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Inhibition of Host Cell Catalase by *Mycoplasma pneumoniae*: A Possible Mechanism for Cell Injury

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This study demonstrates that viable *Mycoplasma pneumoniae* cells inhibit catalase activity in several types of intact human cells as well as in solution. Human erythrocyte catalase was inhibited up to 72%, and the inhibition of catalase in human cultured skin fibroblasts, lung carcinoma epithelial cells, and ciliated epithelial cells from human nasal polyps ranged between 75 and 80%. UV light-killed mycoplasmas failed to inhibit catalase activity both in intact cells and in vitro. After *M. pneumoniae* infection of human cultured skin fibroblasts, the level of malonyldialdehyde, an indicator for membrane lipid peroxidation, was 3.5 times higher than in control fibroblasts. Virulent *M. pneumoniae* completely inhibited catalase activity in solution, whereas the nonvirulent strains had a lesser ability to inhibit catalase activity. These findings suggest that as a result of host cell catalase inhibition by *M. pneumoniae*, the toxicity of the hydrogen peroxide generated by the microorganism and the affected cell is enhanced, thereby inducing host cell damage.

Mycoplasma pneumoniae is a human pathogen causing mostly respiratory tract infections (5, 8, 10, 11). The adherence of the organisms to the epithelial lining of the respiratory tract (5, 12, 16) is known to be a prerequisite for induction of the disease, yet the actual mechanism of *M. pneumoniae* pathogenicity is still unclear (5, 19). Hydrogen peroxide produced by *M. pneumoniae* as a respiratory end product has been suspected to be a virulence factor in *M. pneumoniae* infections (9, 20), but the existence of tissue cell catalase questions its effectiveness in causing injury to the host cell. We have postulated that owing to the mycoplasma infection, host cell catalase is inhibited, failing to decompose the hydrogen peroxide continuously produced both by the host cell and by the intimately adhering mycoplasma. As a result, damage to the host cell is induced. In our work, this hypothesis was studied by examining the effect of *M. pneumoniae* on the activity of catalase in intact human cells and in solution.

MATERIALS AND METHODS

Organisms and growth conditions. An *M. pneumoniae* virulent strain (M129-B16) and its nonvirulent, nonhemadsorbing mutants (HA-3, HA-7) (obtained from J. B. Baseman, University of Texas at San Antonio) were cultured in Roux bottles or petri dishes (90 by 15 mm) containing Hayflick medium (15) supplemented with 0.2% glucose, 10% yeast extract, 10% heat-inactivated horse serum (56°C, 1 h), 600 U of penicillin G per ml, and 0.1 μCi of [^{3}H]palmitate per ml for 3 to 4 days at 37°C. The inoculum was 5 to 10% (vol/vol).

UV irradiation. *M. pneumoniae* lawns attached to the bottoms of petri dishes (90 mm in diameter) or suspensions (3 ml in 35-mm petri dishes) in phosphate-buffered saline (PBS; pH 7.2) were irradiated for 10 min under a UV lamp (Mineralight; model V41; 0.25 A; Ultraviolet Products Inc., San Gabriel, Calif.) at a distance of 5 cm between the dish and the light source. A viability test (CFU counting) indicated 100% killing.

Membrane preparation. *M. pneumoniae* membranes were isolated from cells disintegrated by sonication as previously described (1).

Erythrocytes (RBC). RBC were separated (26) from fresh heparinized blood (blood type O) obtained from normal donors. The RBC were suspended to 2% (vol/vol) in PBS, kept at 4°C, and diluted to 0.008% (vol/vol) in PBS before the experiment.

Infection of human RBC by *M. pneumoniae*. *M. pneumoniae* cultures grown in petri dishes were washed three times with PBS. Fifteen milliliters of 0.008% RBC in PBS was added to each dish. The plates were incubated for different periods of time up to 24 h at 37°C without shaking. When incubations lasted over 4 h, RBC preparations and adherence assays were conducted under sterile conditions. At the end of the incubation, excess RBC were removed by flushing the plates twice with PBS. Cells were scraped off with a rubber policeman into PBS, and free mycoplasma cells were separated from the mycoplasmas which attached to RBC by centrifugation (10 min at 480 × *g*). The pellet was resuspended in PBS and assayed for catalase activity and hemoglobin (Hb). The amount of radioactivity originating from the attached mycoplasmas was assessed by scintillation spectrometry (2).

Human fibroblasts and lung carcinoma epithelial cell cultures. Normal skin fibroblasts and human lung carcinoma epithelial cells (A427, obtained from the Naval Biosciences Laboratory, Naval Supply Center, Oakland, Calif.) were cultured in tissue culture flasks (Falcon Plastics, Oxnard, Calif.) containing 5 ml of nutrient mixture F-10 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO), 0.34% glucose, 0.2 U of insulin (Nordisk Insulin Laboratorium, Copenhagen) per ml, 1 mM glutamine, 100 μM nonessential amino acids (GIBCO), 100 U of penicillin G per ml, and 100 μg of streptomycin per ml (pH 7.4) and incubated at 37°C under humidified air–5% CO_2. The cells were grown in a monolayer and used between passages 2 and 6.

Ciliated epithelial cell cultures. Ciliated epithelial cells from nasal polyps were cultured as previously described (25a) on plates without extracellular matrix. Under these conditions, the cells retained their ciliary activity. The explant tissue pieces were removed before infection experiments.

Infection of cell cultures by *M. pneumoniae*. A lawn of virulent *M. pneumoniae* (M129-B16) cultured in Roux bottles was washed three times with 0.25 M sterile NaCl and scraped off with a rubber policeman into sterile saline. The *M. pneumoniae* suspension in saline (1 ml, 2 mg of cellular protein) was added to each flask containing a monolayer of cells which had been previously washed twice with sterile saline. Afterwards, 3 ml of nutrient mixture F-10 supplemented with 1 mM glutamine and 3% fetal bovine serum (pH 7.4) was added, and the cells were incubated at 37°C under 5% CO_2 for up to 24 h. Incubations were not carried out for longer periods since after 24 h the infected cells tended to peel off. After incubation, the cells were washed twice with saline, scraped off with a rubber policeman into saline solution, and assayed for their catalase activity.

Catalase activity in intact cells. Cell catalase activity was measured at 30°C, using an oxygraph with a 2-ml vessel and an oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio). Cell suspension prepared as described above (1 ml) was added to the oxygraph vessel. When equilibrium was reached, hydrogen peroxide was added to a final concentration of 8 mM, and catalase-accelerated hydrogen peroxide degradation was recorded. Hydrogen peroxide nonenzymatic degradation was also recorded, and the catalase-mediated degradation was corrected accordingly. (Hydrogen peroxide nonenzymatic degradation was about 1% of the catalase-mediated reaction.) Catalase-specific activity was defined for RBC as nanomoles of O_2 generated per minute per microgram of hemoglobin and for cells in culture as nanomoles of O_2 generated per minute per microgram of cell protein.

Lysosomal enzyme activities. Lysosomal enzyme activities in the cultured cells were assayed as previously described (27), using the appropriate 4-methylumbelliferyl substrates.

Lipid peroxidation assay. Lipid peroxides in normal and *M. pneumoniae*-infected fibroblasts were estimated as malonyldialdehyde (MDA; a secondary breakdown product of fatty acids), using the thiobarbituric acid method previously described by Buege and Aust (4).

Catalase inhibition assay in solution. *M. pneumoniae* virulent strain (M129-B16) and nonvirulent, nonhemadsorbing mutants (HA-3, HA-7) grown in Roux bottles were washed three times with 0.25 M NaCl and harvested with a rubber policeman into PBS. Catalase solution (from bovine liver; Sigma Chemical Co., St. Louis, Mo.) in PBS was added (10^{-1} to 10^{-3} U/ml) to the mycoplasma suspension, which was then incubated at 37°C for 30 min. Cells were removed from the assay mixture by centrifugation at 27,000 × *g* for 20 min at 4°C. Catalase activity in the supernatants was measured in duplicate by UV spectrophotometry (at 240 nm), using 20 mM hydrogen peroxide as the substrate, and was determined as the change in absorbance at 240 nm per minute per milliliter.

Hemoglobin determination. RBC were lysed with 2 mM NH_4OH. The lysate was centrifuged for 15 min at 34,000 × *g*. The hemoglobin concentration in the supernatant was calculated from its light absorbance at 412 nm as compared with human hemoglobin standard.

Protein determination. Protein was determined by the method of Lowry et al. (21).

RESULTS

Effect of virulent *M. pneumoniae* on RBC catalase activity. Human RBC were allowed to adsorb to a lawn of virulent *M. pneumoniae* for up to 24 h, and the RBC catalase activity was measured by following hydrogen peroxide-dependent oxygen production. The RBC catalase activity (averaging 7 nmol of O_2 generated/min per μg of Hb) decreased by 55% within 4 h of incubation (Fig. 1), whereas the enzyme activity of the nonadsorbed RBC in the *M. pneumoniae*-containing plates remained unchanged throughout the experiment. When the amount of *M. pneumoniae* organisms in the petri dishes increased (as evaluated by radioactivity measurements), catalase activity of RBC attached to these plates for 24 h decreased by 72 ± 3.5%. UV-irradiated mycoplasmas failed to inhibit RBC catalase activity, indicating that mycoplasma viability is a crucial factor in determining the inhibition phenomenon in intact RBC. We investigated the possibility that the observed decrease in oxygen generation by the RBC catalase was due to shunting its catalatic reaction to its peroxidative reaction (6) by following catalase inhibition as a function of hydrogen peroxide concentration. (At high hydrogen peroxide levels, the peroxidative reaction rate is constant.) The inhibition of RBC catalase in the presence of *M. pneumoniae* was found to be independent of the hydrogen peroxide concentration, indicating that catalase inhibition followed by oxygen production rate does not reflect competition between the peroxidative and catalatic reactions.

Effect of virulent *M. pneumoniae* on catalase activity of cells in culture. The phenomenon of catalase inhibition could be demonstrated in human skin fibroblasts, lung carcinoma epithelial cells, and ciliated epithelial cells. Catalase

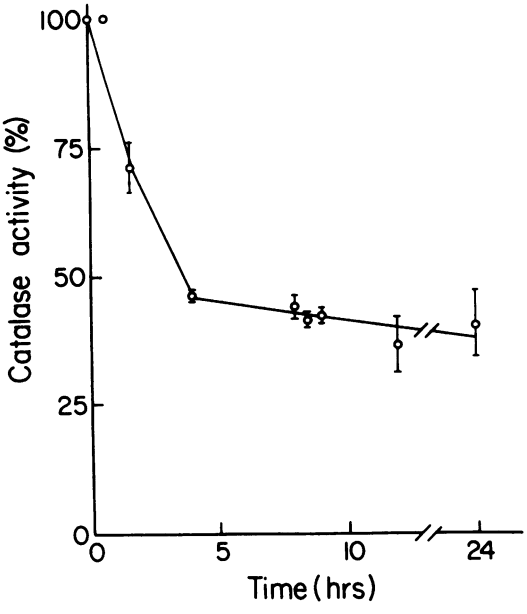

FIG. 1. Inhibition of human RBC catalase activity by viable virulent *M. pneumoniae*. Human RBC were infected for up to 24 h by viable *M. pneumoniae*, and their catalase activity was measured as described in the text. The results (mean ± standard deviation [bars] of three different experiments) are expressed as percentage of control (3.1 to 10.9 nmol of O_2 generated/min per μg of Hb).

activity in each type of cultured cell tested was significantly reduced by 70 to 80% of its initial value after 20 h of incubation with virulent *M. pneumoniae* (Fig. 2). The kinetics of catalase inhibition in cultured skin fibroblasts were similar to those found in RBC.

Lysosomal enzyme activities in fibroblasts and ciliated epithelial cells infected with virulent *M. pneumoniae*. The possibility that catalase inhibition reflects a general cytopathic effect was studied by measuring other cellular enzymatic activities in *M. pneumoniae*-infected fibroblasts and ciliated epithelial cells. Since catalase is a peroxisomal enzyme, i.e., located in single-membrane-limited organelles, we selected as controls lysosomal hydrolases, found in similar structures. The lysosomal enzyme activities measured in *M. pneumoniae*-infected and uninfected cells were similar, in contrast to the 70% decrease in catalase activity of these cells (Table 1).

Lipid peroxidation in fibroblasts infected with virulent *M. pneumoniae*. We assumed that inhibition of cellular catalase could hamper the ability of a cell to decompose intracellular hydrogen peroxide. This, in turn, would cause an oxidative stress on cell constituents, among them the membrane lipids (23). Hydrogen peroxide and oxygen radicals have been shown to peroxidize unsaturated fatty acids (4), which is followed by formation of MDA as a secondary breakdown product. Therefore, MDA was assessed as an indicator of lipid peroxidation in human fibroblasts infected with *M. pneumoniae* for 20 h. The MDA level in infected cells was 3.5 times higher than in control cells (Table 2).

Effect of virulent and nonvirulent *M. pneumoniae* on catalase activity in solution. *M. pneumoniae* mutants HA-3 and HA-7 are nonvirulent owing to the loss of adherence capacity. Therefore, we could test their ability to inhibit catalase activity only in an in vitro experimental system in which adherence is not necessary.

Catalase in solution was incubated with *M. pneumoniae* virulent (M129-B16) and nonvirulent (HA-7, HA-3) strains, and the enzyme activity was measured after removal of the organisms. The virulent strain (1.5 mg of protein per ml) totally inhibited catalase activity within 30 min of incubation, whereas the nonhemadsorbing mutants were less inhibitory (Fig. 3).

The possibility that catalase inhibition correlates with the viability of *M. pneumoniae* cells was studied by killing the organisms by using

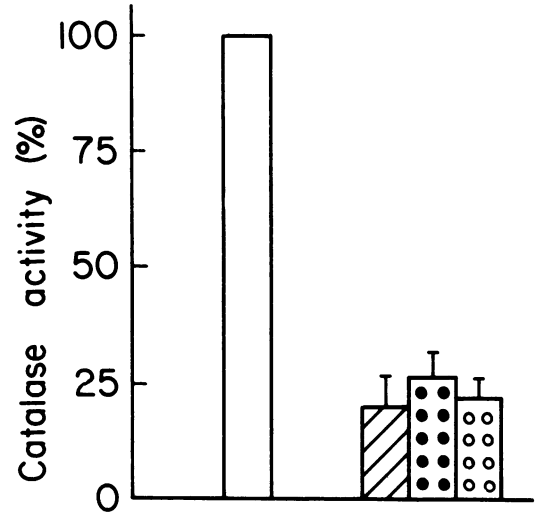

FIG. 2. Inhibition of catalase activity of intact human cells in culture by viable virulent *M. pneumoniae*. □, Uninfected cells. Normal human skin fibroblasts (▨), human nasal ciliated epithelial cells (⊡), and human lung carcinoma epithelial cells (◎) grown in culture were infected for 20 h with viable virulent *M. pneumoniae*, and their catalase activities were measured as described in the text. The results (mean ± standard deviation [bars] of six different experiments) of each culture are expressed as percentage of controls (1.08 ± 0.29, 0.52 ± 0.14, and 0.62 ± 0.15 nmol of O_2 generated/min per μg of protein, respectively).

TABLE 1. Lysosomal enzymes and catalase activities in *M. pneumoniae*-infected human cells grown in culture

Cell type	Lysosomal hydrolases[a]				Catalase[b]
	α-Glucosidase	β-Glucosidase	α-Galactosidase	β-Galactosidase	
Fibroblasts					
Infected[c]	85.5 ± 21.7	54.0 ± 8.2	15.5 ± 3.2	383.9 ± 75.7	0.33 ± 0.12
Control	89.1 ± 8.1	40.7 ± 12.3	14.4 ± 2.4	408.1 ± 53.9	1.08 ± 0.24
Ciliated epithelial cells					
Infected	51.2 ± 11.7	41.4 ± 9.7	67.7 ± 10.3	586.3 ± 15.0	0.17 ± 0.01
Control	59.2 ± 9.4	49.0 ± 3.4	78.5 ± 5.9	590.2 ± 18.5	0.52 ± 0.14

[a] Lysosomal enzymes specific activity was determined as nanomoles of hydrolyzed substrate per hour per milligram of protein. The results are expressed as the mean ± the standard deviation of four experiments.

[b] Catalase activity in the cells was measured as described in the text. The results represent the mean ± the standard deviation of six experiments. The specific activity is expressed in nanomoles of O_2 per minute per microgram of protein.

[c] The cells were infected by *M. pneumoniae* for 20 h.

either UV irradiation or heat inactivation (56°C, 1h). All treatments resulted in almost complete loss of *M. pneumoniae* capacity to inhibit catalase (Table 3), indicating that the inhibition phenomenon is expressed by viable organisms only.

The possibility that catalase inhibition was due to its digestion by proteases (7) released from *M. pneumoniae* was ruled out by the following experiment. *M. pneumoniae* viable organisms were preincubated in PBS (pH 7.2) for 30 min at 37°C. They were then removed by centrifugation at 27,000 × *g* for 10 min, and the supernatant was added to catalase solution and incubated at 37°C for 30 min. No decrease in enzyme activity was measured. When the cells were resuspended in PBS, however, they retained their ability to inhibit the soluble enzyme.

The catalase inhibition capacity of *M. pneumoniae* membranes obtained by ultrasonic treatment was almost completely lost (Table 3). This reinforces the finding that only intact viable organisms exhibit catalase inhibition capacity. Furthermore, it indicates that catalase inhibition is not a result of a membrane-bound proteolytic activity. The 5.3% residual inhibitory activity observed with membrane fractions was probably due to noncomplete disruption of cells by sonication: few *M. pneumoniae* colonies grew from a sonicated suspension.

TABLE 2. Lipid peroxidation in *M. pneumoniae*-infected fibroblasts

Fibroblasts	MDA[a]
Infected	2.08 ± 0.67
Control	0.59 ± 0.08

[a] MDA was assayed as described in the text. The results express the mean ± the standard deviation of four experiments in nanomoles per milligram of protein after 20 h of incubation.

DISCUSSION

Our study demonstrated that viable virulent *M. pneumoniae* markedly inhibited catalase activity in different types of intact human cells and in solution. The first set of experiments was conducted with human RBC. These cells were shown in previous studies to be a suitable model for *M. pneumoniae* interaction with host cells (2, 3, 13, 14, 15, 17–19) and to possess cellular catalase (4). Infection of RBC with viable viru-

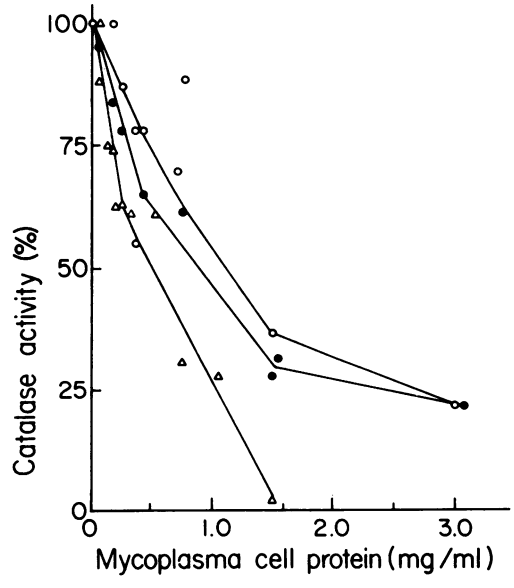

FIG. 3. Inhibition of catalase in solution by virulent and nonvirulent *M. pneumoniae*. Catalase in solution was incubated with various amounts of viable virulent *M. pneumoniae* (M129-B16) (△) or of its nonvirulent, nonhemadsorbing mutants HA-7 (●) and HA-3 (○). Catalase activity (assayed as described in the text) is expressed as percentage of control.

TABLE 3. Inhibition of catalase in solution

M. pneumoniae strain	% of catalase inhibition in vitro[a]			
	Viable cells	UV irradiation	56°C treatment	Membranes
Virulent (M129-B16)	26.5 ± 2.3	1.9 ± 0.5	5.8 ± 0.4	5.3 ± 0.4
Nonvirulent HA-3	8.6 ± 1.4	0	0	ND[b]
HA-7	9.8 ± 1.8	0	0.6 ± 0.1	ND

[a] The results represent the percentage of catalase inhibition after 30 min of incubation with 0.2 mg of mycoplasma cell protein. Mean ± standard deviation of four experiments.
[b] ND, Not done.

lent *M. pneumoniae* resulted in 60% inhibition of catalase activity (Fig. 1), but when the number of bacteria increased, the inhibition rose to 72 ± 3.5%, indicating that by enhancing the efficacy of adherence, the residual activity of catalase can be further reduced. The lack of direct contact between the mycoplasmas and a small percentage of RBC which could not be removed at the end of the incubation period might explain part of the residual activity still observed. Incubation periods longer than 24 h to further reduce catalase activity resulted in hemolysis of RBC, and therefore, the effect of prolonged incubation on catalase activity could not be evaluated.

The ability of viable as opposed to UV light-killed mycoplasmas to inhibit RBC catalase was compared to determine whether nonviable microorganisms that still retain their adherence capacity are also able to inhibit RBC catalase activity. The percent adherence to RBC of both viable and UV light-killed mycoplasmas was estimated from the labeled mycoplasmas located in the RBC fraction. The amount of radioactivity that was found in the RBC fraction was the same for both the viable and the killed mycoplasmas—about 30% of the total radioactivity of the mycoplasma lawn. Similar levels of *M. pneumoniae* attachment to RBC were recently reported by Baseman et al. (3) for the same strain of *M. pneumoniae* and by Banai et al. (2) for *M. pneumoniae* membranes and for UV light-killed *M. gallisepticum*. In our study, the UV light-killed mycoplasmas failed to inhibit RBC catalase activity, indicating that viability of the organisms is crucial for the expression of catalase inhibition capacity. The importance of *M. pneumoniae* viability in causing alterations in host cell metabolism was also stressed by Hu et al. (16).

Since the experiments with the RBC supported our working hypothesis, we selected human skin fibroblasts, lung carcinoma epithelial cells, and ciliated epithelial cells from nasal polyps for additional investigations and confirmation of our findings with RBC. These cells are considered more representative of in vivo infection by *M. pneumoniae*. Of special interest were the nasal epithelial cells, as they demonstrated in culture the well-developed cilia (25a) also seen in other types of respiratory tract cells. The phenomenon of host cell catalase inhibition could be demonstrated in all types of cells, with the highest rate of inhibition in lung carcinoma epithelial cells (Fig. 2). The inhibition of catalase progressed with the incubation time as observed with the RBC (data not shown). It is assumed that inhibition of catalase in these cells by *M. pneumoniae* is specific and not a reflection of a general cytopathic effect. This is suggested from the data in Table 1 which show that the activities of several lysosomal hydrolases in two different types of cultured cells were not significantly altered by infection with *M. pneumoniae*. The lysosomal hydrolases were chosen as controls because of their analogous intracellular location, namely, in single-membrane-limited organelles (lysosomes and peroxisomes, respectively).

Hydrogen peroxide has been implicated as an agent responsible for cell damage (23, 24). Since it is produced by *M. pneumoniae* as a respiratory end product it has been suspected to be a virulence factor in *M. pneumoniae* infection (5, 20). It is suggested that after host cell catalase inhibition, the enzymatic breakdown of the hydrogen peroxide produced by both the host cell and the mycoplasma is reduced, rendering the cell more susceptible to this oxidant. The susceptibility of membrane lipids to oxidative attack in *M. pneumoniae*-infected fibroblasts and controls was studied by measuring MDA, a breakdown product of fatty acids which indicates the extent of lipid peroxidation (4). The higher lipid peroxidation level demonstrated in *M. pneumoniae*-infected cells (Table 2) supported this hypothesis.

In view of the results obtained with virulent *M. pneumoniae*, it was of interest to study the ability of nonvirulent mutants of *M. pneumoniae* to inhibit host cell catalase. Since these mutants are characterized by loss of their adherence capacity (13, 14), it was mandatory to use an in vitro system for this purpose. The nonhemadsorbing mutants were found to inhibit catalase in solution (Fig. 3), indicating that inhibition of catalase and hemadsorption are separable properties of *M. pneumoniae*. The lower capacity of the mutant strains to inhibit catalase in vitro (Fig. 3) is not fully understood. It is assumed

that if the inhibitory activity is membrane related, loss of adherence capacity which is accompanied by alternations in membrane constituents, such as proteins of the binding sites (13, 14, 19) may induce a quantitative change in inhibitory activity of the mutants without a total loss of this property.

As demonstrated with intact RBC, the UV-irradiated mycoplasmas failed to inhibit catalase activity in vitro. These findings suggest (i) that catalase inhibition in vitro is not a result of a nonspecific attachment of the enzyme to the organisms and (ii) that viability is crucial for *M. pneumoniae* organisms to express their inhibitory capacity both in vitro and in intact human cells.

On the basis of data previously reported (1, 3, 5, 7, 10, 13, 14, 16–20, 22, 25) and the experiments presented in this study, we suggest that several factors are involved in the mechanism of *M. pneumoniae* pathogenicity. These include intimate association of the mycoplasmas with their host cell surface and catalase-inhibitory capacity of viable organisms. It is assumed that when host cell catalase is inhibited, the toxicity of hydrogen peroxide produced by both *M. pneumoniae* and the metabolically active host cell is highly enhanced. Experiments are now in progress aiming to identify the inhibitory factor transferred from the viable *M. pneumoniae* to the host cells.

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