Oxidative stress and inflammation contribute to lung toxicity after a common breast cancer chemotherapy regimen

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Received 10 January 2002; accepted in final form 23 March 2002

Abushamaa, Amir M., Thomas A. Sporn, and Rodney J. Folz. Oxidative stress and inflammation contribute to lung toxicity after a common breast cancer chemotherapy regimen. Am J Physiol Lung Cell Mol Physiol 283: L336–L345, 2002.—Delayed pulmonary toxicity syndrome (DPTS) to differentiate this late-onset lung toxicity associated with this specific HDC regimen (44). It should be worth emphasizing that DPTS was described in patients undergoing autologous transplant, as contrasted to allogeneic transplant, in which additional factors, such as graft-vs.-host disease and complications due to the immunotherapy regimen, may come into play (16). Whether the pathogenesis of DPTS is part of the spectrum of idiopathic pneumonia syndrome (10) or is a separate entity remains to be determined.

All three drugs used for the HDC regimen are known to be cytotoxic to the lung at high doses; however, the mechanism by which these drugs cause lung damage either individually or in combination is poorly understood. These drugs may affect cellular glutathione levels. Lung tissue and the alveolar epithelial lining fluid (ELF) contain high levels of antioxidants that presumably protect against oxidants and/or free radicals. The most abundant nonenzymatic antioxidant in ELF is glutathione, and the ELF contains the highest extracellular concentrations of glutathione measured (6, 8). The biological importance that high levels of glutathione play in normal and abnormal lung function is unclear. Elaborate biochemical mechanisms exist to maintain glutathione in a reduced form. Once oxidized, glutathione is efficiently reduced by the action of intracellular glutathione reductase. Thus the lungs may be particularly sensitive to chemotherapeutic regimens that promote an imbalance in oxidative stress and antioxidant reserves.

MATERIALS AND METHODS

Reagents. Glutathione [reduced (GSH) and oxidized (GSSG)], N-ethylmaleimide, dithiothreitol, 5-sulfosalicylic acid, potassium tetraborate, Trizma base, o-phthalaldehyde (OPA), tetraethoxypropane, thiobarbituric acid, glutathione reductase, glutathione peroxidase, glutathione S-transferase, superoxide dismutase, cytochrome c, t-butylhydroperoxide, sodium azide, trypan blue stain, Giemsa stain, crystal violet, glutathione monooethyl ester, and cyclophosphamide were purchased from Sigma (St. Louis, MO). Xanthine oxidase was

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L336
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purchased from Boehringer Mannheim (Indianapolis, IN). Microtainer blood collection tubes (with lithium heparin) were obtained from Becton Dickinson (Franklin Lakes, NJ). Dulbecco’s phosphate-buffered saline (PBS), pH 7.4, was obtained from GIBCO BRL (Gaithersburg, MD). NADPH was purchased from ICN Biomedicals (Irvine, CA). Sodium and potassium phosphate, EDTA, acetic acid, phosphoric acid, and HPLC-grade methanol were purchased from Mallinckrodt (Chesterfield, MO). Platinol-AQ (cisplatin) and BCNU were obtained from Bristol Laboratories (Princeton, NJ). Ethylol (amifostine) was obtained from Alza Pharmaceuticals (Palo Alto, CA).

Animals. Female B6C3 mice of 8–10 wk of age were obtained from Taconic (Germantown, NY). All mice were kept in a specific pathogen-free unit. On days −3, −2, and −1, the mice were injected intraperitoneally with 6 mg/ml of cyclophosphamide and 0.2 mg/ml of cisplatin (in PBS) at doses of 60 and 2.05 mg/kg, respectively. This was followed by intraperitoneal injection of 24.5 mg/kg of 1.67 mg/ml BCNU (in 10% ethanol) on day 0. The doses given were the maximum tolerated doses in mice according to Talmadge et al. (38). At each time point, control mice were injected with vehicle instead of the HDC drugs. Each group consisted of three mice. All animal protocols were preapproved by the Duke University Institutional Animal Care and Use Committee.

For hyperoxia exposure, mice were divided into two groups. One group received the HDC regimen as mentioned above, and the other group received vehicle alone. Both groups of mice were exposed to >99% oxygen for 16, 44, and 66 h as previously described by our laboratory in detail (17).

For experiments involving thiol-sparing agents, 20 mg/ml Ethylol (400 mg/kg) (39), 130 mg/ml glutathione monoethyl ester (2,600 mg/kg) (39), or 115 mg/ml glutathione (2,300 mg/kg) (39) were dissolved in PBS and injected intraperitoneally 1 h before BCNU on day 0. One group of animals (BCNU only) was treated with saline injections on days −3, −2, and −1 followed by BCNU on day 0. Individual experiments contained three or four mice per group. Mice were killed at 4 and 48 h and 6 wk after BCNU injection.

Bronchoalveolar lavage. Mice were euthanized by 50 µg/g ip pentobarbital injection at the following time points post-BCNU administration: 4, 8, 12, 24, 48, and 72 h and 1, 2, 4, 6, and 8 wk. The trachea was cannulated with a 24-gauge blunt needle. Then the peritoneum was opened, and the diaphragm was nicked to collapse the lungs. We performed bronchoalveolar lavage (BAL) by instilling 1 ml of PBS (containing 0.05 mM EDTA) twice into the lungs (total volume 2 ml) followed immediately by gentle aspiration. Recovery was consistently ~90%. The BAL thus obtained was placed immediately on ice until processing. After removing 20 µl of BAL for cell counts and 300 µl for differential cell counts, we centrifuged the remaining BAL at 500 g for 10 min at 4°C. The cell-free BAL supernatant was stored at −80°C until further assay.

BAL and peripheral total and differential cell counts. A 20-µl aliquot of the total BAL fluid was mixed with an equal volume of 0.4% trypan blue, and the total cell count (cells/ml BAL fluid) was obtained using a hemacytometer slide (Fisher Scientific, Pittsburgh, PA). We obtained differential cell counts (for macrophages, lymphocytes, neutrophils, and eosinophils) after centrifuging 300 µl of BAL fluid at 600 g for 6 min in a Cytospin from Shandon (Pittsburgh, PA). Cells were then processed with a Leukostat staining kit from Fisher Scientific (Pittsburgh, PA). A minimum of three hundred cells were counted, and we obtained the numbers of different cell types by multiplying the percentage of cells by the total number of cells in BAL.

For peripheral blood counts, blood was obtained from the retro-orbital plexus by means of a capillary at 1, 2, 4, 6, and 8 wk after BCNU injection. The blood was collected in heparin-treated collection tubes, and 20 µl of blood were then added to 380 µl of 2% acetic acid (containing enough Giemsa stain or crystal violet to give a pale violet color). After being mixed by inversion for about 1 min, cells were counted with a hemacytometer slide, and results were expressed in cells per milliliter of blood.

Protein. BAL protein was measured by either the Micro Protein BCA or Coomassie Plus assay kits commercially available from Pierce (Rockford, IL). Generally, 150 µl of sample were added to 150 µl of reagent, and the absorbance was read on a microplate reader at 562 and 595 nm for Micro Protein BCA and Coomassie Plus assays, respectively. We measured lung tissue protein with the Coomassie Plus assay kit by adding 10 µl of homogenate to 300 µl of reagent.

Lung tissue preparation. Lungs were removed immediately, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis. The lungs were homogenized for 1 min in 0.1 M sodium phosphate buffer, pH 7.6 (containing 0.5 mM EDTA), in a Brinkmann Polytron homogenizer (Brinkmann Instruments, Westbury, NY), followed by sonication at setting number 5 for 1 min in a Virsonic sonicator (Virtic, Gardiner, NY). The samples were placed on ice for at least 30 min and centrifuged at 10,000 g for 20 min at 4°C. The supernatants were then stored at −80°C until the time of analysis.

Glutathione reductase activity. Lung glutathione reductase activity was measured by the method of Carlbeg and Manning (9). One hundred microliters 0.2 M sodium phosphate, pH 7.6 (containing 1 mM EDTA), 25 µl 20 mM GSGS, and 25 µl 2 mM NADPH were incubated at 30°C for 10 min. Fifty microliters of lung homogenate sample were added into a reaction cuvette, and the rate of change of absorbance at 340 nm was measured for 3 min.

Glutathione peroxidase activity. Lung glutathione peroxidase activity was measured by the method of Paglia and Valentine (29). Twenty microliters each of 0.25 M potassium phosphate, pH 7.0 (containing 2.5 mM EDTA and 2.5 mM sodium azide), 6 U/ml glutathione reductase, 10 mM GSH, and 2.5 mM NADPH, and 100 µl of lung homogenate sample were mixed and incubated at 37°C for 10 min. Then 20 µl of 12 mM t-butylhydroperoxide were added, and the rate of change of absorbance at 340 or 366 nm was measured for 3 min.

Glutathione S-transferase activity. Lung glutathione S-transferase activity was measured by the method of Habig et al. (19). Two hundred microliters of a solution of 0.1 M potassium phosphate, pH 6.5 (containing in mM: 1 EDTA, 1 GSH, and 1 1-chloro-2,4-dinitrobenzene), were added to 20 µl of lung homogenate. The change in absorbance at 340 nm was monitored as an indicator of enzyme activity.

Superoxide dismutase activity. Lung and BAL fluid superoxide dismutase activities were measured according to the method of Crapo et al. (13). In brief, to a 3-ml solution of 50 mM phosphate buffer, pH 7.8 (containing in mM: 0.1 EDTA, 0.5 xanthine, and 0.5 cytochrome c), xanthine oxidase was added to give a change in absorbance of 0.02 arbitrary units/min. Then the superoxide dismutase activity in lung homogenates was measured by the change in absorbance at 550 nm. One unit of activity is equivalent to a 50% inhibition of the activity found in the presence of xanthine oxidase alone. For BAL fluid, the same procedure was performed, except for the use of a carbonate buffer, pH 10.0.

Glutathione. Glutathione levels were analyzed by a modification of the HPLC procedure of Liu and Kehrer (24). For
total glutathione, 100 μl of either cell-free BAL fluid or lung homogenate sample were treated with 100 μl of 0.1 M Tris-HCl, pH 8.5, and 50 μl of 25 mM dithiothreitol, pH 7.0. After 30 min on ice, 250 μl of 3.75% 5-sulfosalicylic acid were added to precipitate proteins. The samples were then centrifuged at 11,000 g for 2 min. Then 200 μl of supernatant were removed and treated with 200 μl of OPA solution (50 mg of OPA dissolved in 0.5 ml of methanol and then made up to 10 ml with 0.4 M potassium tetraborate, pH 9.9) for 2 min at room temperature, followed by neutralization with 200 μl of 250 mM sodium phosphate, pH 7.0. One hundred microliters of sample were injected into an HPLC.

For GSSG in BAL fluid, a duplicate sample was treated with 20 mM N-ethylmaleimide before the above-mentioned procedure. In the case of lung tissue, the samples were homogenized in 5% 5-sulfosalicylic acid (10 volumes) containing 20 mM N-ethylmaleimide. Before dithiothreitol reduction, the samples were treated with 1 M Tris-HCl as mentioned above (instead of 0.1 M Tris-HCl).

HPLC was performed using a Supelco LC18 column [15 cm length × 4.0 mm inner diameter, 5 μm] from Supelco (Bellefonte, PA) with a mobile phase of 0.15 M sodium acetate-methanol (92.5:7.5) delivered at 1.5 ml/min. The HPLC was performed on a Hewlett-Packard 1100 system (Hewlett-Packard, Wilmington, DE) equipped with a 1046A fluorescence detector set at 340 nm for excitation and 420 nm for emission.

Lipid peroxidation. Lipid peroxidation in the form of malondialdehyde was measured by HPLC according to the method of Wong et al. (46). One hundred microliters of either cell-free BAL fluid or lung homogenate were treated with 150 μl of 0.44 M phosphoric acid and 50 μl of 0.6% thiobarbituric acid. The samples were heated at 100°C for 1 h and then placed on ice until HPLC injection. Immediately before injection, 300 μl of a solution of 1 M NaOH-methanol (1:11) were added to precipitate proteins and neutralize the samples. The samples were centrifuged at 7,200 g for 5 min at room temperature, and 50 μl of supernatant were injected into the HPLC. Tetraethoxypropane was treated in the same manner as the samples and used for calibration of the method.

The HPLC system and column were as mentioned above for glutathione analysis. The mobile phase used was 50 mM potassium phosphate (pH 6.8)-methanol (60:40) at a flow rate of 1 ml/min. Fluorescence detection was performed at 525 and 550 nm for excitation and emission, respectively.

Histopathology. Lung fixation was performed on untreated, vehicle-injected, and HDC-injected mice at two time points, 48 h (acute) and 6 wk (chronic). Three mice were used for each group. Lung fixation was also performed on the mice exposed to hyperoxia at 16, 44, and 66 h. Fixation of lung tissue was performed by intratracheal instillation of 10% formalin (in PBS) at 20 cm water pressure. Four hours later, the lungs were transferred into 2% formalin at 4°C. Paraffin-embedded sections were made, and microscopic evaluation of the slides was performed.

Statistical analysis. Data statistical analysis was performed using SigmaStat (version 2.0) software on a personal computer. Both two-way ANOVA and Fisher’s least significant difference test were used. Statistical significance was reached using a P value of 0.05. Where indicated, results were expressed as a percentage of average values for vehicle-injected mice at the same time point, only after statistical analysis was performed. In addition, reference values for control mice (untreated mice, i.e., neither HDC nor vehicle) were given in the corresponding figure legends.

RESULTS

BAL protein and body weight. BAL protein levels were measured as indicators of lung injury after HDC. BAL protein levels increased significantly (1.5- to 2.5-fold) in HDC-treated mice at the first time points measured and remained elevated for the entire course of the study (Fig. 1A). In addition, the body weights of
mice treated with the HDC regimen were significantly lower than their vehicle controls. For HDC-treated mice, average differences in weight loss from vehicle-treated mice were 1.46 and 3.23 g at 48 h and 6 wk post-HDC, respectively (P < 0.05).

**BAL total and differential cell counts and peripheral blood counts.** BAL total cell counts over time displayed a multiphasic pattern (Fig. 1B). At 4 and 8 h post-HDC, there was an initial increase (1.6-fold) in total BAL cells (Fig. 1B), which predominantly consisted of macrophages (data not shown). This increase in macrophages was diminished at 12 h post-HDC. For week 1–4, total cell counts were significantly depressed to ~50% of baseline. This was followed by a significant (4.2-fold) increase in total cells at 6 wk (67,330 ± 9,960 vs. 16,000 ± 2,000 cells/ml). The most striking feature was a significant (11-fold) increase in the number of lymphocytes at 6 wk (Fig. 1C) and an increase in the number of neutrophils at 4–6 wk post-HDC (Fig. 1D). BAL eosinophils also showed an elevation at 6 wk, but the total cell numbers were small (3,867 ± 2,467 vs. 133 ± 88 cells).

Peripheral white blood cell (WBC) counts followed a pattern similar to BAL total cell counts. Compared with controls, HDC-treated mice showed significantly reduced peripheral WBC counts at 1 and 2 wk (37.2 ± 0.7% and 60.0 ± 6.6%, respectively). At later time points (4, 6, and 8 wk), peripheral WBC counts returned toward control values (77.9 ± 3.3, 87.8 ± 11.8, and 90.4 ± 6.5%, respectively). The absolute peripheral WBC count for control mice was 7.90 ± 0.16 × 10⁶ cells/ml of blood (mean ± SE).

**Tissue antioxidant profile.** The levels of lung glutathione reductase activity were greatly depressed immediately after HDC (Fig. 2A). At 4 h the glutathione reductase activity was significantly reduced by 3.2-fold compared with vehicle-injected controls (35.6 ± 2.8 vs. 115.2 ± 11.8 mU/mg protein). Glutathione reductase levels returned to normal by 72 h. Glutathione peroxidase activity in the lungs was minimally affected by this drug regimen, showing a mild 1.5-fold elevation 4 wk post-HDC (data not shown). In addition, the activities of glutathione S-transferase and superoxide dismutase in lung tissue were measured at 48 h and 6 wk post-HDC. No changes in their activities were detected at either time point (data not shown). Associated with the inhibition of glutathione reductase activity in the lung was a 3.3-fold increase in GSSG at 4 h post-HDC, and this returned to normal levels by 8 h (Fig. 2B). Total GSH levels in lung tissue were unchanged throughout the course of the study (Fig. 2C). After HDC, there was no evidence of enhanced lipid peroxidation in total lung preparations, as measured by tissue malondialdehyde (MDA) levels (Fig. 2D). To rule out the possibility that GSH in blood may affect lung GSH levels, lung perfusion with PBS was also performed. Perfusion of the lungs before their removal did not alter the levels of glutathione measured (data not shown).

**Extracellular (i.e., BAL) antioxidant profile.** The effects of oxidative stress on BAL constituents were measured at all time points. Total BAL GSH levels showed no evidence for GSH depletion and remained unchanged throughout the 8 wk follow-up, except for a significant elevation at 2 wk post-HDC (Fig. 3A). BAL GSSG levels were significantly elevated two- to threefold between 2 and 6 wk post-HDC (Fig. 3B). BAL lipid peroxidation in the form of MDA was elevated (1.2- to 10.220.33.3 on June 10, 2017 http://ajplung.physiology.org/ Downloaded from
1.6-fold) throughout the first week after HDC but returned to control levels at 2 wk and beyond (Fig. 3C). At 48 h and 6 wk post-HDC, superoxide dismutase activity was measured. No changes were found at either time point (data not shown).

Histopathology. Mouse lungs were examined for histopathology at 48 h and 6 wk after HDC. There were no discernible abnormalities noted at 48 h (data not shown). However, at 6 wk, the sectioned lungs of the cohort treated with the HDC regimen demonstrated distinct histopathological abnormalities compared with vehicle-injected controls, consistent with cytotoxic drug injury. Histopathological findings in the treated animals included the development of alveolar septal capillary congestion (Fig. 4, A and B). Cytopathological features in the treated lung were consistent with a reactive proliferation of bronchiolar epithelial cells characterized by an increase in epithelial cell size with enlargement of the nuclei accompanied by hyperchromatasia and the development of nucleoli (Fig. 4, C and D). These histopathological changes existed in the absence of any sign of infection or pneumonia (based on histopathology on a minimum of 15 mice).

To further explore the hypothesis that mice exposed to HDC are more sensitive to oxidative stress, we performed histopathological examination on hyperoxia-exposed animals. Control lungs (without HDC treatment) were normal at all time points (Fig. 5A). However, in the HDC-treated mice, there was evidence of perivascular interstitial edema with inflammatory cell influx throughout the lung sections of 66-h hyperoxia-exposed mice (Fig. 5B). There was no evidence of lung injury after 16 and 44 h of hyperoxia in HDC-treated mice (data not shown).

Antioxidant therapeutic supplementation. Ethylol, glutathione, and glutathione monoethyl ester were given intraperitoneally to mice 1 h before BCNU injection so we could assess their ability to protect against HDC-induced lung toxicity. Vehicle and BCNU-only animals were used as controls. The protective effects of antioxidant supplementation were studied at 48 h and 6 wk post-BCNU injection. At 48 h after HDC, BAL protein levels were twofold higher ($P < 0.05$) in the HDC-, Ethylol-, and glutathione monoethyl ester-treated animals vs. vehicle controls, whereas GSH prevented the acute rise in BAL protein levels (Fig. 6A). At 48 h, BCNU alone did not show an elevation in BAL protein levels. However, 6 wk post-HDC, all treatment groups had elevated BAL protein levels vs. vehicle controls (Fig. 6B). Furthermore, the BCNU-only treated mice had significantly higher levels of BAL protein than the vehicle control. More importantly, none of the three additional treatments blocked the lung lymphocyte inflammatory response seen at 6 wk post-HDC, although BCNU given alone did not show this response (Fig. 7).

To confirm that these GSH antioxidant supplements affected lung glutathione levels, we measured both total GSH levels in BAL at 4 h post-BCNU injection, a time when the greatest change in GSH would be expected. After Ethylol and GSH treatment, glutathione levels increased about twofold in BAL fluid, with no change in the oxidized fraction (Table 1). This was a transient elevation in GSH, because the levels of GSH in BAL at 48 h and 6 wk did not reveal any significant elevations in GSH after Ethylol and GSH administration (data not shown). However, glutathione monoethyl ester did not have any effect on extracellular glutathione status in BAL fluid. None of these three agents altered glutathione status in lung tissue at 4 h post-BCNU (data not shown).
DISCUSSION

HDC used for the treatment of breast cancer is known to cause severe pulmonary toxicity (44) and can be life threatening (31). To more fully understand this toxicity, we developed a mouse model using doses of HDC drugs equivalent to the doses given to humans on a weight basis and just at the maximally tolerated dose (8). Although our mouse model has obvious limitations when one attempts to extrapolate to human studies, it does offer the advantage of allowing one to study the effect of HDC alone, with no additional concomitant factors (e.g., prior standard chemotherapy, bone marrow transplant, concomitant radiotherapy, individual susceptibilities to the chemotherapy drugs, age, platelet counts, or differences in individual prior medical histories) (3).

All three drugs used in this HDC regimen have been previously shown to be individually cytotoxic to the lung. BCNU can inhibit lung glutathione reductase activity (21, 36) and deplete epithelial lining fluid glutathione levels (5, 7, 28). BCNU toxicity is generally attributed to multiple dosage regimens (21), but there are reports indicating lung toxicity from a single injection (23). This could consequently lead to higher levels of GSSG and/or depletion of GSH. Cyclophosphamide can cause lung injury and has also been shown to
deplete glutathione stores (11, 30, 42), increase the generation of reactive oxygen species by alveolar macrophages (12), inhibit prostaglandin E2 production by macrophages (11), and modulate BAL cell populations (12). Acrolein, a cyclophosphamide metabolite, can also react directly with glutathione, forming irreversible conjugates (18). Cisplatin has been shown to increase the production of superoxide anions (26) and to decrease the activity of lipid peroxide protecting enzymes, such as catalase, Cu/Zn superoxide dismutase, glutathione peroxidase, and glutathione S-transferase, in the liver and kidney of rats (33).

Our mouse model shows evidence supporting a modest oxidative stress, which may favor the development of lung injury. There was an initial transient increase in lung tissue GSSG, which was associated with inhibition of glutathione reductase activity in the lung at 4 h post-HDC. This elevated GSSG subsided as glutathione reductase activity was restored to normal levels by 72 h. Additionally, evidence for lipid peroxidation in BAL fluid, but not lung tissue, was detected in our mouse model. ELF also showed increases in GSSG starting at 2 wk post-HDC, a time point that approximates the influx of inflammatory cells. This could be due to an increase in glutathione synthesis via induction of cystine transport as has been found in endothelial cells exposed to BCNU (14), or, alternatively, the changes could reflect the influx of cells into BAL fluid rather than a direct effect on lung tissue (12).

To determine whether the oxidative stress seen in our lung injury model was physiologically relevant, we exposed mice (with and without HDC) to hyperoxia at various time points, before the development of hyper-

Table 1. Glutathione levels in BAL fluid after administration of glutathione-sparing agents

<table>
<thead>
<tr>
<th>Group</th>
<th>BAL Total GSH, μM</th>
<th>BAL, %GSSG</th>
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</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>13.35 ± 3.39</td>
<td>12.00 ± 2.44</td>
</tr>
<tr>
<td>HDC</td>
<td>25.52 ± 4.35</td>
<td>8.60 ± 1.02</td>
</tr>
<tr>
<td>HDC + Ethyl</td>
<td>51.38 ± 11.59*</td>
<td>7.00 ± 0.65</td>
</tr>
<tr>
<td>HDC + EthGSH</td>
<td>22.01 ± 1.87</td>
<td>9.83 ± 0.62</td>
</tr>
<tr>
<td>HDC + GSH</td>
<td>52.66 ± 0.78*</td>
<td>5.90 ± 0.40</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. The glutathione-sparing agents [Ethyl, glutathione monoethyl ester (EthGSH), or reduced glutathione (GSH)] were given to mice 1 h before 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) injection. Glutathione levels were measured 4 h after BCNU injection. Control mice received either vehicle or high-dose chemotherapy (HDC) alone. Each group consisted of 3–6 mice. BAL, bronchoalveolar lavage; GSSG, oxidized glutathione; %GSSG = [(2 × GSSG)/total GSH] × 100. *Significant difference from both vehicle and HDC-treated groups (P < 0.05).
oxia-induced lung injury (17). Hyperoxia-induced lung injury is a well-documented form of oxidative injury and has been shown to be detectable after 72 h of hyperoxia exposure (31). Therefore, we elected to study the histopathological changes taking place in the lung at 16, 44, and 66 h of hyperoxia exposure. After 66 h of hyperoxia exposure, HDC-treated mice showed signs of lung injury as indicated by perivasculary interstitial edema with inflammatory cell influx, whereas control mice (no HDC) showed no evidence for pathological injury. These data suggest that our HDC regimen is sufficient to cause a disturbance in the oxidant/antioxidant balance in the lung such that HDC-treated mice are more susceptible to hyperoxia.

To further test our hypothesis that oxidative stress occurs in the lung, we chose to supplement mice with various pharmaceutical agents that can offer protection against oxidative stress. Glutathione monoethyl ester is one of the best agents for protecting against mitochondrial glutathione depletion (2, 25, 37, 39) and could provide clues as to the cellular compartmentalization of damage. On the other hand, glutathione administration is known to greatly raise the glutathione concentration in the extracellular fluids (2, 25, 37, 39). Ethyl (amiostine) is Food and Drug Administration approved for use as a cytoprotective drug and must be dephosphorylated by cells that contain membrane-bound alkaline phosphatase (43, 47). We used these thiol scavengers to examine the importance of glutathione in this mouse HDC model. As expected, total GSH in BAL fluid was elevated at 4 h after a single intraperitoneal administration of Ethyl and GSH. However, these elevations in GSH were not maintained at 48 h and 6 wk. Administration of glutathione monoethyl ester did not affect fluid or lung tissue GSH levels, a finding reported by others (22, 32). Of these, only animals supplemented with glutathione showed lung-specific protection at 48 h after HDC.

Enzymes other than glutathione reductase could be more critical to lung tissue integrity. It is now known that BCNU can inhibit other tissue enzymes, which include lipoamide dehydrogenase (1), thioredoxin reductase (4), nicotinamide mononucleotide-adenyllyltransferase (15), as well as multidrug resistance protein (41). Others have also indicated that it is the decrease in the level of NADPH that is responsible for slowing down the reduction and recycling of GSSG (24). Therefore, the inhibition of glutathione reductase alone may not be enough to lead to lung injury.

An interesting and unexplained feature of lung toxicity seen in humans given the same drug combination is the delayed onset of pulmonary toxicity occurring 6–12 wk after the HDC regimen (44). Our animal model demonstrates marked increases in BAL inflammatory cell types at two time points: a response immediately after HDC as well as a delayed response beginning at ~4–6 wk. The early inflammatory response consists primarily of macrophages and occurs from 4 to 8 h. This initial macrophage recruitment may be due to cyclophosphamide’s ability to release low-molecular-weight chemotactic factors for macrophages (12). The delayed inflammation is marked by alveolar infiltrates consisting primarily of lymphocytes and secondarily by an increase in the number of neutrophils. We postulate that these inflammatory cells mediate an enhanced inflammatory response that can lead to progressive pulmonary dysfunction. In addition, the influx of inflammatory cells in BAL fluid at 4–6 wk correlated with the restoration of white blood cells counts to baseline values in peripheral blood. Blunting of this inflammatory response with prednisone may explain the improvement in lung function seen in individuals who develop DPTS (44). Furthermore, the combination of HDC drugs appears to be important for this response, as BCNU alone does not lead to delayed lung inflammation in our mouse model. Which cytokines or chemokines drive this response is not yet known. Some investigations have shown changes in cytokines to be a major contributor of lung toxicity (34, 35). Transforming growth factor-β may predict the development of pulmonary drug toxicity and veno-occlusive disease (27). It is not yet known why the BAL cell influx detected in this study occurs in a delayed fashion, but this combination of drugs clearly affects cell populations (20, 38, 45).

Our results indicate that there are at least two distinct phases associated with this HDC-induced lung damage. There is an initial acute phase of lung injury as shown by early elevations in BAL protein and lactate dehydrogenase. This lung injury pattern, as measured by BAL protein levels, persisted throughout the entire course of the study. The second phase is manifest by an inflammatory cellular infiltrate composed primarily of lymphocytes and neutrophils occurring 4–6 wk after HDC. We propose that this delayed inflammatory infiltrate augments the chronic low-grade toxicity that is seen early on. These findings suggest that pharmacological manipulations that block or attenuate the delayed inflammatory response may prove beneficial in protecting against the development of lung toxicity after this HDC regimen.

The authors thank Drs. William Petros and Stephen Young for critical review of this manuscript, Randy Fehdrau for providing the chemotherapy drugs, and Tom Tlusty for assistance with obtaining peripheral blood counts.

This work was funded, in part, by National Institutes of Health Grants HL-55166 and ES/HL-08698 and an American Heart Association Grant-in-Aid. During the early phases of this work, R. J. Folz was a Parker B. Francis Fellow in Pulmonary Research.

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