, 517 (1968).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. G. A. Levvy, A. J. Hay, I. Strachen, *Biochim. Biophys. Acta* **222**, 333 (1970).
17. W. Stoffel, F. Chu, E. H. Ahrens, *Anal. Chem.* **31**, 307 (1959).
18. Y. D. Karklanis, J. Y. Zeltner, J. Jackson, D. J. Carlo, *ibid.* **85**, 595 (1978).
19. N. Sharon, *Complex Carbohydrates; Their Chemistry, Biosynthesis and Functions* (Addison-Wesley, London, 1975).
20. G. C. Rovozzo, R. E. Luginbuhl, C. F. Helmboldt, *Cornell Vet.* **53**, 560 (1963).
21. J. L. Ryan, R. D. Arbeit, H. B. Dickler, P. A. Henkart, *J. Exp. Med.* **142**, 814 (1975).
22. J. H. Jorgensen and R. F. Smith, *Appl. Microbiol.* **26**, 43 (1973).
23. H. Kusama and R. H. Suss, *Infect. Immun.* **5**, 363 (1972).
24. D. C. Morrison and L. F. Kline, *J. Immunol.* **118**, 362 (1977).
25. J. A. G. Rhodin, *Histology, A Text and Atlas* (Oxford Univ. Press, New York, 1974), p. 102.
26. S. G. Cohen and T. M. Sapp, *Exp. Mol. Pathol.* **2**, 74 (1963).
27. L. Pillemer, M. D. Schoenberg, L. Blum, L. Wurz, *Science* **122**, 545 (1955).
28. H. Eisen, *Immunology. An Introduction to Molecular and Cellular Principles of the Immune Responses* (Harper & Row, Hagerstown, Md., 1974), p. 522.
29. I. Kahane and H. Brunner, *Infect. Immun.* **18**, 273 (1977).
30. M. C. Goel and R. M. Lemcke, *Ann. Microbiol.* **126**, 299 (1975).
31. We thank J. L. Ryan for assistance in mitogenesis testing. A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology, Miami, Fla., 1979. Scientific Contribution No. 860, Storrs Agricultural Experiment Station.
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