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# MYCOPLASMA PNEUMONIAE: A PROKARYOTE WHICH CONSUMES OXYGEN AND GENERATES SUPEROXIDE BUT WHICH LACKS SUPEROXIDE DISMUTASE

Robert E. Lynch and Barry C. Cole

Department of Medicine, University of Utah College of Medicine

Salt Lake City, Utah 84132

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Summary: Superoxide dismutase and catalase were not detected in M. pneumoniae and several other species of Mycoplasma some of which consume oxygen and secrete $H_2O_2$. M. pneumoniae in suspension formed $O_2^-$ in the presence of NADH and flavins and extracts of M. pneumoniae formed $O_2^-$ in the presence of either NADH or NADPH. The lack of superoxide dismutase in M. pneumoniae could not be attributed to superoxide dismutase in the complex medium in which the organ isms were grown because organisms grown in medium in which the superoxide dismutase had been inactivated by heat still contained undetectable amounts. Mycoplasmas appear to be an exception to the rule that organisms which consume $O_2$ synthesize superoxide dismutase.

Superoxide dismutases, a group of metalloenzymes which catalyze the disproportionation of superoxide anions to $H_2O_2$ and $O_2$, are thought to be indispensable to aerobic creatures which reduce $O_2$. Those which do not reduce $O_2$ synthesize little or no superoxide dismutase (1). Species which, though aerotolerant, fail to reduce $O_2$ also lack superoxide dismutase. When facultative anaerobes shift from anaerobiosis to aerobic metabolism, the synthesis of superoxide dismutase is induced (2). From these and other observations McCord, Fridovich, and co-workers advanced the hypothesis that $O_2^-$ was an inevitable by-product of the biologic reduction of $O_2$ (summarized in ref. 3). They proposed that $O_2^-$ was an agent of cytotoxicity and that the biologic function of superoxide dismutase was to prevent the potentially lethal effects of $O_2^-$ by scavenging it. However, the blame for apparent cytotoxicity of $O_2^-$ has shifted to species which may arise from $O_2^-$ and its spontaneous dismutation product $H_2O_2$, namely OH. and $^1O_2$. However, an argument which challenges the importance of superoxide dismutase as an indispensable defense against the

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toxicity of oxygen has been based on the presence of superoxide dismutase in anaerobic bacteria (4, evidence contained in 5-8).

The mycoplasmas are prokaryotes which possess a genome one-half the size of the smallest bacterium and which grow only in very complex medium. Many of these organisms which exist in nature in a parasitic relationship with a eukaryotic host consume $O_2$ (9), and some form $H_2O_2$ (10-13). We therefore sought to determine whether they possess those enzymes which seem to protect other cells from the toxic effects of the reactive intermediates produced when $O_2$ is reduced by one or two electrons to form $O_2^-$ and $H_2O_2$ rather than by 4 electrons to form water. For comparison we also studied Acholeplasma laidlawii, a related member of the class Mollicutes which is less nutritionally demanding and possesses a larger genome than the mycoplasmas.

## Materials and Methods

Reagents. Cytochrome c type III (Sigma), nitroblue tetrazolium (Sigma), xanthine (Sigma), bovine erythrocyte superoxide dismutase (Truett, Diagnostic Data), flavin mononucleotide (Sigma), flavin adenine dinucleotide (Sigma), riboflavin (Nutritional Biochemicals), were obtained from commercial sources. Xanthine oxidase was purified from unpasteurized cream avoiding proteolysis (14).

Assays. Superoxide dismutase was assayed by its ability to inhibit the reduction of cytochrome c mediated by $O_2^-$ generated by the enzymic action of xanthine oxidase (15). Acrylamide gels were stained for superoxide dismutase activity (16), for catalase activity (17), and peroxidase activity (17) after electrophoresis by established methods. Catalase was assayed in solution by the rate of change in absorbance at 240 nm of a solution of $H_2O_2$ 20 mM in 16.7 mM potassium phosphate buffer pH 7.0 (18). Peroxidase activity was determined with o dianisidine as chromogenic substrate (19). The production of superoxide was estimated from the rates of reduction of cytochrome c ($5 \times 10^{-5}$ M) determined spectrophotometrically at 550 nm or of nitroblue tetrazolium ($4.1 \times 10^{-4}$ M) at 560 nm. The portion attributed to reduction by $O_2^-$ was the part which superoxide dismutase inhibited. Bands of NAD(P)H - NBT oxidoreductase activity in samples electrophoresed in acrylamide gels were located by soaking the gels first in a solution of nitroblue tetrazolium 200 mg/dl for 45 min at room temperature in the dark followed by soaking the gel in a solution of either NADH or NADPH (1 mM) in 50 mM potassium phosphate pH 7.8, $10^{-4}$ M EDTA in the dark until good contrast is obtained. When inhibition of the reduction of NBT by $O_2^-$ was attempted superoxide dismutase (10 µg/mL) was included in the solution of NBT. NADH and NADPH oxidase activities were measured spectrophotometrically at 340 nm at 25 degrees in solutions of 33 uM substrate in 0.15 M NaCl, 10 mM potassium phosphate pH 7.65. Protein was determined by the method of Lowry (20). Acrylamide gel electrophoresis was by a modification (21) of the methods of Davis (22) and Maurer (23).

Mycoplasma strains and culture procedures. The sources of the mycoplasmas used in this study are as follows: M. fermentans (M713), M. gallisepticum (M722), M. hominis (M711) and A. laidlawii type A (M728), M.F. Barile, Bureau of Biologies, NIH, Bethesda, MD: M. pneumoniae strains 1428 and TW 48-5,

J.G. Tully, Laboratory of Infectious Diseases, NIH, Bethesda, MD; M. pneumoniae (Josè), N.L. Somerson, Ohio State University, Columbus, Ohio; M. pulmonis, JB, D. Taylor-Robinson, Harrow, England; M. arthritidis (158 P1OP9), B.C. Cole, University of Utah, Salt Lake City, UT; A. laidlawii (T305), R.N. Gourlay, ARC Inst. Res. Anim. Dis., Compton, England.

The M. pneumoniae strains were grown as lawns in 850 $cm^2$ roller bottles (Falcon, 3027) in Difco PPLO broth supplemented to final concentrations of 40% (vol/vol) unheated horse serum, 1% (wt/vol) glucose, 10% (vol/vol) fresh yeast extract, 0.01% (wt/vol) NAD and 1000 units/ml penicillin (24). The lawns were harvested by agitation with glass beads. All other mycoplasmas were grown in suspension cultures. Mycoplasma arthritidis and M. hominis were cultured in Difco PPLO broth with supplemented 12 to 15% (vol/vol) inactivated horse serum, 5% (vol/vol) fresh yeast extract, 0.5% (wt/vol) L arginine HCl and 1000 units/ml penicillin. M. gallisepticum and M. fermentans were grown in broth supplemented with 20% (vol/vol) heated horse serum, 7.5% yeast extract, 0.5% glucose and 1000 units/ml penicillin, M. pulmonis received an additional supplement of 0.01% NAD. Acholeplasma laidlawii was cultured in Tryptose phosphate broth (Difco) supplemented with 2% (vol/vol) Difco PPLO serum fraction and 1000 units/ml penicillin. The identity of each mycoplasma species was confirmed using the growth inhibition test (25) and by epi-immunofluorescence (26) employing reference antisera.

## Results

Acholeplasma laidlawii produced superoxide dismutase confirming recent studies (27) but catalase was not found (Table 1). In contrast, neither superoxide dismutase nor catalase were found in any of the Mycoplasma species tested. The lack of production of catalase and superoxide dismutase by organisms which live in air and reduce $O_2$ is surprising. Possibly the conditions of growth were not suitable for the synthesis of superoxide dismutase. It might be argued that there was insufficient $O_2$ present in the cultures to promote superoxide dismutase production. However, it should be noted that M. pneumoniae did not produce this enzyme when grown under aeration using roller bottles whereas A. laidlawii did produce superoxide dismutase under conditions of lesser aeration (still culture).

The possibility that the growth medium contained sufficient superoxide dismutase to render the synthesis of superoxide dismutase by mycoplasmas unnecessary was considered. By autoclaving the yeast extract and the base broth, heating the horse serum at 94.5°C, and removing the material coagulated by heat by centrifugation, a medium was obtained in which we could not detect either superoxide dismutase or catalase. In the extracts prepared from M. pneumoniae grown in this medium we found no superoxide dismutase

Table I

ACTIVITIES OF SUPEROXIDE DISMUTASE AND OF CATALASE IN EXTRACTS OF MYCOPLASMA SPECIES AND OF ACHOLEPLASMA LAIDLAWII

| SPECIES | SUPEROXIDE DISMUTASE ASSAYED IN: | | | CATALASE ASSAYED IN: | | |
|---|---|---|---|---|---|---|
| | SOLUTION | | GELS | SOLUTION | | GELS |
| | Specific Activity Units/mg | µg Protein Assayed | | Specific Activity units/mg | µg Protein Assayed | |
| M. hominis | 0.04* | 1880 | - | 0 | 940 | - |
| M. pulmonis | 0.1 * | 740 | - | 0 | 370 | - |
| M. arthritidis | 0.02* | 1780 | - | 0 | 890 | - |
| M. gallisepticum | 0.1 | 1500 | - | 0 | 750 | - |
| M. fermentans | 0.2 | 1460 | - | 0 | 730 | - |
| M. synoviae | 0.01* | 500 | - | 0 | 250 | - |
| A. laidlawii | | | | | | |
| Strain M728 | 32.8 | 38 | + | 0 | 1900 | - |
| Strain 1305 | 32.9 | 32 | | 0.004 | 1580 | |
| M. pneumoniae | | | | | | |
| Strain José | 0, 0.01* | 540-730 | - | 0 | 270-730 | - |
| Strain TW 48-5 | 0* | 200 | | 0 | 100 | |
| Strain 1428 | 0* | 478 | | 0 | 236 | |

The activity of superoxide dismutase was measured by its ability to inhibit the reduction of oxidized cytochrome c mediated by $O_2^-$.

* Values marked with an asterisk were not significantly different from the uninhibited rate and should probably be considered zero.

(<0.02 µg) or catalase. Our assays would have detected <5.4 ng superoxide dismutase and <5.7 ng catalase.

One explanation for the absence of SOD from M. pneumoniae is that they make no $O_2^-$. Consequently the ability of extracts of M. pneumoniae prepared in a French press, as well as the sediment obtained by 39,000 xg centrifugation, and the cytosol after removal of membranes to generate superoxide from NADH was assessed. The production of $O_2^-$ was determined from the portion of the reduction of oxidized cytochrome c, measured spectrophotometrically at 550 nm, which superoxide dismutase inhibited. In the presence of NADH cytochrome c was reduced. The majority of the reduction was attributable to $O_2^-$ and most remained in the supernate after centrifugation. Near-

Table II

Superoxide production from NADH[b] in lysate, cytosol, and membrane fractions of M. pneumoniae

| "FRACTION" | RATES OF REDUCTION OF CYTOCHROME c[a] | | PERCENT INHIBITION |
|---|---|---|---|
| | WITHOUT SOD | WITH SOD[c] | BY SOD[c] |
| A. Lysate (French press) | 0.42 | 0.16 | 62 |
| B. Cytosol (Supernate from A) | 0.44 | 0.13 | 71 |
| C. Membranes | 0.136 | 0.064 | 53 |

a $A_{550}$/min/mL

b 33 μM

c 6.7 μg/mL

A washed suspension of M. pneumoniae in 0.15M NaCl, 10 mM potassium phosphate pH 7.65 was lysed by passage through a French pressure cell and divided into two equal parts. One, the lysate, was not processed further. The other was subjected to centrifugation for 30 min at 4 degrees at 39,000 g. The resulting supernatant was the cytosol (B). The resulting pellet was resuspended in a volume of 0.15M NaCl, 10 mM potassium phosphate pH 7.65 equal to ½ the original volume of the lysate. Assays were performed in 0.15MNaCl, 10 mM potassium phosphate pH 7.65 at 25°.

ly all of the activity in the oxidation of NADH (95%) and NADPH (89%) was also soluble (Table II).

The production of $O_2^-$ was also measured in suspensions of organisms employing both cytochrome c and NBT as spectrophotometric indicators of $O_2^-$. The latter was considered to be present when reduction of either was inhibited by superoxide dismutase. An experiment with NBT (Table III) indicated that production of $O_2^-$ occurred in suspensions of cells as well as in extracts. In the suspensions of cells, as was later shown in extracts, flavins markedly stimulated the formation of $O_2^-$ in the presence of NADH. In all cases a substantial fraction of the reduction of NBT was attributable to $O_2^-$. Although some late lysis occurred in the suspension of organisms, 80% of the activity in the reduction of NBT in the presence of NADH and FMN was sedimentable at 39,000 g for 30 min whereas in lysates 95% of the NADH oxidase activity was soluble. When cytochrome c was used to detect $O_2^-$ in sus-

Table III

PRODUCTION OF SUPEROXIDE BY SUSPENSIONS OF MYCOPLASMA PNEUMONIAE

| SUBSTRATES | | | RATE OF NBT REDUCTION[a] | | % INHIBITION BY SOD[b] |
|---|---|---|---|---|---|
| NADH (100 uM) | FAD (40 uM) | FMN (40 uM) | NO SOD | WITH SOD[b] | |
| + | + | | 0.061 | 0.019 | 62 |
| + | | | 0.029 | 0.012 | 68 |
| | + | | 0.003 | 0.002 | - |
| + | | + | 0.110 | 0.043 | 57 |
| + | | | 0.028 | 0.019 | 62 |
| | | + | 0.001 | - | - |

Mycoplasma pneumoniae, thrice washed in 0.15 M NaCl, 10 mM potassium phosphate pH 7.65, was incubated at 25 degrees in a solution containing 4.1 x $10^{-4}$ M NBT, 0.15 M NaCl, 10 mM potassium phosphate pH 7.65 with the indicated additions, and the absorbance at 560 nm was recorded with in time in a Cary 118C recording spectrophotometer with the cuvette positioned immediately in front of the photomultiplier tube to minimize the effect of light scattering by the turbid suspensions.

a $A_{560}$/min/mg protein

b SOD = superoxide dismutase, 33 ug/mL

pensions of organisms, inhibition by superoxide dismutase was less (27-40% in different experiments).

The NADH oxidase of mycoplasmas can be stained in acrylamide gels by soaking the gel in a solution of nitroblue tetrazolium (NBT) and then in a solution of NADH. The oxidase is detected as it removes electrons from NADH and transfers them to NBT, thereby forming a dark blue deposit of insoluble formazan. If the NADH oxidase does not reduce NBT directly but instead first forms $O_2^-$ which reduces NBT, then superoxide dismutase should inhibit the formation of the blue formazan. To test this possibility soluble supernatants, prepared from disrupted M. pneumoniae grown in roller bottles, were electrophoresed into acrylamide gels. Two identical gels were stained for NADH oxidase activity. One gel was stained in the usual way and the other was soaked first in a solution which contained NBT and superoxide dismutase (10 µg/mL). The blue band of formazan deposited by the NADH oxidase (Rm 0.27) was diminished in the gels treated with superoxide dismutase. This suggests that NADH oxidase is one source of $O_2^-$ in M. pneumoniae.

## Discussion

If mycoplasmas produce $O_2^-$ how can they survive without the synthesis of superoxide dismutase? The production of $O_2^-$ might be compartmentalized protecting the vulnerable parts of the cell. Conceivably mycoplasmas could have evolved a composition resistant to the attack by $O_2^-$ and its progeny. In nature these organisms might even avail themselves of the superoxide dismutase and catalase of their eukaryotic hosts: the close association of mycoplasmas with animal cells is well known and supports this notion (28). Other pathways of detoxification of either $O_2^-$ or $H_2O_2$ are possible. We detected no activity of an o-dianisidine peroxidase in extracts of M. pneumonia. It remains to be determined whether glutatione peroxidase or an NADH peroxidase (29) or still another pathway of inactivation of peroxide (11) or of superoxide exists.

The pathogenic role of $O_2^-$ in disease processes remains to be established although preliminary studies by us have failed to show that the lytic effects of M. pneumoniae on guinea pig erythrocytes or human lung fibroblasts can be abrogated in the presence of superoxide dismutase.

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