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# Toxic Membrane Fractions from *Mycoplasma fermentans*[1]

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A recent isolate of *Mycoplasma fermentans* (strain K10, from human leukemic bone marrow) induced a lethal toxicity syndrome in mice. High doses of both viable and inactivated cells were toxic when injected intraperitoneally. Whole lysates and membranes from osmotically shocked cells killed mice, but cytoplasm did not. When membranes were dissolved in detergents and reaggregated by dialysis in the presence of $Mg^{2+}$, the lipid-protein complex thus formed was toxic. Lipids extracted from membranes with chloroform-methanol did not kill mice. Protein-rich fractions (obtained by reaggregation plus acetone washes or ammonium sulfate precipitation of dissolved membranes) were also not toxic. No qualitative differences in proteins from three toxic isolates and three nontoxic laboratory strains of *M. fermentans* were detectable by polyacrylamide gel electrophoresis. The toxic factor contained in reaggregated membranes was heat-stable but sensitive to Pronase, trypsin, and lipase.

Acute toxicity followed by death within several hours can be induced in laboratory animals by high doses (ca. $10^{10}$ colony-forming units, CFU) of *Mycoplasma neurolyticum*, *M. gallisepticum*, *M. arthritidis*, and *M. pulmonis* (20, 21). Lethality occurs only when viable organisms are injected intravenously into their natural animal hosts. However, the nature of the host-parasite relationships for many of the mycoplasmas pathogenic for animals has not been well defined. In 1967, Murphy et al. (7) reported a lethal toxicity induced in mice by high doses of *M. fermentans* strains recently isolated from patients. Laboratory strains of *M. fermentans*, as well as several other human species, were not toxic when tested under identical conditions (7). The syndrome, host spectrum, and pathology of the disease have been described (Gabridge, Abrams, and Murphy, J. Infec. Dis., *in press*). The disease is distinct from that induced by the toxigenic animal strains of mycoplasma. Unique features include: induction of a shock-type syndrome after intraperitoneal injection of $10^{10}$ CFU of toxic strains; hepatocellular necrosis and thymic involution; toxicity in a murine host when a human mycoplasma species is used; and death 24 to 48 hr after intraperitoneal injections of $10^{11}$ nonviable cells (M. G. Gabridge and W. H. Murphy, Bacteriol. Proc., p. 70, 1971).

This study examines the nature of the toxic factor and is an integral part of our attempt to elucidate the basic mechanism of pathogenesis in this experimental mycoplasma disease.

## MATERIALS AND METHODS

**Media.** Broth and agar media were similar to those described previously (7), but contained 10% horse serum, 10% calf serum, 500 units of penicillin G per ml, and 0.02% thallium acetate.

**Mycoplasma.** Toxic *M. fermentans* strains (K10, Z62, E10) were isolated from bone marrow specimens from leukemic patients (6). Nontoxic control strains (PDMF, WMF, NIHMF) were obtained from R. Brackett, Parke-Davis and Co., Detroit; M. Butler, Wellcome Research Laboratories, Kent, England; and N. Somerson, Ohio State University, Columbus, respectively. Strains were adapted to our media by several passages and cloned before they were stored in multiple samples at −70 C. Frozen stocks were used to inoculate 20-ml seed cultures. These were used at the log phase of growth to inoculate (by using a 1 to 2% inoculum size) large quantities of media for individual experiments.

**Mice.** BALB/wm mice (8), 1 month of age, were obtained from our own inbred colony.

**Reagents.** Phosphate-buffered saline (PBS) contained: NaCl, 8.0 g; KCl, 0.2 g; $Na_2HPO_4$, 1.15 g; $KH_2PO_4$, 0.20 g; and 800 ml of distilled water.

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After autoclaving, sterile solutions of 0.1 g of $CaCl_2$ in 100 ml of distilled water, and 0.1 g of $MgCl_2 \cdot 6\ H_2O$ in 100 ml of distilled water were added. Concentrated β-buffer contained 7.8 mM NaCl, 2.5 mM tris(hydroxymethyl)aminomethane (Tris), and 0.5 mM β-mercaptoethanol in deionized water; the final *p*H was 7.4 (11). Buffer was diluted 1:20 in deionized water before use. Enzymes were obtained from the following sources: Pronase (B grade), Calbiochem, Los Angeles; lipase (pancreatic), Nutritional Biochemicals, Corp., Cleveland; and trypsin, Difco, Detroit.

**Preparation of membranes.** Organisms at the log phase of growth (48 to 72 hr) were collected by centrifugation at 12,000 × *g* for 40 min. Cells from 6 liters of media were washed twice in PBS (*p*H 6.8), resuspended in 5 ml of 2 M glycerol (16), incubated for 30 min at 37 C, and injected into 300 ml of deionized water. This mixture was incubated for 30 min at 37 C. The cytoplasmic fraction was obtained after removing membranes and cell debris by centrifugation at 34,000 × *g* for 30 min. A purified membrane fraction was obtained as follows. Cells were disrupted as described above, and the suspension was centrifuged at 1,900 × *g* for 3 min (12). The supernatant fluid containing the membranes and cytoplasm was saved. The pellet, consisting of clumps of unbroken cells, was resuspended in 15 ml of deionized water and sonically treated for 15 min at 7 C (Raytheon sonic oscillator, 10 kc, 1.1 amps). The suspension was centrifuged at 1,900 × *g* for 3 min, and the pellet was discarded. The supernatant fluid thus obtained was pooled with the supernatant fluid from the original lysate. Membranes were collected by centrifugation at 34,000 × *g* for 30 min and washed six times in alternate washes of 0.05 M NaCl and β-buffer. The final membrane pellet was resuspended in 100 to 200 ml of β-buffer and stored at −20 C.

**Preparation of reaggregated membranes.** Membranes were dissolved in a combination of sodium dodecyl sulfate (SDS, 0.5 mg per mg of protein) and sodium deoxycholate (DOC, 2.0 mg per mg of protein) and incubated for 30 min at 37 C (17). The partially cleared solution was dialyzed at 4 C against four daily changes of 250 volumes of β-buffer containing 0.02 M $MgCl_2$ and Merthiolate (1:10,000). Flocculant material which formed in the dialysis bag was collected by centrifugation at 34,000 × *g* for 30 min. The pellet was washed six times with β-buffer containing Merthiolate (1:10,000) and was stored at 4 C.

**Isolation of lipid and protein.** Lipids were extracted with chloroform-methanol (2:1, v/v) by using the procedure of Razin et al. (14) and were washed by the method of Folch (3). Because of the toxic nature of the solvents used for lipid extractions, the following procedure was developed to suspend the extracted lipids in an innocuous vehicle. Lipids extracted as described above were heated at 55 C under a stream of $N_2$ to evaporate the solvents. The lipid residue remaining from 20 ml of membranes was dissolved in 20 ml of ethanol at 55 C (19). The ethanol was evaporated under $N_2$ to a volume of 1 ml, and 15 ml of Tween 80 (0.66%) was then added. This lipid extract was evaporated (55 C) under $N_2$ to a volume of 10 ml and was stored at 4 C.

Protein-rich fractions were obtained by: (i) reaggregation of unextracted, dissolved membranes as described above; (ii) washing reaggregated membranes with cold (4 C) 90% acetone (15); or (iii) three cycles of dissolving and precipitating membranes with detergents and ammonium sulfate at 12% saturation (17).

**Assays.** Protein was determined by the Lowry method (4) with a crystalline egg albumin standard. Lipid content was determined gravimetrically after extraction and washing. Nucleic acids were extracted by the Schneider method (18), and ribonucleic acid (RNA) was assayed by using an orcinol test (2) with a yeast RNA standard. Carbohydrate was determined by the Dubois (1) test with a reagent glucose standard.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gels containing 7.5% acrylamide, 35% acetic acid, and 5 M urea were prepared as described by Razin (13). Proteins were extracted from whole cells by using phenol-acetic acid-water (2:1:0.5, w/v/v), mixed with sucrose, and layered on top of the gel in tubes (6 by 100 mm) (13). Upper and lower (cathode) buffer reservoirs contained 10% (v/v) acetic acid. Samples containing 300 to 500 μg of protein were subjected to electrophoresis for 2.5 hr at 4 ma/tube. Gels were stained for 30 min in 1% Amido Black 10B in 7% acetic acid. After being washed in tap water, gels were kept overnight in 7% acetic acid. Complete destaining was accomplished by repeated washes in 7% acetic acid over a 48-hr period. Stained gels were scanned at 500 nm with a spectrophotometer (Gilford model 240) equipped with a linear transport.

## RESULTS

**Toxicity of cell fractions.** *M. fermentans*, strain K10, was representative of the toxic strains recently isolated from patients and consistently killed mice when injected intraperitoneally (ip) in high doses (Table 1). The $LD_{50}$, when deaths were scored 72 hr after ip injection, was $2.5 \times 10^{10}$ CFU. Previous studies have shown that only high doses of recent isolates of *M. fermentans* are toxic and that standard laboratory control strains rarely kill mice when injected in equivalent amounts (ca. $10^{10}$ CFU). Doses of $10^9$ CFU, as

TABLE 1. *Toxicity of M. fermentans fractions*

| Fraction[a] | Toxicity | |
|---|---|---|
| | Dead/total[b] | Per cent |
| Viable cells | 36/36 | 100% |
| Whole lysate | 12/12 | 100% |
| Cytoplasm | 0/36 | 0% |
| Membranes | 34/36 | 95% |

[a] Each fraction contained approximately $10^{11}$ CFU equivalents.

[b] Number of mice dead/number injected.

TABLE 2. *Relationship of composition and toxigenicity of M. fermentans membrane fractions*

| Fraction | Method of isolation | Composition (% dry wt) | | | | Toxicity[d] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Protein | Lipid | CHO | RNA | 0.5 mg | 1.0 mg | 2.0 mg | 4.0 mg |
| Lipid | Chloroform-methanol | | 100 | | | | 0/6 | 0/7 | 1/5 |
| Protein | Membranes | 60.3 | 29.0 | 9.2 | 3.6 | 2/10 | 6/14 | 8/8 | |
| | Reaggregation[a] | 72.9 | 22.2 | 3.2 | 0.7 | 0/13 | 6/15 | 15/15 | |
| | Reaggregation, acetone[b] | 92.3 | | | | | 0/6 | 0/6 | 1/6 |
| | Ammonium sulfate ppt[c] | 94.0 | | | | | | 0/16 | 0/15 |

[a] Membranes were dissolved in detergents and reaggregated when dialyzed in the presence of $Mg^{2+}$.
[b] Membranes were reaggregated and washed in cold acetone.
[c] Membranes were dissolved in detergents, and proteins were precipitated with ammonium sulfate.
[d] Number of mice dead/number injected.

well as culture supernatant fluids from both fresh isolates and control strains, are not toxic.

Suspensions of cells of strain K10 lysed by osmotic and sonic shock were lethal. Plate counts indicated that the lysis procedure killed >99.9% of the cells. When suspensions were fractionated by centrifugation, the cytoplasm failed to kill mice. However, membrane fractions were highly toxic at a dose of $10^{11}$ CFU equivalents, i.e., material collected from $10^{11}$ viable cells. Final membrane preparations contained <0.03 $LD_{50}$ per ml of viable cells. A series of control tests revealed that equivalent amounts of $\beta$-buffer were not toxic, thus indicating that the activity resided in the membranes and not the suspending medium.

**Composition of membranes.** Chemical analysis of membranes (Table 2) indicated that they consisted of 60.3 ± 6.2% protein, 29.0 ± 1.2% lipid, 9.2 ± 0.67% carbohydrate, and 3.6 ± 0.21% RNA (mean values ± standard error of the mean as per cent dry weight of K10 membranes; results are based on eight individual determinations using two different membrane preparations). These results are consistent with values reported by others (12, 14) for parasitic mycoplasma and indicate relatively little cytoplasmic contamination. Electron microscopy (*unpublished data*) revealed that the membrane preparations contained collapsed vesicles or sac-like structures with very few normal cells.

**Toxicity of membrane lipids.** Lipids, one of the major membrane components, were extracted with chloroform-methanol, resuspended in Tween 80, and injected ip into mice (Table 2). Controls indicated that the Tween 80 (1%) was nontoxic. Mice, after receiving various doses of lipids from two different membrane preparations, did not show signs of toxicity, and only one death occurred in 18 treated animals. Thus, lipids were essentially nontoxic.

**Toxicity of membrane proteins.** Proteins, the other major membrane component, were extracted by several methods. Protein-rich fractions were obtained by: (i) dissolving membranes in SDS plus DOC, followed by dialysis in the presence of $Mg^{2+}$ to form reaggregated membranes; (ii) washing reaggregated membranes in cold aqueous acetone to extract remaining lipid; and (iii) dissolving untreated membranes in SDS plus DOC and salting out protein with ammonium sulfate. Each of these methods resulted in increased protein content (Table 2).

Controls indicated that the $\beta$-buffer used to resuspend proteins was innocuous. Reaggregated membranes, enriched for protein but still containing significant amounts of lipid, induced the shock-type signs and were toxic for mice. The $LD_{50}$ (1.0 mg of protein) was about equal to that of normal membranes. Reaggregated membranes washed in acetone failed to kill mice. Likewise, ammonium sulfate-precipitated proteins were nontoxic at doses up to 4 mg/animal. Thus, neither purified protein or lipid fractions alone were toxic, whereas reaggregated membranes containing protein and lipid (plus minor carbohydrate and RNA components) were quite toxic. The finding that protein was not the sole determinant of toxicity was consistent with electrophoretic comparisons (*see below*) of nontoxic laboratory strains and toxic isolates of *M. fermentans*.

**Electrophoretic comparison of toxic and nontoxic strains.** Toxic (K10, E10, Z62) and control (PDMF, WMF, NIHMF) strains of *M. fermentans* were examined by disc electrophoresis in polyacrylamide gels. All six strains were grown in media prepared from the same lots of serum, yeast extract, and broth base to minimize variation. Comparisons (Fig. 1) of gel patterns obtained from the same electrophoresis run failed to reveal a protein fraction distinctive of the toxigenic strains. Some protein bands differed slightly in intensity, but essentially all of the major pro-

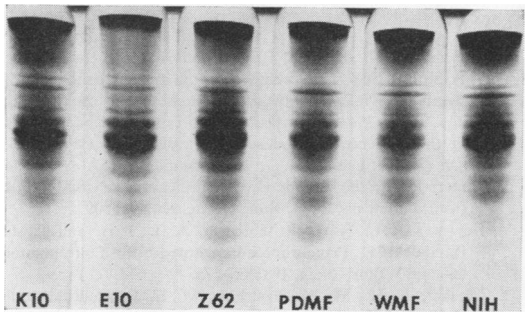


FIG. 1. *Disc electrophoresis of toxic (K10, E10, Z62) and control (PDMF, WMF, NIH) strains of Mycoplasma fermentans in polyacrylamide gels. Gels were stained for 30 min with 1% Amido Black 10B and destained by 48 hr in 7% acetic acid. Anode was at the top.*

tein species detectable under the test conditions were present in both toxic and control strains. The similarities of the six protein patterns also were evident in densitometer tracings (Fig. 2) done from stained gels.

**Nature of toxic factor in reaggregated membranes.** Reaggregated membranes consisted of 72.9 ± 5.7% protein, 22.2 ± 2.5% lipid, 3.2 ± 0.35% carbohydrate, and 0.7 ± 0.08% RNA (mean, ± standard error of the mean, from three to six determinations from three different membrane preparations). Plate counts revealed that no viable cells were present.

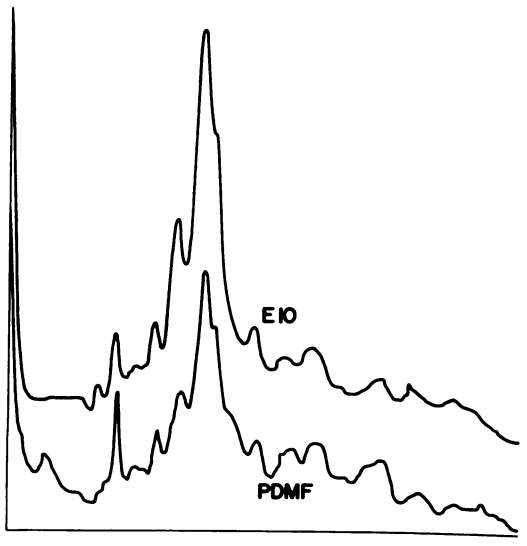


FIG. 2. *Densitometer tracings of polyacrylamide gels from representative toxic (E10) and control (PDMF) strains of Mycoplasma fermentans. Relative intensity of band staining is on the ordinate; distance from top of gel (left) is on the abcissa.*

TABLE 3. *Stability of toxic factor in reaggregated membranes*

| Treatment | Fraction of mice killed |
|---|---|
| Control[a] | 9/10 |
| Heat (55 C, 15 min) | 9/10 |
| Pronase (250 μg/ml, 37 C, 20 hr) | 0/10 |
| Trypsin (250 μg/ml, 37 C, 15 min) | 2/10 |
| Lipase (100 μg/ml, 37 C, 30 min) | 0/10 |

[a] Untreated reaggregated membranes. Other controls indicated that Pronase, trypsin, and lipase were not toxic and that incubation at 37 C did not destroy toxicity.

The relative stability of the toxic factor in reaggregated membranes was determined by thermal and enzymatic inactivation (Table 3). The toxic factor withstood an exposure to 55 C for 15 min with no loss in toxicity. It was quite sensitive to trypsin, with 2/10 deaths from trypsin-treated reaggregated membranes, compared to controls with 9/10 deaths. Both lipase and Pronase completely abolished toxicity. The toxic factor thus was heat-stable and sensitive to lipid- and protein-degrading enzymes.

## DISCUSSION

The lethal toxicity of recently isolated strains of *M. fermentans* for mice has been introduced as a new experimental disease, based on the effective route of injection of organisms, signs of disease, host spectrum, and histopathology. The disease in question has no known counterpart in human infections, because its induction, like other mycoplasma-induced lethal toxic diseases, requires an extremely high number of organisms. It is distinct from the other animal infections, however, because the induction of lethal toxicity syndromes by *M. neurolyticum*, *M. gallisepticum*, *M. pulmonis*, and *M. arthritidis* requires viable organisms. Sonic lysates (20) and freeze-thaw extracts (21) of organisms are not toxic. Our results indicate that *M. fermentans* is toxic when nonviable cells (approximately $10^{11}$ CFU equivalents) are injected ip into mice. This dose corresponds to the titer presumed to arise in vivo from two to three replicative rounds by a normal $LD_{50}$ inoculum of viable cells (M. G. Gabridge, Ph.D. thesis, Univ. of Michigan, 1971).

When osmotically shocked cell preparations of *M. fermentans* isolates were fractionated, the cytoplasm and buffer controls were not toxic, whereas the membrane fraction consistently killed mice. Membranes, consisting mainly of lipid and protein, and reaggregated membranes,

which have an increased protein content, both induced the lethal toxicity syndrome. Toxicity was not observed when purified lipids or proteins were used, and the lethal effect thus was limited to membrane preparations containing both lipid *and* protein. To our knowledge, this is the first instance of a pathologic response attributed to a mycoplasma cell fraction.

The toxic factor in reaggregated membranes was heat-stable, but sensitive to trypsin, Pronase, and lipase. This toxin is therefore distinct from the *M. neurolyticum* toxin (22), which is a thermolabile protein. It is also distinct from the *M. mycoides* toxin, which is a polysaccharide (10) capable of inducing allergic (5) reactions, and from the toxin of *M. agalactiae*, which can induce endotoxin reactions (23). Piercy (9) recently noted that heat-inactivated *M. mycoides* preparations could induce synovitis when injected into limb joints of calves. He postulated that the toxicity of nonviable cells was due to some undefined, integral part of the cell "analogous to the endotoxins of gram negative bacteria."

The toxin of recent isolates of *M. fermentans* is similarly contained in a cell component, identified in this instance as the membrane fraction. Lethal toxicity induced by *M. fermentans* evidently results when cells in the inoculum replicate until some critical mass of membranes has accumulated, thereby inducing the shock-type syndrome. While the precise mechanism of pathogenesis must await final purification of the toxic product, the data just presented should serve as an adequate basis for future studies on mycoplasma-induced lethal toxicity. While the lethal toxicity syndrome does not directly reflect any known natural infection, studies of this artificial system may have implications for human disease such as primary atypical pneumonia. One could speculate that a focal accumulation of organisms on the respiratory epithelium could have a direct toxic effect attributable to a mycoplasma membrane component similar to the one described in this report.

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