Role of Superoxide Anion in Host Cell Injury Induced by *Mycoplasma pneumoniae* Infection

A Study in Normal and Trisomy 21 Cells

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Abstract. The role of *Mycoplasma pneumoniae*-generated superoxide and hydrogen peroxide in inducing host cell injury was studied in normal and trisomy 21 human cells. As a result of *M. pneumoniae* infection, catalase activity in infected normal skin fibroblasts and ciliated epithelial cells decreased by 74–77% as compared with uninfected controls. Addition of superoxide dismutase to the infected cultured cells totally prevented the inhibition whereas addition of catalase or catalytically inactivated superoxide dismutase had no protective effect. Trisomy 21 erythrocytes and cultured skin fibroblasts in which CuZn-superoxide dismutase content is 50% greater than in normal cells were infected by *M. pneumoniae*. The inhibition of catalase activity in these cells was 7–33% and 0–20.5%, respectively, as compared with 65–72% and 48–68% inhibition in normal infected controls. Following *M. pneumoniae* infection, the levels of malonyldialdehyde, an indicator for membrane lipid peroxidation were raised in trisomy 21 cultured fibroblasts by 10–32% while in normal cells malonyldialdehyde increased by 140–870%. Externally added superoxide dismutase, but not catalase, reduced the extent of lipid peroxidation in normal infected cells. Lactate dehydrogenase release from normal infected cells was time correlated with the increase in their malonyldialdehyde formation. It is suggested that superoxide generated during *M. pneumoniae* infection is involved in the inhibition of host cell catalase activity. The inactivation of this cellular antioxidative defense mechanism results in progressive oxidative damage to the *M. pneumoniae*-infected cells.

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Introduction

In the past decade, *Mycoplasma pneumoniae* has received increasing attention not only as a pathogen of the human respiratory tract, but also as an agent capable of inducing severe extrapulmonary complications. *M. pneumoniae* infection accounts for ~20% of all pneumonia cases in the general population and should be considered in the differential diagnosis of severe pulmonary disease, myocardial and cerebral involvement, hemolytic anemia, and dermatological disorders (1).

M. pneumoniae infection is associated with metabolic and morphologic changes in the host cell (2–6); however, the precise mechanism of pathogenicity is still the subject of continuing studies. The adherence of *M. pneumoniae* to its host cell is a mandatory process for the initiation of cell injury, although adherence capacity and virulence are separable properties of this extracellular pathogen (7). *M. pneumoniae* is known to produce H_2O_2 (9–11) and superoxide (O_2^-)[1] (12), but lacks catalase and superoxide dismutase (SOD) activity (12, 13). These powerful oxidants (14, 15), were implicated as possible factors in inducing host cell damage (10, 12, 16). However, the relatively small amounts of H_2O_2 and O_2^- produced by *M. pneumoniae* and the existence of detoxifying enzymes in the host cell (catalase and SOD) bring their effectiveness as toxic agents into question. We have recently demonstrated that viable virulent *M. pneumoniae* inhibits catalase activity in different types of human host cells. It has been assumed that as a result of host cell catalase inhibition the toxicity of H_2O_2 is greatly enhanced, thereby causing cell injury (17). Since viable *M. pneumoniae* generates O_2^- (12), a radical capable of inhibiting catalase activity in vitro (18), we postulate that the inhibition of host cell catalase activity is mediated by the O_2^- produced by the infecting my-

1. *Abbreviations used in this paper:* Hb, hemoglobin; LDH, lactate dehydrogenase; MDA, malonyldialdehyde; O_2^-, superoxide anion; RBC, erythrocyte(s); SOD, superoxide dismutase.

coplasmas. This hypothesis was studied using normal cells and trisomy 21 cells, which possess higher than normal levels of CuZn-SOD, due to the presence of CuZn-SOD gene on chromosome 21.

Methods

Organisms and growth conditions

Virulent *M. pneumoniae* (strain M129-B16) were cultured for 3–4 d at 37°C in Roux bottles or petri dishes (90 × 15 mm) containing Hayflick medium (19) supplemented with 0.2% glucose, 10% yeast extract, 10% horse serum, 600 U/ml of penicillin G, and 0.1 μCi/ml [^{3}H]palmitate.

Infection of normal and trisomy 21 human erythrocytes (RBC) by M. pneumoniae

RBC were separated from fresh heparinized blood obtained from normal donors and trisomy 21 patients (20). The RBC were diluted to 0.008% (vol/vol) in sterile phosphate-buffered saline pH 7.4 (PBS). Infection of RBC by *M. pneumoniae* was performed as previously described (17), by adding a suspension of 0.008% RBC in PBS to lawns of *M. pneumoniae* cultures grown in petri dishes. The cells were incubated for 20–24 h, collected into PBS, and free mycoplasmas were separated from mycoplasmas that adhered to the RBC by centrifugation (10 min at 480 *g*). The pellet was resuspended in PBS and assayed for catalase activity. The hemoglobin (Hb) content was determined as previously described (17). The amount of radioactivity originating from the attached mycoplasmas was assessed by scintillation spectrometry (21).

Human cell cultures

Ciliated epithelial cell cultures. Ciliated epithelial cells derived from nasal polyps were cultured as previously described (22), on plates without extracellular matrix, retaining their ciliary activity. The explant tissue pieces were removed before infection by *M. pneumoniae.*

Human fibroblast cultures. Normal skin fibroblasts (females: 159, 213; male: 226) were obtained from healthy donors. Fetal skin fibroblasts (female, K34) were obtained from a spontaneously aborted fetus at 5 mo of gestation. Trisomy 21 skin fibroblasts (females, K222, K193) were obtained from fetuses prenatally diagnosed by amniocentesis as trisomy 21 and aborted at 5 mo of gestation. Skin fibroblasts (male, K256) were also obtained from a newborn diagnosed as having a trisomy 21. All skin biopsies were obtained following a written informed consent. The cells were cultured in tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) containing 5 ml of nutrient mixture F-10 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Laboratories), 0.34% glucose, 0.2 U/ml insulin (Nordisk Insulin Laboratoriumn, Copenhagen), 1 mM glutamine, 100 μM nonessential amino acids (Gibco Laboratories), 100 U/ml penicillin G, and 100 μg/ml streptomycin at pH 7.4, and incubated at 37°C, under humidified air with 5% CO_2. The cells were grown in a monolayer and used between passages 4 and 8.

Infection of cell cultures by M. pneumoniae

A lawn of virulent *M. pneumoniae* cultured in Roux bottles was washed three times with 0.25 M sterile NaCl and scraped off with a rubber policeman into sterile saline. This suspension (1 ml, 1 mg cellular protein) was added to a flask containing a monolayer of cells that had been washed twice with sterile saline. 3 ml of nutrient mixture F-10 or medium RPMI 1640 (Gibco Laboratories) supplemented with 1 mM glutamine and 3% fetal bovine serum at pH 7.4 were added, and the cells were incubated at 37°C under 5% CO_2 for 20 h. For lactate dehydrogenase assays (LDH) cells were cultured in a serum-free media for 2–20 h.

Incubations were not carried out for longer periods since after 20 h the infected cells tended to peel off. SOD from bovine blood (69 U/ml, 25 μg/ml), catalase from beef liver (900 U/ml, 25 μg/ml), or 25 μg/ml albumin from bovine serum (Sigma Chemical Co., St. Louis, MO) added to the medium had been previously sterilized by filtration. After incubation, the cells were washed twice with saline, scraped off with a rubber policeman into saline and assayed for catalase activity, malonyldialdehyde (MDA) levels, and protein content. Protein was determined by the method of Lowry et al. (23).

Catalase activity in intact cells

Cell catalase activity was measured as previously described (17) by following H_2O_2-dependent oxygen production at 30°C, using an oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, OH).

MDA assay

Lipid peroxides in control and *M. pneumoniae*-infected fibroblasts were estimated as MDA (a secondary breakdown product of fatty acids) using the thiobarbituric acid method previously described (24).

SOD activity in RBC and in cultured skin fibroblasts

RBC were lysed by deionized water and fractionated as previously described (25). Fibroblasts were collected into PBS, freeze-thawed six times and homogenized for 1 min in Teflon pestle homogenizer (Arthur H. Thomas Co., Philadelphia, PA). SOD activity was measured by the inhibition of superoxide-induced reduction of cytochrome *c* (25).

SOD inactivation

SOD was catalytically inactivated using diethyldithiocarbamate as previously described (26).

LDH activity in cultured skin fibroblasts and their medium

Fibroblasts were collected into PBS and freeze-thawed six times. The cells and their medium were centrifuged for 15 min at 15,000 *g*. LDH activity in the supernatants was measured as previously described (27).

Results

Effect of added SOD on catalase inhibition in M. pneumoniae-infected cells. The inhibition of host cell catalase due to *M. pneumoniae* infection was demonstrated in cultured ciliated epithelial cells as well as in normal cultured fibroblasts (Fig. 1). The enzyme activity was significantly reduced by 74 and 77% of its initial value, respectively, within 20 h of incubation with virulent *M. pneumoniae.*

Addition of SOD to the medium at initiation of infection provided complete protection of catalase activity in the infected cells (100% residual activity) whereas the addition of the enzyme 4 h after infection resulted in partial protection (65% residual activity). Inactivated SOD did not prevent the inhibition of catalase activity in the host cells (Fig. 1 *B*).

The amount of radioactivity associated with the infected cells in the presence and absence of SOD was similar (~30% of the total counts). The addition of SOD, therefore, did not interfere with the attachment of *M. pneumoniae* to the host

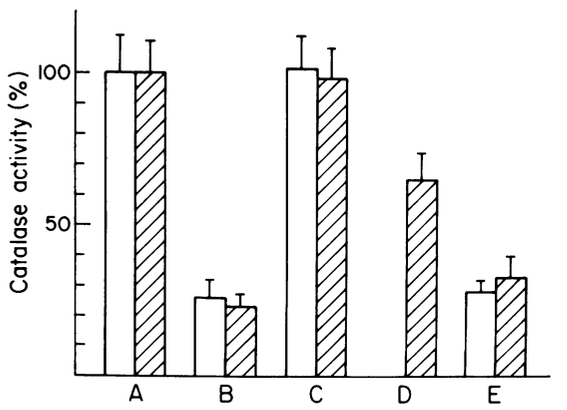

Figure 1. The effect of external SOD and catalase on the activity of intracellular catalase in cells infected by *M. pneumoniae.* Cultured ciliated epithelial cells derived from nasal polyps (□) and normal cultured skin fibroblasts (▨) were incubated with virulent *M. pneumoniae* for 20 h, after which their catalase activity was measured. The results represent the means±SD of four experiments. *A,* uninfected controls; *B,* infected cells supplemented with inactivated SOD (25 μg/ml); *C,* infected cells supplemented with SOD (25 μg/ml; 69 U/ml) at the onset of infection; *D,* infected cells supplemented with SOD 4 h after onset of infection; *E,* infected cells supplemented with catalase (25 μg/ml, 900 U/ml) at the onset of infection.

cells. Contrary to the findings with SOD, the addition of catalase to the medium of the infected cells had no effect on the percent inhibition of intracellular catalase (Fig. 1 *E*). The inability of this enzyme to protect host cell catalase could not be attributed to its inactivation since its activity in the medium after 20 h of incubation was not significantly reduced. Bovine serum albumin added to the incubation medium had no effect on catalase inhibition. This finding rules out the possibility of a nonspecific protein effect.

Inhibition of catalase activity in M. pneumoniae-infected normal and trisomy 21 RBC. Since addition of SOD to *M. pneumoniae*-infected cells resulted in protection of their catalase activity (Fig. 1), it was of interest to study trisomy 21 RBC known to possess elevated levels of CuZn-SOD. (The gene for CuZn-SOD is located on chromosome 21).

SOD activities in eight samples of RBC obtained from trisomy 21 patients and the percent degree of catalase inhibition in these cells due to *M. pneumoniae* infection are summarized in Table I. Catalase activity in all trisomy 21 RBC infected by *M. pneumoniae* was inhibited by 7–33% while in normal infected RBC, catalase inhibition ranged between 65 and 72%. To both types of RBC the attachment of *M. pneumoniae* was similar (Table I). SOD activities in trisomy 21 uninfected RBC were, as expected, 1.5 times higher than in controls (Table I).

Inhibition of catalase activity in M. pneumoniae-infected normal and trisomy 21 cultured fibroblasts. The percent inhibition of catalase activity in trisomy 21 fibroblasts infected by *M. pneumoniae* ranged from 0 to 20.5% as compared with 48–78% inhibition in control fibroblasts (Table I). SOD activity in trisomy 21 uninfected fibroblasts was 14% higher than in the controls (Table I).

MDA levels in control and trisomy 21 cultured fibroblasts infected by M. pneumoniae. The levels of MDA, which indicate the extent of lipid peroxidation, were measured in *M. pneumoniae*-infected fibroblasts obtained from normal and trisomy 21 subjects. In the infected trisomy 21 cells the levels of MDA were raised by only 10–32% after 20 h of incubation with *M. pneumoniae* while in the infected control cells, MDA levels increased by 140–870% (Table II, Fig. 2 *C*). In both types of cells, a corresponding rise was found in MDA levels and in the percent inhibition of catalase activity due to *M. pneumoniae* infection (Fig. 3). Addition of SOD to the medium of two samples of control cultured cells infected by *M. pneumoniae* resulted in reduction of the extent of lipid peroxidation by 50 and 100%. Addition of catalase to the medium did not affect the MDA levels in the infected control cells (Table II).

LDH activities in normal cultured skin fibroblasts infected by M. pneumoniae. The activity of LDH in *M. pneumoniae*-infected fibroblasts decreased by ~80% during 20 h of incubation as compared with 10% in controls. (Fig. 2 *A*). A parallel significant rise in LDH activity was observed in the media of the infected cells (Fig. 2 *B*) accompanied by a concomitant rise in the intracellular MDA levels (Fig. 2 *C*).

Table I. SOD Activity and Catalase Inhibition in Trisomy 21 and Normal Cells

	SOD activity* in uninfected cells	Catalase inhibition‡ in *M. pneumoniae*-infected cells
	U/mg protein	%
Trisomy 21 RBC‡ (n = 8)	1.74±0.32	19.5±8.9
Control RBC§ (n = 6)	1.04±0.09	68.5±5.0
Trisomy 21 fibroblasts		
K256	0.417±0.021	19.0
K222	0.431±0.027	20.5
K193	0.473±0.034	0
Control fibroblasts		
K34	0.386±0.014	48
213	0.388±0.014	78

* The results are expressed as mean±SD.
‡ RBC were infected by virulent *M. pneumoniae* for 24 h and cultured skin fibroblasts were infected for 20 h. The results represent the mean percent inhibition of the enzyme activity±SD.
§ The adherence of *M. pneumoniae* to the RBC was calculated from the amount of radioactivity associated with the RBC and was found to be 156±35 cpm/μg Hb for trisomy 21 infected RBC and 135±32 cpm/μg Hb for normal infected RBC.

Table II. MDA Levels in Trisomy 21 and Normal Cultured Skin Fibroblasts Infected by M. pneumoniae

	MDA*			
	Noninfected	Infected‡		
			+SOD§	+Catalase§
Trisomy 21				
K256	1.05±0.22	1.26±0.34		
K222	1.20±0.38	1.59±0.12		
K193	0.81±0.16	0.91±0.20		
Control				
159	0.59±0.08	2.08±0.67		
213	0.81±0.29	7.84±2.69	4.60±1.02	
K34	0.55±0.05	1.33±0.17	0.57±0.11	
226	0.51±0.07	1.40±0.21	0.67±0.10	1.83±0.32

* MDA levels are expressed as nanomoles per milligram protein, and represent the mean±SD of three experiments.
‡ Cells were infected by *M. pneumoniae* for 20 h before the measurement of their MDA levels.
§ SOD (69 U/ml, 25 μg/ml) or catalase (900 U/ml, 25 μg/ml) were added to the cultured cells at onset of infection.

Discussion

The purpose of the present study was to evaluate the role of O_2^- and H_2O_2 generated by *M. pneumoniae* in inducing host cell injury. In a recent study (17) we demonstrated that following *M. pneumoniae* infection, host cell catalase activity is markedly inhibited. We now present data suggesting that the inhibitory factor is the O_2^- produced during *M. pneumoniae* infection.

The effect of O_2^- on host cell catalase inhibition was studied by evaluating the role of SOD (which greatly accelerates the spontaneous dismutation of O_2^-) in two experimental systems for *M. pneumoniae* infection: (*a*) normal cultured skin fibroblasts and ciliated epithelial cells supplemented with SOD, and (*b*) trisomy 21 cells, in which CuZn-SOD content is increased by 50% as compared with normal cells (28–34) due to the presence of the CuZn-SOD gene on chromosome 21 (gene dosage effect) (35).

As shown in Fig. 1, infection of normal cultured cells by *M. pneumoniae* caused marked inhibition of the intracellular catalase activity. However, the addition of external SOD to the infected cells provided protection to the enzyme activity. Since SOD does not cross the cell membrane, it probably inactivates the *M. pneumoniae*-derived O_2^- before the penetration of this radical into the host cell. SOD confers protection against *M. pneumoniae* infection only if provided in its active form. A 100% protection is achieved if the enzyme is added at the onset of infection, whereas its addition 4 h after infection resulted in only partial protection (Fig. 1). These findings indicate that although SOD protects host cell catalase activity in *M. pneumoniae*-infected cells, it is unable to reverse the inhibition. Similar protective effects of SOD against O_2^--induced catalase inhibition in vitro and O_2^--induced RBC cytotoxicity were reported by Kono and Fridovich (18) and by Weiss (36), respectively.

An attempt to inhibit intracellular catalase activity by the xanthine-xanthine oxidase system in the presence of extracellular catalase (37) was unsuccessful. This can be explained by the inefficient O_2^- penetration into the cell due to the lack of intimate association between the enzymatic O_2^--generating system and the host cell membrane.

To further evaluate the corresponding effect of O_2^- and SOD on host cell catalase activity, trisomy 21 RBC and cultured skin fibroblasts were studied. These cells were especially suitable for this purpose as they represent a cell model in which the amount of CuZn-SOD is "naturally" high. As previously reported (28–32), we found a 50% increase in CuZn-SOD activity in trisomy 21 RBC (Table I). The <50% increase found in trisomy 21 skin fibroblasts (Table I) can be attributed to the presence of mitochondrial Mn-SOD in the examined whole cell homogenate. (The gene for Mn-SOD is located on chromosome 6, and therefore Mn-SOD is not increased in trisomy 21 cells). As expected from the experiments with the "external" SOD, the percent inhibition of catalase in *M. pneumoniae*-infected trisomy 21 RBC and cultured fibroblasts was significantly lower than in normal controls (Table I). These results suggest an inverse correlation between the activity of intracellular SOD and the extent of catalase inhibition due to *M. pneumoniae* infection. It is

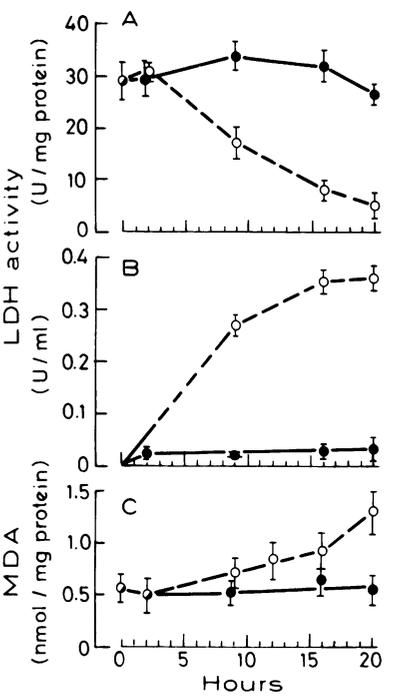

Figure 2. LDH activity and MDA formation in normal cultured fibroblasts infected by *M. pneumoniae*. Cell cultures were infected by virulent *M. pneumoniae* for 2–20 h. Cellular MDA levels and LDH activities were determined. LDH activity was concomitantly measured in the media. *A*, LDH activity in cells; *B*, LDH activity in media; *C*, MDA levels in cells. Uninfected cells (●); infected cells (○).

therefore assumed that the relatively high amounts of intracellular SOD in trisomy 21 cells provide additional protection against the inhibitory effect of O_2^- on host cell catalase activity.

Since the inhibition of catalase activity may result in oxidative damage to the host cell, it was of interest to compare the levels of MDA (an indicator of lipid peroxidation) in normal and trisomy 21 cells infected by *M. pneumoniae*. As shown in Table II, MDA levels in uninfected trisomy 21 cells were higher than in the uninfected controls. This supports other observations (38) of accelerated peroxidative processes within trisomy 21 cells, attributed to enhanced H_2O_2 production due to the increase in their CuZn-SOD activity. Infection of trisomy 21 cells, which possess higher than normal levels of CuZn-SOD, resulted in only minor changes in their MDA levels, contrary to the markedly elevated MDA levels observed in infected normal controls (Table II). Extracellular supplementation of SOD to the normal infected fibroblasts resulted in a significant decrease in their MDA content, whereas addition of catalase did not induce a similar effect (Table II). Inhibition of MDA formation by SOD in rat liver mitochondria was also reported by Zimmerman et al. (39). These results suggest that (*a*) the degree of oxidative damage induced in the host cells by *M. pneumoniae* is dependent on the amount of SOD available to the infected cells both from external and internal sources (Table II), and on the extent of intracellular catalase inhibition (Fig. 2, Table I); (*b*) H_2O_2 produced by the infecting mycoplasmas does not play a direct role in the induction of the oxidative damage to the host cell membrane (at least during the first 20 h of infection). It may, however, contribute to the toxic effect of the intracellularly generated H_2O_2.

The parallel increase in MDA formation and in LDH release to the medium by the *M. pneumoniae*-infected cells suggests that there is a negative correlation between membrane lipid peroxidation and the viability of the infected cells (Fig. 2).

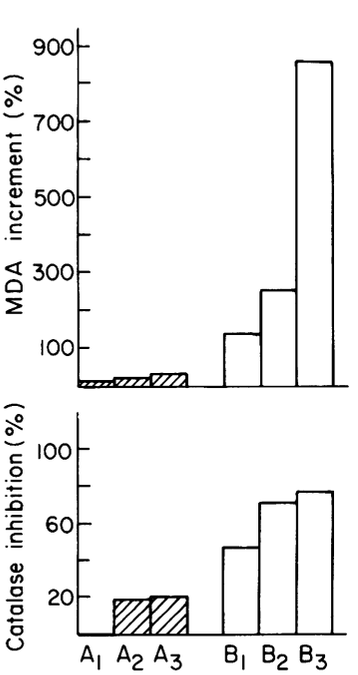

Figure 3. Correlation between percentage of catalase inhibition and increment in MDA levels in normal and trisomy 21 cultured fibroblasts infected by *M. pneumoniae*. Cell cultures were infected by virulent *M. pneumoniae* for 20 h and their catalase activity and MDA levels were determined. A_{1-3} trisomy 21 cells. A_1, K193; A_2, K256; A_3, K222. B_{1-3} normal cells. B_1, K34; B_2, 159; B_3, 213.

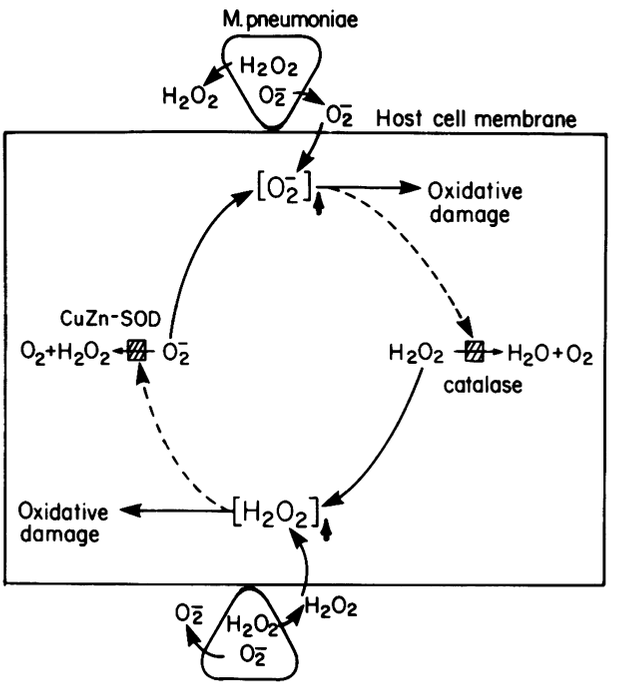

Figure 4. Schematic representation of the suggested role of O_2^- and H_2O_2 in inducing injury to *M. pneumoniae*-infected cells.

Based on previous reports (8–18, 39–42) and on the data presented in our study, the following pathogenetic events of *M. pneumoniae* infection are suggested (Fig. 4): *M. pneumoniae* adheres to the host cell membrane, after which O_2^- and H_2O_2 continuously generated by the microorganisms are introduced into the host cell. As a result of O_2^- penetration, gradual irreversible inhibition of host cell catalase is induced, thereby causing intracellular H_2O_2 accumulation. This, in turn, may cause product inhibition of cellular CuZn-SOD (35, 38–40). This process is self-perpetuated and results in increased intracellular levels of O_2^- and H_2O_2, which induce progressive oxidative damage to vital cell constitutents.

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