Protective Effects of the Glutathione Redox Cycle and Vitamin E on Cultured Fibroblasts Infected by Mycoplasma pneumoniae

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The role of the glutathione (GSH) redox cycle and vitamin E as antioxidant defense systems was studied in normal human cultured skin fibroblasts infected by virulent Mycoplasma pneumoniae. In cells infected for 20 h, catalase activity was inhibited by 75% and the intracellular GSH decreased to 32% of its normal values. GSH peroxidase and oxidized glutathione (reductase activities in the infected cells were unaffected.) GSSG glutathione in the medium of the infected cells rose in accordance with the intracellular GSH decrease. The observed elevation in GSSG/GSH ratio was attributed to the increase in intracellular H2O2 content in M. pneumoniae-infected cells due to the marked inhibition in their catalase activity. The protective effect of the GSH redox cycle in infected cells was studied by depletion of cellular GSH, prior to their infection with M. pneumoniae, using buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamyl cysteine synthetase. After 16 h of incubation with BSO, the GSH levels were reduced to 38% of their normal value and recovered to 55% during 24 h after removal of the inhibitor. BSO had no effect on GSH peroxidase and catalase activities in either infected or noninfected cells. The level of malonyl dialdehyde (an indicator of membrane lipid peroxidation) in BSO-treated cells infected by M. pneumoniae was 1.8 times higher than in infected controls. Cells enriched with 0.25 and 2.25 μg of vitamin E per mg of protein prior to their infection by M. pneumoniae revealed the following: (i) a lesser degree of catalase inhibition, 46 and 30%, respectively, versus 64% in infected control cells that were not supplemented with vitamin E; (ii) lower levels of malondialdehyde, 55 and 20% increments, respectively, versus a 140% increment in infected controls; (iii) higher residual activity of lactate dehydrogenase, 76 and 96%, respectively, versus 58% in infected controls. Our data indicate that the oxidative damage induced in M. pneumoniae-infected cells due to the increase in intracellular levels of H2O2 and O2− is limited by the host cell GSH redox cycle and by supplementation with vitamin E.

Mycoplasma pneumoniae, a specific pathogen to humans, is responsible for respiratory disease as well as for a wide range of extrapulmonary complications (8). The pathogenic mechanisms associated with M. pneumoniae infection are the subject of continuous studies (2, 3, 10, 12, 17). In our recent work with cultured human cells we have demonstrated that superoxide anions (O2−) generated during M. pneumoniae infection induce marked inhibition in host cell catalase activity, resulting in cellular oxidative damage (2, 3, 19). It has been suggested that the oxidative injury may result from the increase in intracellular levels of H2O2 due to the inhibition of catalase activity in M. pneumoniae-infected cells (2, 3). The protection of these cells from oxidative injury may, therefore, depend mostly upon the glutathione (GSH) redox cycle which catalytically scavenges H2O2 (9) and upon the presence of nonenzymatic cellular antioxidants which react with free radicals (5, 7).

In this work the role of the GSH redox cycle and vitamin E as antioxidants in M. pneumoniae-infected cells was studied by (i) depletion of cellular GSH by inhibition of its biosynthesis with buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamyl cysteine synthetase (14, 15), and (ii) enrichment of cellular vitamin E content by supplementation of the vitamin in the medium of the cultured cells.

MATERIALS AND METHODS

Reagents. Yeast glutathione reductase (type IV), NADPH, GSH, oxidized glutathione (GSSG), and d-α-tocopherol succinate were obtained from Sigma Chemical Co., St. Louis, Mo. Perhydrol (30% H2O2) was purchased from E. Merck AG, Darmstadt, Federal Republic of Germany, and d-α-[3H]tocopherol (11 Ci/mmol) and [3H]palmitic acid (100 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. BSO was synthesized according to the procedure previously described (14, 15).

Organisms and growth conditions. Virulent M. pneumoniae (strain M129-B17) organisms were cultured as previously described (3) for 3 to 4 days at 37°C in Roux bottles containing modified Hayflick medium supplemented with 0.1 μCi of [3H]palmitate per ml.

Human cell cultures. Normal human skin fibroblasts were obtained from healthy donors. The cells were cultured in tissue culture flasks (25 cm2; Becton Dickinson Labware, 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 μU of insulin (Nordisk Insulin Laboratorium, Copenhagen, Denmark) per ml, 1 mM glutamine, 100 μM nonessential amino acids (GIBCO), 100 μU of penicillin G per ml, and 100 μg of streptomycin per ml at pH 7.4. The cells were incubated at 37°C in 5% CO2, grown in a monolayer, and used between passages 2 and 6.

GSH depletion. A freshly prepared sterile solution of 20 mM BSO (in saline) or 20 mM NaCl was added to the medium of the cultured cells to a final concentration of 0.2 mM. The cultures were incubated at 37°C in 5% CO2 for 4 to 24 h. BSO was removed from the medium by washing the cells twice with saline.
VOL. 52, experiments) infected by vitamin. Vitamin E and vitamin levels, resulting in their redox state in M. pneumoniae-infected cells. Since the enzymatic activities which operate the intracellular GSH redox cycle (i.e., GSH peroxidase and GSSG reductase) were not affected by M. pneumoniae infection (Table 1), this system was suitable for measuring the GSH/GSSG ratio as an indicator for intracellular H₂O₂ formation (29) in M. pneumoniae-infected cells. The intracellular GSH decreased.

**TABLE 1. Effect of BSO treatment on intracellular catalase, GSH peroxidase, and GSSG reductase activities in control and M. pneumoniae-infected human fibroblasts**

<table>
<thead>
<tr>
<th>Fibroblasts</th>
<th>Catalase (nmol of O₂/μg of protein per min)</th>
<th>GSH peroxidase (ΔA₄₄₀/mg of protein per min)</th>
<th>GSSG reductase (ΔA₄₃₀/mg of protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>0.53 ± 0.04</td>
<td>32.9 ± 6.8</td>
<td>21.9 ± 2.7</td>
</tr>
<tr>
<td>BSO³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.48 ± 0.04</td>
<td>33.5 ± 6.1</td>
<td>21.9 ± 2.7</td>
</tr>
<tr>
<td>× g. LDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noninfected</td>
<td>1.81 ± 0.49</td>
<td>34.8 ± 5.2</td>
<td>20.2 ± 2.4</td>
</tr>
<tr>
<td>BSO³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.78 ± 0.38</td>
<td>33.5 ± 4.9</td>
<td>20.2 ± 2.4</td>
</tr>
</tbody>
</table>

* Cells were incubated with 0.2 mM BSO for 16 h, washed, and incubated in a fresh medium for 20 h with or without M. pneumoniae.

RESULTS

Catalase, GSH peroxidase, and GSSG reductase activities in M. pneumoniae-infected cells. Infection of cultured human skin fibroblasts by virulent M. pneumoniae induced marked inhibition in their catalase activity. The inhibition was time dependent, resulting in 20 to 25% residual enzymatic activity after 20 h of incubation (Fig. 1, Table 1). The specific activities of GSH peroxidase and GSSG reductase were not significantly changed in M. pneumoniae-infected cells as compared with uninfected controls (Table 1).

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GSH peroxidase activity was measured according to the method of Paglia and Valentine (27). GSSG reductase was assayed by the method of Roos et al. (28). For lactate dehydrogenase (LDH) assays, cells were collected into phosphate-buffered saline (pH 7.4), freeze-thawed six times, and centrifuged for 15 min at 15,000 × g. LDH activity in the supernatants was measured as previously described (20).

GSH and GSSG assays. Cells suspended in saline were deproteinized by an equal volume of 2 M perchloric acid containing 4 mM EDTA. As previously described (1), intracellular GSH was measured by the O-phthalaldehyde method and GSSG in the medium of the cultured fibroblasts was determined with GSSG reductase.

Lipid peroxidation (MDA formation) assays. 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 (6).

Cellular vitamin E measurement. Cultured cells were washed twice with saline and scraped off with a rubber policeman into sterile saline. This suspension (1 ml, 1 mg of cellular protein) was added to a flask containing a monolayer of cells that had been washed twice with sterile saline. A 3-ml portion of nutrient mixture F-10 supplemented with 1 mM glutamine and 3% fetal bovine serum at pH 7.4 was added, and the cells were incubated at 37°C under 5% CO₂ for 2 to 20 h. Incubations were not carried out for longer periods since after 20 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, and assayed for enzymatic activities, for GSH, malonyldialdehyde (MDA), and vitamin E levels, and for protein content.

Enzyme assays. Catalase activity in intact fibroblasts was measured as previously described (3) by following hydrogen peroxide-dependent oxygen production at 30°C, using an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). For GSH peroxidase and GSSG reductase assays, the cells were freeze-thawed six times and homogenized for 1 min in a Teflon pestle homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.). GSH peroxidase activity was measured according to the method of Paglia and Valentine (27). GSSG reductase was assayed by the method of Roos et al. (28). For lactate dehydrogenase (LDH) assays, cells were collected into phosphate-buffered saline (pH 7.4), freeze-thawed six times, and centrifuged for 15 min at 15,000 × g. LDH activity in the supernatants was measured as previously described (20).

Vitamin E supplementation. Cells obtained from a normal donor were cultured for 6 days in medium supplemented with 1 or 3 μg of D-α-tocopherol succinate dissolved in 0.25% dimethyl sulfoxide per ml of medium.

Since both vitamin E and mycoplasmas were ³H radiolabeled, only the fibroblasts that were not to be infected were cultured in the presence of the labeled vitamin. Cells that were to be infected by M. pneumoniae were supplemented prior to their infection by the same amounts of nonradioactive vitamin.

Infection of cultured fibroblasts 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 of cellular protein) was added to a flask containing a monolayer of cells that had been washed twice with sterile saline. A 3-ml portion of nutrient mixture F-10 supplemented with 1 mM glutamine and 3% fetal bovine serum at pH 7.4 was added, and the cells were incubated at 37°C under 5% CO₂ for 2 to 20 h. Incubations were not carried out for longer periods since after 20 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, and assayed for enzymatic activities, for GSH, malonyldialdehyde (MDA), and vitamin E levels, and for protein content.

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FIG. 1. Catalase activity in normal human cultured fibroblasts infected by M. pneumoniae. Cultured cells were incubated with virulent M. pneumoniae for up to 20 h, and their catalase activity was measured. The results (mean ± standard deviation of four experiments) are expressed as percentage of uninfected controls (1.19 ± 0.32 nmol of O₂ generated/min per μg of protein). Catalase activity in uninfected controls was unchanged during the 20 h of the experiment.
to 40% (18.2 ± 4.3 nmol/mg of protein) during 8 h of infection (Fig. 2) and further decreased to 32% after 20 h. This was followed by a gradual increase of GSSG in the medium of the infected cells: 2.5 ± 0.5 and 25 ± 2 nmol per mg of protein after 8 and 20 h of infection, respectively.

**Effect of BSO on intracellular GSH.** Incubation of cultured fibroblasts with BSO resulted in a gradual decline in cellular GSH (Fig. 3A). After 16 h of incubation, intracellular GSH was reduced to 38% of normal values. Further exposure to BSO for up to 24 h had no significant effect on GSH levels.

After 16 h of incubation with BSO, the inhibitor was removed and the recovery of GSH during 24 h was measured. GSH levels slowly increased to 55% of normal values within 24 h (Fig. 3B). The slow recovery of GSH enabled infection of GSH depleted cells by *M. pneumoniae* in the absence of BSO during infection.

**MDA levels, catalase inhibition, and GSH peroxidase activity in GSH-depleted cells infected by *M. pneumoniae.* The levels of MDA in BSO-treated cells infected by *M. pneumoniae* were 1.8 times higher than in cells infected in the absence of the inhibitor. BSO treatment did not significantly change the MDA levels in uninfected cells (Table 2). GSH peroxidase activity and the percentage of catalase inhibition in GSH-depleted cells infected by *M. pneumoniae* were similarly unaffected (Table 1).

**Catalase inhibition, MDA levels, and LDH activity in vitamin E-enriched cells infected by *M. pneumoniae.* The basal cellular content of vitamin E in the cultured fibroblasts ranged from 0.08 to 0.12 µg/mg of protein, similar to previously reported values (30). The cellular uptake of a d-α-tocopherol was not linearly proportional to its concentration in the medium; cells cultured in medium supplemented with 1 µg of d-α-tocopherol succinate per ml contained 0.25 µg of tocopherol per mg of protein, whereas in those cultured in the presence of 3 µg/ml vitamin E levels were 2.25 µg/mg of protein (Fig. 4).

Infection of the vitamin E-enriched cells with *M. pneumoniae* for 20 h resulted in a lesser degree of catalase inhibition, lower levels of MDA, and higher residual LDH activity compared with infected controls (Fig. 4): in cells containing 0.25 and 2.25 µg of vitamin E per mg of protein catalase activity was inhibited by 46 and 30%, respectively, compared with 64% inhibition observed in infected control cells. MDA levels in cells containing 0.25 µg of vitamin E per mg of protein increased by 55% and by only 20% in cells enriched with 2.25 µg of vitamin E per mg of protein, whereas in the infected controls MDA levels were raised by 140%. Cellular LDH activity was 76 and 96% in *M. pneumoniae*-infected cells enriched with 0.25 and 2.25 µg of vitamin E per mg of protein, respectively, and 58% in infected controls. Vitamin E had no effect on the degree of *M. pneumoniae* attachment to the cells (the adherence of *M. pneumoniae* to the control cells was 238 ± 35 cpm/µg of protein, it was 243 ± 37 cpm/µg of protein to cells supplemented with 1 µg of vitamin E per ml, and it was 283 ± 53 cpm/µg of protein to cells supplemented with 3 µg of vitamin E per ml) or on catalase activity and MDA levels in uninfected cells.

**DISCUSSION**

In our previous studies with human cells infected by *M. pneumoniae* we demonstrated that host cell catalase activity

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### TABLE 2. MDA levels in control and GSH-depleted cells infected by *M. pneumoniae*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg of protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected</td>
</tr>
<tr>
<td>BSO</td>
<td>3.01 ± 0.55</td>
</tr>
<tr>
<td>Control</td>
<td>1.71 ± 0.34</td>
</tr>
</tbody>
</table>

* The results represent the mean ± standard deviation of three experiments.
* Cells were incubated with 0.2 mM BSO for 16 h, washed, and incubated in a fresh medium for 20 h with or without *M. pneumoniae.*
attributed mainly to GSSG efflux (1), whereas its further rise in the medium can be explained by leakage and autooxidation of cellular GSH due to progressive damage to the host cell membrane.

In view of the cellular events that follow *M. pneumoniae* infection and result in host cell injury due to catalase inhibition and H\textsubscript{2}O\textsubscript{2} increase, it seemed that *M. pneumoniae*-infected cells are dependent to a great extent upon GSH peroxidase activity for the enzymatic detoxification of H\textsubscript{2}O\textsubscript{2} (21). Previous studies have demonstrated that inhibition of the GSH redox cycle enhanced oxidative cytolysis in different types of cells (4, 11, 16, 23–25, 28). Therefore, it was of interest to study the role of the GSH redox cycle as a defense mechanism in human cells infected by *M. pneumoniae*. This could be accomplished by specific depletion of cellular GSH, using BSO (14, 15). It should be noted that, of the agents commonly used to alter intracellular levels of GSH or its redox cycle, BSO is the least toxic and the most specific (4, 15). The observed 62% decrease in GSH content of normal cells after 16 h of incubation with BSO and its slow recovery (Fig. 3) suggests a relatively slow turnover of GSH in these cells. Similar results were obtained with human endothelial cells (16).

BSO had no effect on the specific activities of catalase and GSH peroxidase in normal cells (Table 1). Similar observations were reported by Arrick et al. (4). In *M. pneumoniae*-infected cells pretreated by BSO (resulting in GSH depletion), an enhanced oxidative damage was observed, expressed by a significant rise in MDA levels as compared with infected controls in which GSH synthesis was not inhibited (Table 2). This could not be attributed to a nonspecific effect of BSO since GSH peroxidase activity was unaffected by the inhibitor and the percent inhibition of catalase was similar to infected controls. On the basis of these findings it is suggested that the detoxification of the elevated levels of H\textsubscript{2}O\textsubscript{2} in *M. pneumoniae* cells (in which catalase activity is inhibited) may depend mostly upon the GSH redox cycle.

Although the GSH redox cycle can limit the H\textsubscript{2}O\textsubscript{2}-induced oxidative injury in the infected cells, it is unable to prevent the toxic effect of O\textsubscript{2}\textsuperscript{-} on the host cell. Under these conditions, natural nonenzymatic antioxidants capable of scavenging oxygen radicals (vitamin E) may offer further protection against the oxidative damage in *M. pneumoniae*-infected cells.

Vitamin E functions in vivo as a lipid antioxidant and as a free radical scavenger (5, 7). Being lipid soluble, this vitamin is associated with the cell membranes. As the host cell membrane is the initial site of the parasite-host interaction (17, 18), it was of interest to study the effect of *M. pneumoniae* infection on vitamin E-enriched cells.

In *M. pneumoniae*-infected cells increased cellular levels of vitamin E were associated with decreased inhibition of catalase activity (Fig. 4). This was accompanied by a parallel decrease in MDA formation and by a rise in residual cellular LDH activity. The effect of vitamin E could not be attributed to its interference with the attachment process since the adherence of *M. pneumoniae* to vitamin E-enriched cells was similar to controls. It is therefore suggested that the protective effect of vitamin E in *M. pneumoniae*-infected cells reflects its ability to scavenge H\textsubscript{2}O\textsubscript{2} or O\textsubscript{2}\textsuperscript{-} or both (7, 13).

The increased cellular levels of vitamin E were associated with a decrease in the extent of oxidative damage induced by *M. pneumoniae* to the host cells (Fig. 4). However, although the cellular vitamin E content was 20- to 25-fold higher than normal, the cellular damage induced by *M. pneumoniae* is specifically inhibited by O\textsubscript{2}\textsuperscript{-} generated by the infecting mycoplasmas. It has been suggested that the inhibition of catalase activity may result in increased intracellular levels of H\textsubscript{2}O\textsubscript{2}, inducing oxidative damage to the host cell (2).

Infection of normal human cultured cells by *M. pneumoniae* resulted in marked inhibition of intracellular catalase activity (Fig. 1). The expected rise in H\textsubscript{2}O\textsubscript{2} content in these cells was studied by following the intracellular GSH redox state. This system was suitable for our study since it is specifically associated with maintenance of intracellular H\textsubscript{2}O\textsubscript{2} steady-state concentration (24, 26, 29), and the activities of GSH peroxidase and GSSG reductase (which operate the redox cycle) were not affected by *M. pneumoniae* infection (Table 1).

The data presented in Fig. 2 and the observed rise in extracellular GSSG demonstrate that, following *M. pneumoniae* infection, the GSSG/GSH ratio is increased, reflecting increased H\textsubscript{2}O\textsubscript{2} levels due to the inhibition of catalase activity. However, the conversion of GSH to GSSG was not stoichiometric since GSH may also be incorporated into mixed disulfides (1), which were not assayed. The extracellular GSSG observed during the first 8 h of infection can be

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**FIG. 4.** Catalase inhibition, LDH activity, and MDA formation in vitamin E-enriched cells infected by *M. pneumoniae*. Cultured cells were incubated with virulent *M. pneumoniae* for 20 h. Cellular catalase and LDH activities, MDA levels, and vitamin E content were measured. The cells were cultured prior to their infection in medium supplemented with: (A) 0.25% dimethyl sulfoxide; (B) 1 μg of vitamin E per ml dissolved in 0.25% dimethyl sulfoxide; (C) 3 μg of vitamin E per ml dissolved in 0.25% dimethyl sulfoxide. The results represent the mean ± standard deviation of four experiments. LDH and catalase activities in uninfected controls were 26 ± 4 U/mg of protein and 1.20 ± 0.33 nmol of O\textsubscript{2}/min per μg of protein, respectively.

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could not be completely prevented. One possible explanation may be the change induced in the membrane fluidity due to the marked uptake of vitamin E which may affect the permeability of the host cell membrane (13).

Our studies indicate that in M. pneumoniae-infected cells in which catalase activity is inhibited, the enzymes that operate the GSH redox cycle are not affected. Therefore, oxidative damage induced in these cells by the increased H2O2 levels is significantly reduced by the GSH redox cycle. Our data also indicate that vitamin E, being an effective antioxidant, when supplemented to the host cells prior to their infection by M. pneumoniae confers further antioxidative protection against this pathogen. The GSH redox cycle and vitamin E may similarly play an important role in vivo in limiting the direct damage induced by M. pneumoniae to the host cell.

LITERATURE CITED