Glutathione Protects Mice from Lethal Sepsis by Limiting Inflammation and Potentiating Host Defense

Pia Villa,1,2 Alessandra Saccani,1 Antonio Sica,1 and Pietro Ghezzi1

Glutathione (GSH) is the main endogenous thiol antioxidant and plays an essential role in protection from reactive oxygen species. GSH has somewhat opposite effects in immunity: it is often regarded as anti-inflammatory, because it inhibits the production of several inflammatory cytokines and chemokines and their action [1–3], but GSH also is essential for several immune functions, including interleukin (IL)–2 production, IL-2 responses, and cytotoxic T cell activity [4–7]. In particular, lower GSH levels in patients with AIDS have been correlated with immune deficiency [4], and alcohol intoxication, a condition that is associated with increased susceptibility to infections [8], also results in GSH depletion [9].

The aim of the present study was to investigate the role of GSH in the innate immunity responses mediated by neutrophil (polymorphonuclear leukocyte [PMNL]) infiltration at the site of infection in a model of polymicrobial sepsis induced by cecal ligation and puncture (CLP) [10]. To reduce GSH, diethylmaleate (DEM), an agent that depletes GSH by endogenous enzymatic conjugation [11], was used in combination with buthionine sulfoximine (BSO), a specific inhibitor of GSH synthetase [12], and the effect of GSH depletion was evaluated on PMNL migration, bacterial colonies in the peritoneum, and survival. To investigate the mechanisms of GSH regulation of PMNL migration, we measured both the levels of total chemotactic agents and of the CXC chemokine KC in this experimental model and the migratory response of PMNL to IL-8 in the mouse air pouch, an in vivo model of chemotaxis used to assess the chemotactic activity of various chemotactic agents [13].

Although GSH levels were depleted with DEM/BSO for these experiments, we have reported elsewhere that CLP induces oxidative stress and depletes GSH and that N-acetyl-l-cysteine (NAC), a GSH-repleting agent and an antioxidant that has been the subject of clinical trials in sepsis-associated adult respiratory distress syndrome [14], improved survival [10]. The effect of NAC was also studied on PMNL migration in the peritoneal cavity and in the lung.

Materials and Methods

Animals and surgery. Male (25–30 g) Crl:CD-1 (ICR)BR mice (Institute of Cancer Research) were fed ad libitum and housed under controlled conditions (22°C ± 0.5°C, 55% relative humidity, cycle of 12 h of light/12 h of dark). CLP was performed as described elsewhere [15]. Mice were anesthetized, bled, and killed at the indicated time. The peritoneal cavity was washed with 6 mL of sterile PBS, and the lavage fluids were collected. PMNL were counted in a hemocytometer after the lavage fluid was diluted 1:5 in Turk’s solution. The peritoneal lavage fluid was serially diluted in saline, plated on tryptic soy agar (Difco), and incubated at 37°C for 24 h, after which the colonies were counted. Data were expressed as total peritoneal bacterial colony-forming units.

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Procedures involving animals and their care were conducted in conformity with institutional guidelines, which are in compliance with the national laws of Italy (Decreto Legislativo 116, Gazzetta Ufficiale, Suppl 40, 18 February 1992; Circolare 8, Gazzetta Ufficiale, 14 July 1994) and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358,1, 12 December 1987; “Guide for the Care and Use of Laboratory Animals,” US National Research Council, 1996).

Reprints or correspondence: Dr. Pia Villa, Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy (villa@marionegri.it).

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The air pouch model was performed as described elsewhere [13]. Mice were anesthetized with ether, and 3 mL of air was injected under the skin on their backs. After 3 days, the pouches were re-injected with 3 mL of air. On day 6, a stimulus was injected into the pouch. In a set of experiments, 0.25 mL of cell-free lavage fluid obtained from vehicle- or BSO/DEM-treated mice 2 h after CLP was given to naive mice. In another set of experiments, 1 μg of recombinant human (rh) IL-8 dissolved in 1 mL of 0.5% carboxymethylcellulose in saline was injected in the air pouch of mice 90 min after treatment with vehicle or BSO/DEM. Four hours after the stimulus, the mice were anesthetized, and the pouches were washed with 5 mL of saline. The lavage fluid was immediately cooled on ice, and the PMNLs were counted.

In a different set of experiments, to prepare peritoneal PMNL for chemotaxis assay, mice were treated at first intraperitoneally with 1.5 mL of thioglycollate 3% and, after 4 h, with BSO (700 mg/kg subcutaneously [sc]) and DEM (0.8 mL/kg sc) or NAC (1 g/kg orally). After 2 h the animals were killed, the peritoneal cavity was washed with 6 mL of sterile PBS, and the neutrophils were used for the in vitro chemotaxis and GSH and superoxide anion assays (see “Miscellaneous assays” below).

**Treatments.** BSO (700 mg/kg sc; Sigma) and DEM (0.8 mL/kg sc; Sigma) were given 90 min before CLP. When BSO and DEM were given 90 min before the injection of rhIL-8 in the air pouch, they were administered intraperitoneally. NAC was given orally (1 g/kg; Sigma) 45 min before CLP.

**Miscellaneous assays.** GSH was measured in liver homogenates as nonprotein sulphydryl groups, according to the method of Sedlak and Lindsay [16], and in PMNL, according to the enzymatic method of Griffith [17]. Lung myeloperoxidase (MPO) was determined spectrophotometrically in tissue homogenates by use of o-dianisidine as substrate [18]. KC was measured in the serum and peritoneal fluid with a commercial ELISA kit (R&D Systems). Superoxide anion release was assayed as described elsewhere [19]. In brief, superoxide-dependent cytochrome c (Sigma) reduction was determined spectrophotometrically at 550 nm by incubation of 1 × 10⁶ cells with phorbol myristate acetate (PMA; Sigma). The final concentrations in Hanks’s balanced salt solution were 0.05 mM cytochrome c and 10⁻⁶ M PMA. Cell migration was evaluated by use of a chemotaxis microchamber technique, as described elsewhere [20].

**Statistical analysis.** Significance was assessed by Student’s t test and Tukey’s test for multiple comparisons. Colony-forming unit data are shown as “box and whiskers” graphs that indicate the median and the 25th and 75th percentiles, with minimum and maximum values at the extremes of the “whiskers.” Statistical analysis of colony-forming unit data was performed by use of the Wilcoxon rank sum test. Survival curves were compared by log-rank test.

**Results**

**GSH depletion decreases PMNL migration to the site of infection and worsens sepsis.** Sepsis induced a marked PMNL migration in the peritoneum 8 h after surgery, which was reduced 70% by treatment with BSO/DEM (figure 1A). This impairment of PMNL infiltration was paralleled by an increased number of bacteria infecting the peritoneal cavity, from 44 × 10⁵ (median value) in CLP alone to 1092 × 10⁵ in BSO/DEM + CLP (figure 1B). In the same mice, CLP induced PMNL infiltration in the lungs, as assessed by MPO activity, a marker of neutrophil sequestration, which was significantly increased by GSH depletion (figure 1C). The outcome was an increased mortality of septic mice given GSH-depleting agents (P < .01 by log-rank test) (figure 1D).

Although GSH was depleted by chemicals in these experiments, sepsis induces GSH depletion, as judged by liver GSH levels, which in CLP mice were 40% of those of naive mice, although depletion was not as marked as that induced by BSO/DEM (mean GSH levels ± SE) were 2.20 ± 0.05 mg/g liver in naive mice, 1.36 ± 0.05 mg/g liver 8 h after CLP, and 0.24 ± 0.02 mg/g liver after BSO/DEM).

The fact that septic mice have lower GSH levels prompted us to study the effect of NAC, which was administered orally at 1 g/kg 45 min before CLP. NAC augmented PMNL migration to the site of infection (figure 2A) and significantly decreased bacterial colonies in the peritoneal cavity (median values were 2534 × 10³ cfu with CLP and 339 × 10³ cfu with NAC + CLP; figure 2B). Under these conditions, pulmonary PMNL infiltrate was not increased but was slightly inhibited by ~25% (figure 2C). Consequently, survival of septic mice was markedly improved by NAC (P < .01 by log-rank test; figure 2D).

We evaluated the effect of NAC on liver GSH levels 8 h after CLP, when a 40% reduction occurs, but, by this time, no significant effect by NAC was observed (data not shown). However, in peritoneal PMNL, the GSH content was significantly (P < .05 by Student’s t test) increased 2 h after NAC treatment in thioglycollate-pretreated mice (mean ± SE, 7.7 ± 0.5 and 9.6 ± 0.3 nmol/mg protein in vehicle- and NAC-treated mice, respectively).

Because of the variability of bacterial counts, which depend on oxidant status, antimicrobial capacity, and bacterial flora, these experiments were repeated at least 1 year after the set of experiments shown in figures 1 and 2, to ensure their reproducibility. The results of a new set of 3 separate experiments are given: Median values and 25th and 75th percentiles (n = 11) were 57 × 10⁵ cfu (25%, 9; 75%, 126) for CLP/vehicle and 1310 × 10³ cfu (25%, 364; 75%, 6367) for CLP/BSO/DEM, which were significantly different from the results for CLP/vehicle by the Wilcoxon rank sum test (P < .01). For the NAC experiments, results were 210 × 10³ cfu (25%, 30; 75%, 582) for CLP/vehicle and 5.7 × 10³ cfu (25%, 3; 75%, 75) for CLP/NAC, which were significantly different from the results for CLP/vehicle by the Wilcoxon rank sum test (P < .05).

The trends for the effects of BSO/DEM and NAC on peritoneal PMNL also were confirmed. PMNL counts (×10⁶ cells; mean ± SE) were as follows: CLP/vehicle, 12.9 ± 0.5, and CLP/BSO/DEM, 3.3 ± 0.6 (P < .01 vs. CLP/vehicle by Student’s t test); CLP/vehicle, 10.5 ± 1.0, and CLP/NAC, 14.8 ± 1.0 (P < .01 vs. CLP/vehicle by Student’s t test).
GSH depletion impairs chemokine production and response in vivo. The impaired migration of PMNL to the site of infection in GSH-depleted mice could be due to many factors, and the effect of GSH depletion on response to chemokines and on their production was investigated in particular. To that purpose, the levels of chemotactic factors were measured in the peritoneal cavity of septic mice at an early time point (2 h), before significant cell infiltration occurred. In one set of experiments, the total chemotactic activity of the peritoneal lavage fluid from donor naive or septic mice (with or without BSO/DEM) was evaluated by injecting 0.25 mL of cell-free lavage fluid into the air pouch of naive recipient mice. CLP induced the appearance of a strong chemotactic activity, which was decreased by 60% with GSH depletion in septic mice (table 1). Almost identical results were obtained when KC levels were measured in peritoneal lavage fluid by ELISA: CLP induced very high KC levels, and these were decreased by 60% with BSO/DEM pretreatment (table 1). Because it was reported that, in some cases, a reduction in the ratio of peritoneal to plasma KC can decrease local PMNL accumulation by counteracting the chemotactic gradient [21], the ratio of peritoneal to serum KC was also measured in our experimental model. As shown in table 1, this ratio was not modified by BSO/DEM.

We also investigated the possibility that GSH depletion alters not only the production of chemokines (or other chemotactic factors) but also the in vivo PMNL migration in response to them. In fact, GSH depletion by BSO/DEM pretreatment significantly reduced, by 60%, the migration of PMNL measured 4 h after the local injection of 1 μg of rhIL-8 into the air pouch of mice (table 2).

On the other hand, we can exclude that GSH depletion affects PMNL response to chemotactic stimuli ex vivo. Peritoneal PMNL were prepared from thioglycollate-pretreated mice, and the GSH levels and ability to migrate of the PMNL were evaluated in a standard in vitro chemotaxis assay that used Boyden chambers. A 2-h BSO/DEM pretreatment decreased
cellular GSH by 50% (control, 7.7 ± 0.4 nmol/mg protein; BSO/DEM, 4.1 ± 0.6 nmol/mg protein; \( P < .01 \) by Student’s \( t \) test). When PMNL from BSO/DEM-pretreated mice were tested for their chemotactic responsiveness to IL-8, their migratory capacity was augmented rather than inhibited (table 3). To assess another functional activity of PMNL from GSH-depleted mice, we also evaluated their capacity to produce superoxide anion, which is an essential element of their antimicrobial activity. Superoxide production was not different from that of PMNL from control mice (data not shown).

**Table 1.** Chemotactic activity and levels of the CXC chemokine KC in peritoneal lavage fluid from septic mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>In vivo chemotaxis, PMNL count ( \times 10^6 ) cells/mL</th>
<th>Peritoneal KC, ng/mL</th>
<th>Serum KC, ng/mL</th>
<th>Peritoneum KC: serum KC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>0.32 ± 0.05(^b)</td>
<td>0.02 ± 0.01(^b)</td>
<td>&lt;0.2</td>
<td>—</td>
</tr>
<tr>
<td>Control CLP</td>
<td>3.76 ± 0.37</td>
<td>6.00 ± 0.48</td>
<td>21</td>
<td>0.29</td>
</tr>
<tr>
<td>BSO/DEM-CLP</td>
<td>1.74 ± 0.23(^b,c)</td>
<td>2.37 ± 0.72(^b,c)</td>
<td>7</td>
<td>0.34</td>
</tr>
</tbody>
</table>

*NOTE.* Buthionine sulfoximine (BSO; 700 mg/kg) and diethylmaleate (DEM; 0.8 mL/kg) were given subcutaneously 90 min before cecal ligation and puncture (CLP). Two hours after CLP, the mice were killed, and the peritoneal lavage fluid was collected to measure KC levels and the chemotactic activity of polymorphonuclear leukocytes (PMNL) in the air pouch of naive mice. The results are means ± SE for 5–7 mice.

\(^a\) Pooled values from 5 mice.

\(^b\) P < .01 vs. control CLP mice (Tukey’s test).

\(^c\) P < .05 vs. naive mice (Tukey’s test).
GSH Status Significantly Influenced This Antibacterial Response

Our findings that GSH might be important to PMNL migration to the primary site of infection or inflammation in sepsis, but not in the lung, and thus possibly preventing oxidative damage.

Although the decreased production of KC and possibly of other chemoattractants by GSH-depleting agents might explain our findings, the possibility that GSH depletion also impairs migration in response to chemokines was considered. In fact, when rhIL-8 was injected into the air pouch of GSH-depleted mice, a lower migratory response was observed.

Similar results from a different model were reported in a study that showed that GSH decreases intratracheal lipopolysaccharide (LPS)–induced pulmonary PMNL infiltration [23], which was explained by reduced intercellular adhesion molecule–1 (ICAM-1) expression after DEM. Thus, DEM impairs intratracheal LPS-induced [23] but not CLP-induced (the present study) pulmonary PMNL infiltration. The same discrepancy was observed when ICAM-1 was blocked: anti–ICAM-1 antibodies or ICAM-1–targeted gene disruption did not inhibit pulmonary PMNL infiltration after CLP [24] but did inhibit lung PMNL accumulation after local or intraperitoneal LPS [25, 26]. Thus, it is possible that the differential regulation by GSH reported here might reflect different mechanisms, in terms of soluble mediators and adhesion molecules, implicated in LPS- and sepsis-induced pulmonary PMNL accumulation. In most studies that have reported that GSH inhibits cytokine or chemokine production, LPS, rather than a true infection, was used to trigger cytokine production.

The implication of the present study is that GSH depletion, which is often associated with sepsis, might be detrimental impairing host response to infection and by augmenting PMNL-mediated lung damage. By inhibiting inflammation but potentiating innate immunity mechanisms, treatment with thiol antioxidants and GSH-repleting agents might be preferred to treatments that inhibit overall PMNL migration. In fact, there is a delicate balance between host defense and inflammation, and we are not aware of pharmacological approaches to selectively inhibit the latter. Studies that have used LPS or bolus injection of live bacteria have shown that inhibition of TNF has protective effects [27, 28]. However, in a model of CLP-induced sepsis, anti-TNF antibodies can worsen the survival outcome [29, 30]. Our data suggest that thiol antioxidants and GSH-repleting agents might help reorient PMNL migration in a way that is more favorable to the host and that this strategy can be complementary to supplementation with glutamine, which seems to play an important role in neutrophil function [31].

Table 3. In vitro migration of polymorphonuclear leukocytes (PMNL) obtained from control or glutathione-depleted mice.

<table>
<thead>
<tr>
<th>Pretreatment stimulus</th>
<th>Control</th>
<th>BSO/DEM treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.7 ± 0.7</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>IL-8 (300 ng/mL)</td>
<td>6.0 ± 1.7</td>
<td>41.5 ± 6.5</td>
</tr>
<tr>
<td>IL-8 (600 ng/mL)</td>
<td>21.8 ± 5.6</td>
<td>78.3 ± 16.2</td>
</tr>
</tbody>
</table>

NOTE. Mice were first treated intraperitoneally with 3% thioglycolate and, after 4 h, subcutaneously with buthionine sulfoximine (BSO; 700 mg/kg) and diethylmaleate (DEM; 0.8 mL/kg). After 2 h, the animals were killed, and the peritoneal PMNL were collected by lavage and their ability to migrate was measured. The results are means ± SE of triplicate samples. IL, interleukin.

aP < .01 vs. control (Student’s t test).
References


