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Thiol-Mediated Regulation of ICAM-1 Expression in Endotoxin-Induced Acute Lung Injury


The intracellular redox state regulates several aspects of cell function, suggesting that strategies directed toward altering the cellular redox state may modulate cell activation in inflammatory states. As the most abundant intracellular thiol, glutathione plays a critical role as an intracellular redox buffer. Using diethylmaleate (DEM) as a glutathione-depleting agent, we evaluated the effects of GSH depletion in a rodent model of polymorphonuclear neutrophil (PMN)-dependent acute lung injury. Rats received 500 μg of LPS by intratracheal challenge, inducing a 5.5-fold increase in lung permeability and sixfold increase in lung PMN content. Pretreatment with DEM prevented the LPS-induced increase in lung PMN influx and lung permeability. Northern analysis and immunohistochemical studies suggest that this effect may be mediated by preventing up-regulation of lung ICAM-1 mRNA and protein expression. This effect is specific to ICAM-1, because lung cytokine-induced neutrophil chemoattractant and TNF-α mRNA levels are unaffected. This finding is not unique to the lung, because a similar effect on PMN influx was recapitulated in a rodent model of chemical peritonitis. Further, in vitro studies demonstrated that pretreatment of HUVEC monolayers with DEM prevented both ICAM-1 up-regulation and PMN transendothelial migration. These data indicate the presence of a thiol-sensitive mechanism for modulating ICAM-1 gene expression and suggest a potential novel therapeutic strategy for diseases characterized by PMN-mediated tissue injury. The Journal of Immunology, 1998, 160: 2959–2966.
reduction in PMN sequestration. In the present studies, we demonstrate that GSH depletion prevents the up-regulation of endothelial cell ICAM-1 expression in vivo and, in so doing, mitigates PMN influx into the lung. In vitro studies demonstrate that this is a direct effect on the endothelium and is sufficient to prevent PMN migration across an endothelial cell monolayer. Finally, the effect is not specific to the pulmonary capillary bed, since neutrophil migration into the peritoneal cavity following induction of chemical peritonitis is prevented by DEM pretreatment.

Materials and Methods

Reagents

Powdered Brewer’s thioglycollate and phenol-extracted Escherichia coli O111:B4 LPS were both from Difco Laboratories (Detroit, MI). Thioglycollate was dissolved in H2O, autoclaved, and stored in the dark at room temperature until uniformly green and clear. Murine TNF-α was purchased from Genzyme (Cambridge, MA). DEM, PGE1, human serum albumin (HSA), o-phenyldiamine hydrochloride (OPD), FMLP, and glutathione were all obtained from Sigma Chemical Company (St. Louis, MO). Hexadeccyltrimethylammonium bromide (HTAB) and phorone (disopropylidine acetone) were purchased from Fluka (Switzerland). N,N-dimethylformamide (DMF) and trypsin-EDTA were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 5-sulfosalicylic acid (SSA) were both purchased from Aldrich Chemical Co. (Milwaukee, WI). 32P-labeled albumin was obtained from Merck Frosst (Montreal, Quebec) and Na215CrO4 from Amersham (Oakville, ON). RPMI 1640 and calcium- and magnesium-free HBSS were obtained from Life Technologies (Grand Island, NY). Rabbit aortic smooth muscle biotinylated IgG was used as a secondary Ab for immunohistochemistry and was purchased from Dimension Laboratories (Mississauga, ON).

Induction of LPS-induced acute lung injury and systemic GSH depletion

All animal studies were performed in accordance with guidelines set forth by the Toronto Hospital Animal Care Committee and the Canadian Council on Animal Care. Lung injury induced by intratracheal LPS challenge has been well characterized. It is dependent on neutrophil influx mediated by up-regulation of ICAM-1 on the pulmonary capillary endothelium (6) as well as alveolar macrophage induction of TNF-α, IL-1, and CINC (18–20). Male Sprague Dawley rats weighing 250 to 275 g were obtained from Charles River Laboratories (Constate, Quebec). Animals were housed in standard wire bottom cages, fed standard rat chow and water ad libitum, and were allowed to acclimatize before use. To accomplish systemic GSH depletion, animals were administered various doses of DEM or phorone by i.p. injection following an overnight fast. One hour following the administration of the GSH-depleting agent, animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and a tracheostomy was performed. At the time of kill, lungs were inflated with approximately 10 ml of methanol/acetic acid (95%/5%) and sections obtained for immunohistochemical analysis. Tissues were stained with anti-ICAM-1 mAb clone 1A29, followed by a biotinylated goat anti-mouse IgG (Dimension Laboratories, Mississauga, ON). After rinsing, a streptavidin-horseradish peroxidase conjugate was added. The peroxidase reaction was developed by immersion in a freshly prepared solution of 0.02% 3,3′-diaminobenzidine and 0.005% H2O2 in 0.05 M Tris buffer, pH 7.6, followed by hematoxylin counterstaining. Appropriate negative controls were performed using secondary Ab alone.

Assessment of lung injury

Pulmonary transcapillary albumin transit was assessed by injection of 1 μCi of 125I-labeled albumin into the inferior vena cava 30 min before kill. At the end of the experimental protocol, rats were ventilated, heparin (100 U/kg) was injected into the right ventricle, and 1 ml of blood was withdrawn by cardiac puncture. Following exsanguination, lungs were perfused blood-free by cannulating the pulmonary artery and infusing 10 ml of a low-potassium dextran solution containing 0.5 μL of PGE1. The left ventricle, left atria, and mitral valve were opened widely to allow free drainage of effluent. The right lung was immediately frozen in liquid nitrogen for assay of myeloperoxidase activity, nonprotein thiol, and evaluation of ICAM-1 and TNF-α mRNA expression. The left lung was used to calculate a permeability index (PI) as follows: PI = (lung cpm / g tissue) / (blood cpm / ml).

Myeloperoxidase assay

Lung samples were thawed and approximately 0.2 g of tissue was homogenized in 25 ml of potassium phosphate (10 mM, pH 7.4) for 1 min using a Brinkman Polytron (model PT10/35, Brinkman Instruments, Inc., Westburg NY). The homogenate was centrifuged at 12 000 × g for 20 min at 4°C. The supernatant was discarded and the pellet resuspended and homogenized in 25 ml potassium phosphate (50 mM, pH 6.0) containing 0.5% HTAB. The homogenate was frozen overnight at −70°C, rehomogenized for 1 min, and sonicated (model VC 50T, Sonics and Materials Inc., Danbury, CT) at 60 W for 1 min. After centrifugation as above, the supernatant was collected and used for both MPO and protein assay.

MPO activity was assessed at pH 5.4 and 37°C. The change in absorbance at 655 nm during the first 3 min was measured using the Cobas FARA II Chemistry System (Roche Diagnostic Systems, Montclair, NJ). Each cuvette contained 25 μl of sample, 25 μl of 16.33,5,5′-tetramethylbenzidine dissolved in N,N-dimethylformamide and 175 μl of 220 mM potassium phosphate buffer containing 110 mM NaCl. The reaction was initiated by addition of 25 μl of 3.0 mM H2O2, and the change in absorbance during the first 3 min was measured. The absorbance change per minute was used as a measure of MPO activity. Results are expressed as MPO activity per microgram of protein. Protein concentrations were determined using the Pierce BCA protein assay (Pierce, Rockford, IL).

Glutathione assay

Quantitation of total lung and endothelial nonprotein sulfhydryls (NPSH) was assessed using a DTNB-based assay as described by Jocelyn (21). Lung tissue was thawed and weighed, and approximately 0.25 g was homogenized in 2 ml of 5% 5-sulfosalicylic acid (SSA). The homogenate was sonicated for 30 s and centrifuged for 10 min at 10,000 × g. The resultant acid thiol extract was assayed for nonprotein sulfhydryls by quantitating the reduction of DTNB through its conversion to 5-thio-2-nitrobenzoic acid (TNB) at 412 nm using a spectrophotometer. Sample values were then calculated from a standard curve generated using known amounts of GSH and are expressed as GSH equivalents per gram of lung tissue.

ICAM-1 immunohistochemistry

At the time of kill, lungs were inflated with approximately 10 ml of methanol/acetic acid (95%/5%) and sections obtained for immunohistochemical analysis. Tissues were stained with anti-ICAM-1 mAb clone 1A29, followed by a biotinylated goat anti-mouse IgG (Dimension Laboratories, Mississauga, ON). After rinsing, a streptavidin-horseradish peroxidase conjugate was added. The peroxidase reaction was developed by immersion in a freshly prepared solution of 0.02% 3,3′-diaminobenzidine and 0.005% H2O2 in 0.05 M Tris buffer, pH 7.6, followed by hematoxylin counterstaining. Appropriate negative controls were performed using secondary Ab alone.

Lung RNA extraction and Northern blot analysis

Total RNA from lungs was obtained using the guanidium-isothiocyanate method (22). Briefly, lungs were harvested from treated animals and immediately frozen in liquid nitrogen. Lungs were then thawed and homogenized in 2 ml of 5% 5-sulfosalicylic acid (SSA). The homogenate was assessed using a DTNB-based assay as described by Jocelyn (21). Lung tissue was thawed and weighed, and approximately 0.25 g was homogenized in 2 ml of 5% 5-sulfosalicylic acid (SSA). The homogenate was sonicated for 30 s and centrifuged for 10 min at 10,000 × g. The resultant acid thiol extract was assayed for nonprotein sulfhydryls by quantitating the reduction of DTNB through its conversion to 5-thio-2-nitrobenzoic acid (TNB) at 412 nm using a spectrophotometer. Sample values were then calculated from a standard curve generated using known amounts of GSH and are expressed as GSH equivalents per gram of lung tissue.

ICAM-1 expression in vitro

Rat heart endothelial cells (RHEC) were isolated from 5-day-old rats according to the method of Kasten (24) and allowed to grow to confluence in 75-cm2 flasks. HUVEC were isolated as previously described (25). The endothelial cells were harvested using trypsin-EDTA treatment and seeded into 96-well, flat-bottom microtiter plates in minimal essential media with 20% FCS. When the monolayers had reached confluence (approximately 4 days) cells were pretreated with DEM for 30 min followed by activation with 20% LPS. After 6 h, supernatants were collected for measurement of lactate dehydrogenase (LDH) activity. Cells were washed twice with RPMI 1640/85% FCS and incubated with mouse anti-rat (clone 1A29) or anti-human ICAM-1 (clone R11/1) mAb for 1 h, washed, and then incubated with peroxidase-conjugated goat anti-mouse IgG for 1 h. Cells were
washed again and color development initiated by the addition of the substrate, o-phenyldiamine hydrochloride (OPD). After 18 min, the reaction was stopped with 3 M sulfuric acid, and the optical density at 492 nm was determined using a microtiter plate reader. Data are expressed as the change in absorption from baseline. Quantitation of supernatant LDH activity as a measure of cytotoxicity was determined using spectrophotometric methods as previously described (26). Previous reports have demonstrated that LDH activity is not affected by DEM or other sulfhydryl-reactive agents (27).

**PMN transendothelial migration**

Human PMN were isolated from healthy volunteers by collecting blood into heparinized tubes. Neutrophils were isolated by dextran sedimentation and centrifugation through a discontinuous Ficoll gradient (28). PMN purity as assessed by size and granularity on flow cytometry was consistently greater than 95%. PMN were labeled with Na251CrO4 and then resuspended in RPMI 1640 containing 0.5% HSA and 10 mM HEPES (pH 7.4) at a concentration of 10 × 106/ml for 30 min and then washed. Transwell chambers with polycarbonate membranes of 3.0 μM pore size (Costar, Cambridge, MA) were coated for 1 h with fibronectin (50 μg/ml) and then seeded with 1 × 105 HUVEC. Cells were incubated in MEM with 20% FCS until confluence as demonstrated by prevention of 125I-labeled albumin diffusion across the endothelial monolayer. PMN transendothelial migration was assessed as described previously (29). Briefly, after treatment of endothelial cells with LPS ± DEM, the upper and lower surfaces of the HUVEC filter units were washed with medium and then transferred to a new, clean well (lower compartment). To this well, 0.6 ml of RPMI 1640 (10 mM HEPES, pH 7.4, 0.5% HSA) was added containing the chemo- tactic peptide FMLP (10-7 M). Before immersion of the HUVEC filter unit, 0.1 ml of medium containing 2 × 105 labeled PMN was added above the HUVEC. After a 45-min incubation, migration was stopped by washing the upper compartment twice with 0.1 ml of RPMI 1640 to removed non-adherent PMN. The undersurface of the filter was then vigorously rinsed with 2 ml of ice-cold PBS, 0.2% EDTA solution and collected into the lower compartment. The cells that migrated into the lower compartment or that were detached from the undersurface of the filter were lysed by the addition of 0.5% Triton X-100 and analyzed for 51Cr using a Wallac 1410 Wizard automatic gamma counter (Turku, Finland). The results are expressed as the percentage of the total 51Cr PMN added above the HUVEC monolayer that migrated through the HUVEC filter unit. All the stimulation conditions were performed with triplicate replicates.

**Statistical analysis**

Results are expressed as mean ± SEM of the indicated number of studies. Statistical significance among the group means was assessed by one-way analysis of variance. Post hoc testing was performed using the Bonferroni modification of the t test.

**Results**

**Effect of glutathione depletion on LPS-induced lung injury**

Intratracheal challenge with LPS caused a significant increase in the lung permeability index (Control: 0.04 ± 0.02 vs LPS: 0.22 ± 0.03; \( p < 0.001 \)) as assessed by the transcapillary flux of 125I-labeled albumin (Fig. 1A). Pretreatment with a single dose of DEM (6 mmol/kg, i.p.) 1 h before intratracheal challenge had no effect on lung permeability in control animals, yet completely prevented the increase in permeability associated with LPS challenge. This protective effect was not evident at doses less than 6 mmol/kg (Fig. 1B). Two lines of evidence suggest that the effect of DEM was related to its ability to deplete glutathione. First, DEM conferred protection at 6 mmol/kg, a dose that was capable of reducing GSH by more than 80%, but not at lower doses that had limited ability to reduce GSH levels (Fig. 1B). Second, we evaluated the effect of phorone, another rapidly acting GSH-depleting agent. Phorone (250 mg/kg) administered by i.p. administration 1 h before intratracheal LPS challenge induced a comparable reduction in lung GSH levels to that seen with DEM (Fig. 2A). As shown in Figure 2B, treatment with phorone recapitulated the effect on lung permeability demonstrated with DEM, with almost complete prevention of the increase in lung permeability induced by LPS challenge.

**Effect of DEM on lung PMN accumulation following intratracheal LPS**

We had previously shown reduced numbers of PMN in the bronchoalveolar lavage fluid of animals treated with DEM before LPS challenge. Histologic evaluation of lung tissue following LPS treatment in the presence or absence of DEM provided some insight into the mechanism whereby DEM exerted its effect (Fig. 3). LPS challenge causes thickening of the interalveolar septa due to interstitial edema and influx of PMN into the lung interstitium. By contrast, in animals pretreated with DEM, PMN are absent from both within the alveolar spaces and the interstitium. Further, PMN do not appear to be trapped within the pulmonary capillaries. The reduction in PMN sequestration in the lung is also evident upon evaluation of lung MPO activity. Unlike the quantitation of bronchoalveolar lavage fluid neutrophils, lung MPO activity evaluates total lung PMN content and is a quantitative measure of PMN demonstrated on histologic sections. As shown in Figure 4, LPS induces a 6-fold increase in lung MPO activity. DEM has no effect on MPO activity in control animals, yet significantly attenuates the increase in lung MPO following LPS challenge. We have previously demonstrated that DEM induces a neutrophilia, thus excluding systemic neutrophil depletion as a possible mechanism for this effect (17). Taken together, these data suggest that DEM prevents LPS-induced PMN sequestration into the lung and the ensuing transmigration into the interstitium and alveolar spaces.

**FIGURE 1.** A. Effect of DEM on lung injury as assessed by the transcapillary flux of 125I-labeled albumin. Animals were pretreated with DEM (6 mmol/kg) or saline by i.p. injection 1 h before intratracheal endotoxin (500 μg) or vehicle challenge and killed 4 h later. Data are expressed as mean ± SEM of at least four animals per group. \( * p < 0.001 \) vs no LPS, \( \dagger p < 0.001 \) vs LPS, no DEM. B. Effect of increasing the administered dose of LPS on lung injury and lung thiol levels. Data are expressed as mean ± SEM of four animals per group. Bars, permeability index; solid line, lung thiol levels. \( * p < 0.001 \) vs LPS, no DEM (0 mmol/kg), \( \dagger p < 0.001 \) vs LPS, no DEM.
Effect of DEM on lung ICAM-1 expression

Lung PMN sequestration is dependent on interactions between adhesive ligands on the PMN and the pulmonary capillary endothelium. Abs directed against $\beta_2$ integrins or ICAM-1 have been reported to prevent lung PMN influx in LPS-induced acute lung injury (6). Having previously demonstrated that PMN up-regulation of the $\beta_2$ integrin CD11b/CD18 was unaffected by GSH depletion (17), we assessed whether pretreatment with DEM attenuated LPS-induced up-regulation of its complementary endothelial ligand, ICAM-1. Analysis of lungs obtained from sham animals stained with an anti-ICAM-1 Ab demonstrated low level ICAM-1 expression within the alveolar septa and the epithelial surface (Fig. 5), a reflection of low level constitutive ICAM-1 expression on the pulmonary capillary endothelium and alveolar epithelial cells, respectively. Four hours following intratracheal challenge with LPS, ICAM-1 protein expression is markedly up-regulated. By contrast, up-regulation of ICAM-1 expression following LPS challenge was not evident in animals pretreated with DEM, suggesting that GSH depletion may be preventing lung leukosequestration through an inhibitory effect on ICAM-1 expression.

Differential effect of DEM on pulmonary expression of ICAM-1, CINC, and TNF-α mRNA

To determine whether the observed effect of DEM on LPS-induced ICAM-1 up-regulation was due to modulation of ICAM-1 gene expression, we evaluated lung ICAM-1 mRNA expression using Northern analysis. As demonstrated in Figure 6A, constitutive ICAM-1 mRNA expression was detectable in whole lungs obtained from control animals. In animals receiving LPS there was a

FIGURE 2. A, Effect of phorone on lung thiol levels. Animals were pretreated with phorone (250 mg/kg) or saline by i.p. injection 1 h before intratracheal endotoxin (500 $\mu$g) or vehicle challenge and killed 4 h later. Data are expressed as mean ± SEM of four animals per group. *p < 0.001 vs no phorone. B, Effect of phorone on LPS-induced lung injury. Animals were pretreated with phorone (250 mg/kg) or saline by i.p. injection 1 h before intratracheal endotoxin (500 $\mu$g) or vehicle challenge and killed 4 h later. Data are expressed as mean ± SEM of four animals per group. *p < 0.001 vs no LPS, †p < 0.001 vs LPS, no phorone.

FIGURE 3. Composite photomicrograph showing representative histologic section of lungs from animals pretreated with DEM compared with controls. Animals were pretreated with DEM (6 mmol/kg) or saline by i.p. injection 1 h before intratracheal endotoxin (500 $\mu$g) or vehicle challenge and killed 4 h later. Sections were stained with hematoxylin and eosin. Representative of one of three animals per group. Magnification, ×300.

FIGURE 4. Effect of DEM on lung PMN influx as assessed by quantitation of total lung MPO activity. Animals were pretreated with DEM (6 mmol/kg) or saline 1 h before intratracheal endotoxin (500 $\mu$g) or vehicle challenge and killed 4 h later. Data are expressed as mean ± SEM of four to eight animals per group. *p < 0.001 vs no LPS, no DEM. †p < 0.001 vs LPS, no DEM, p < 0.01 vs no LPS, DEM.

FIGURE 5. Effect of DEM on lung ICAM-1 expression. Immunohistochemical analysis of lungs obtained from animals pretreated with DEM compared with controls. Animals were pretreated with DEM (6 mmol/kg) or saline by i.p. injection 1 h before intratracheal endotoxin (500 $\mu$g) or vehicle challenge and killed 4 h later. Lungs were fixed by intratracheal instillation of methanol/acetic acid and stained with an anti-ICAM-1 mouse anti-rat mAb as described in Materials and Methods. Representative of one of three animals per group. Magnification, ×300.
significant increase in ICAM-1 mRNA expression. However, consistent with its effects on tissue expression of ICAM-1 protein, DEM pretreatment almost completely prevented up-regulation of ICAM-1 mRNA expression following challenge with LPS. The effect of DEM on the expression of two other genes whose products are considered to contribute to lung injury was next evaluated. The LPS-stimulated increase in mRNA levels of CINC, a chemotactic protein clearly shown to be important in LPS-induced neutrophil sequestration (20), was modestly reduced by DEM pretreatment. ICAM-1 mRNA expression following challenge with LPS. In preliminary studies, DEM at 250 µM was sufficient to impair PMN transmigration in vitro. To determine whether the effect of DEM on ICAM-1 expression was species specific, this parameter was similarly evaluated in HUVEC. As is evident in Figure 7C, HUVEC pretreated with DEM, followed by activation with LPS, demonstrated a similar attenuation in ICAM-1 up-regulation.

**Effect of DEM on PMN transendothelial migration in vitro**

Transendothelial migration in vitro is a functional measure of PMN-endothelial cell interactions. To determine whether the reduction in ICAM-1 expression mediated by thiol depletion was sufficient to impair PMN transmigration, we evaluated the effects of endothelial cell thiol depletion on PMN transmigration in vitro. HUVEC monolayers atop a 3-µm pore polycarbonate filter in a dual compartment chamber were pretreated with DEM for 30 min followed by activation with LPS (1 µg/ml) for 6 h. Human peripheral blood neutrophils were then placed above the monolayer, and the rate of transendothelial migration into the bottom chamber in response to FMLP (10^{-7}) was evaluated. As shown in Figure 8, there is an increase in the number of PMN transmigrating across the filter following activation of the endothelium by LPS. By contrast, DEM pretreatment attenuates this increase such that the rate of transmigration is equivalent to that seen under basal conditions. In parallel studies, pretreatment with anti-ICAM-1 Ab resulted in inhibition of transmigration comparable to that observed for DEM (data not shown), suggesting that the reduction in ICAM-1 expression is sufficient to account for the observed effect.

**Effect of DEM on thioglycollate-induced PMN influx into the peritoneal cavity**

Antibody strategies aimed at preventing PMN sequestration at various extravascular sites have demonstrated site specificity. To determine whether the effect of GSH depletion on PMN trafficking was specific to the pulmonary microvasculature, we evaluated the effect of DEM on thioglycollate-induced PMN influx into the peritoneal cavity. Four hours following the administration of thioglycollate by i.p. injection, the number of peritoneal exudate neutrophils increased from 0.71 ± 0.9 x 10^7 to 4.1 ± 5.5 x 10^7 (p < 0.01). This increase was almost completely attenuated by pretreatment with DEM (Fig. 9). DEM had no effect on the basal cell number within the peritoneal cavity.
The present studies demonstrate that pharmacologic agents characterized by their ability to deplete intracellular thiols are able to reduce the magnitude of lung injury in an experimental model of ARDS. The major histopathologic correlate of this effect was reduced PMN sequestration in the lung as evidenced both by histology and levels of total lung MPO activity. This finding suggested that these agents might act by inhibiting the PMN-endothelial interactions necessary to initiate the inflammatory process. Since we had previously shown that PMN $\beta_2$ integrin up-regulation and transmigration in response to chemotactic stimuli was unaffected by thiol depletion (17), we postulated that the salutary effect on lung injury might be due to altered endothelial cell activation. The major observation in the present studies is that DEM treatment prevented ICAM-1 up-regulation in response to inflammatory stimuli. In vivo, the increase in ICAM-1 protein and gene expression in the lung induced by LPS was totally abrogated by prior treatment with DEM. This was not a global effect on LPS-induced gene expression, since the rise in lung TNF-$\alpha$ and CINC mRNA was unaffected. Further, DEM exerted a similar effect on rat and human endothelial cells in vitro without cytotoxicity or an alteration in barrier function. This finding suggests that these agents might exert their actions directly on endothelial cells in vivo, although it does not rule out the possibility that DEM might inhibit other cells in vivo from releasing cytokines that induce endothelial cell ICAM-1 expression. However, as mentioned above, one candidate mediator, TNF-$\alpha$, did not differ between untreated and DEM-treated animals at the protein or mRNA level.

Although the principal effect of DEM in vivo is GSH depletion, nonspecific effects of this agent have been reported. For example DEM may inhibit protein synthesis, a phenomenon that is an unlikely contributor to the current observations because phorone recapitulates the effect of DEM but has no effect on protein synthesis (31). Further, BALF levels of TNF-$\alpha$ are equivalent in DEM-treated and control animals. DEM has also been reported to reduce

**Discussion**

The present studies demonstrate that pharmacologic agents characterized by their ability to deplete intracellular thiols are able to reduce the magnitude of lung injury in an experimental model of ARDS. The major histopathologic correlate of this effect was reduced PMN sequestration in the lung as evidenced both by histology and levels of total lung MPO activity. This finding suggested that these agents might act by inhibiting the PMN-endothelial interactions necessary to initiate the inflammatory process. Since we had previously shown that PMN $\beta_2$ integrin up-regulation and transmigration in response to chemotactic stimuli was unaffected by thiol depletion (17), we postulated that the salutary effect on lung injury might be due to altered endothelial cell activation. The major observation in the present studies is that DEM treatment prevented ICAM-1 up-regulation in response to inflammatory stimuli. In vivo, the increase in ICAM-1 protein and gene expression in the lung induced by LPS was totally abrogated by prior treatment with DEM. This was not a global effect on LPS-induced gene expression, since the rise in lung TNF-$\alpha$ and CINC mRNA was unaffected. Further, DEM exerted a similar effect on rat and human endothelial cells in vitro without cytotoxicity or an alteration in barrier function. This finding suggests that these agents might exert their actions directly on endothelial cells in vivo, although it does not rule out the possibility that DEM might inhibit other cells in vivo from releasing cytokines that induce endothelial cell ICAM-1 expression. However, as mentioned above, one candidate mediator, TNF-$\alpha$, did not differ between untreated and DEM-treated animals at the protein or mRNA level.

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**FIGURE 7.** Effect of DEM on endothelial ICAM-1 expression in vitro. A, Expression of ICAM-1 on RHEC pretreated with DEM (250 $\mu$M) for 30 min before addition of LPS (1 $\mu$g/ml) for 6 h. ICAM-1 expression was determined by ELISA as described in Materials and Methods. Data are mean ± SEM of duplicate wells from four experiments. *, $p < 0.01$ vs control. †, $p < 0.05$ vs no DEM. B, Effect of delayed addition of DEM on RHEC ICAM-1 expression. LPS (1 $\mu$g/ml) was added to the culture medium at time 0. DEM (250 $\mu$M) was added from 30 min prior ($-30$) to 60 min following ($+60$) addition of LPS. ICAM-1 expression was evaluated 6 h following addition of LPS. Data are mean ± SEM of duplicate wells from three experiments. *, $p < 0.01$ vs control. †, $p < 0.05$ vs no DEM. C, Expression of ICAM-1 on HUVEC pretreated with DEM (250 $\mu$M) for 30 min before addition of LPS (1 $\mu$g/ml) for 6 h. Data are mean ± SEM of duplicate wells from three experiments. *, $p < 0.01$ vs no LPS. †, $p < 0.05$ vs no DEM. Note nonuniform origin.

**FIGURE 8.** PMN transendothelial migration across DEM-pretreated HUVEC monolayers. HUVEC monolayers were grown to confluence on 3-$\mu$m pore polycarbonate filters, pretreated with DEM (250 $\mu$M) for 30 min, followed by activation with LPS (1 $\mu$g/ml) for 6 h. Human PMN transmigration across the monolayers in response to FMLP (10$^{-7}$ M) was assessed as described in Materials and Methods. Data are mean ± SEM of triplicate wells from three experiments. *, $p < 0.05$ vs no DEM. †, $p < 0.05$ vs no LPS, DEM.

**FIGURE 9.** Effect of DEM on thioglycollate-induced cell influx into the peritoneal cavity. Animals were pretreated with DEM (6 mmol/kg) or saline 1 h before administration of thioglycollate or saline (10 ml) by i.p. injection. Animals were killed 4 h later. Data are expressed as mean ± SEM of three animals per group. *, $p < 0.001$ vs saline, no DEM. †, $p < 0.001$ vs thioglycollate, no DEM.
core body temperature by approximately 3.0°C (31). This hypothermic effect is an unlikely explanation for these data because core body temperature was maintained through the use of warming blankets.

Several lines of evidence suggest that the protective effects of DEM are related specifically to the thiol-depleting properties of this agent. First, the effect of DEM correlated with its ability to deplete lung GSH. Specifically, concentrations of DEM able to deplete GSH were protective, while lower concentrations had no effect. Secondly, in a previous report we have demonstrated that the beneficial effects of DEM parallel the kinetics of GSH deple-
tion (17). Finally, phorone, an unrelated thiol-depleting agent, had a similar protective effect, excluding the possibility that this phe-
nomenon was due to a conjugate or metabolite of DEM. Taken together, these data suggest that the salutary effects of DEM are related to the ability of this agent to deplete cellular glutathione.

The mechanism whereby thiol depletion might prevent ICAM-1 up-regulation and thus exert its beneficial effect in vivo requires further study. Northern analysis of total lung mRNA demonstrates that thiol depletion mediates its effect through prevention of the LPS-induced increase in ICAM-1 mRNA expression. ICAM-1 mRNA expression is regulated at the transcriptional level and by modulating the rate of mRNA degradation (32, 33). In other cell systems, both of these processes have been shown to be modulated by changes in intracellular thiol concentrations. The promoter region of ICAM-1 contains a consensus binding motif for NF-kB. Although thiol oxidation has been reported to increase nuclear translocation of NF-kB, recent evidence suggests that the overall effect may be more complex due to redox-sensitive events at the level of DNA binding (34). In the nucleus, thiol oxidation has been shown to prevent the binding of NF-kB to its DNA binding site by forming mixed disulfides at a redox-reactive cysteine in the DNA binding region of NF-kB, potentially leading to a reduction in NF-kB-dependent gene expression. Relevant to this proposed mechanism, alveolar macrophage expression of TNF-α and CINC is also dependent on NF-kB activation and is responsive to redox manipulation (16, 35). The lack of any significant effect on these macrophage products suggests either a greater resistance of mac-
rophages to glutathione depletion or the presence of a greater po-
tential for redox buffering in these cells. Alternatively, the system-
ically administered DEM may achieve greater or more sustained levels in endothelium as compared with macrophages.

Alternatively, thiol depletion may play a role in modulating ICAM-1 mRNA stability. The 3’ untranslated region of ICAM-1 mRNA has several reiterations of the pentamer adenosine-uridine-uridine-uridine-adenosine (32). This adenosine-uridine-rich element (ARE) is a relatively well-conserved motif in several labile transcripts. Recently several transl-acting mRNA binding proteins that interact with the ARE have been identified. The binding ac-
tivity of these proteins may either proportionally or inversely correlate with transcript stability (36). Further, critical thiol groups at the mRNA binding site may modulate binding to the ARE and thus alter mRNA stability (37), suggesting that a reduction in ICAM-1 mRNA stability may account for the reduction in steady state ICAM-1 mRNA observed following thiol depletion.

ICAM-1 is an important ligand for the β2 integrin family of leukocyte membrane glycoproteins. Interactions between these complementary surface molecules mediate firm adhesion of the PMN to the endothelial cell and are a prerequisite for PMN trans-
migration across endothelial cell monolayers in vitro and into the interstitium in vivo (38). The observed effects of GSH depletion on PMN influx into the lung and peritoneal cavity are thus consistent with the importance of ICAM-1 in these processes. However, the profound reduction in lung neutrophil sequestration in animals pre-
treated with DEM suggests that this agent may have additional effects on PMN-endothelial interactions apart from the effect on ICAM-1 expression demonstrated in the present studies. We previously reported that DEM administration in vivo had no effect on the levels of CD11b/CD18 on the surface of circulating neutrophil.

Recent studies have demonstrated that CD11a/CD18 in ad-

tion to CD11b/CD18 may play a significant role in PMN seque-
tration during inflammation (6). Further, these studies did not specifically address alterations in the adhesive interactions of these cells. Alternatively, DEM may have additional effects on other endothelial cell adhesion molecules involved in lung leukoseque-
tration including E-selectin and VCAM-1. These possibilities require further study.

A limited number of other studies have demonstrated beneficial effects associated with GSH depletion, although the mechanisms for these effects were not evaluated. Both buthionine sulfoximine (BSO), an agent that prevents de novo GSH synthesis, and DEM reduced mortality following cerebral ischemia-reperfusion injury (39). Thiol depletion has also been reported to be protective in a murine model of endotoxin/galactosamine-induced hepatic necro-

sis (40). The common pathogenetic link between these reported observations and the current studies is the central role of PMN-
endothelial cell interactions leading to PMN sequestration and tar-
gent organ injury. The present studies provide sound evidence that the salutary effect observed in each model may have been related to impaired up-regulation of endothelial cell adhesion molecules.

Importantly, this information suggests that treatment with thiol-
depleting agents may be generally effective in pathologic processes mediated by endothelial cell-PMN interactions involving ICAM-1, a phenomenon demonstrated by the abrogation of thioglycollate-
elicted PMN influx into the peritoneal cavity in animals pretreated with DEM in the current study.

There is substantial evidence to suggest that alterations in the redox state of the cell may modulate cellular and gene activation. The data presented herein demonstrate that cellular redox manip-
ulation achieved by depletion of intracellular thiol levels may prove useful in modulating the broad range of disease processes characterized by neutrophilic infiltration. While lowering of the predominant endogenous cellular antioxidant may seem an un-
likely therapy for disease processes characterized by neutrophil-
mediated tissue injury, this strategy appears to be effective by virtue of its ability to prevent neutrophil sequestration at the inflamma-
tory site. This suggests that redox manipulation may be most effective if directed toward the modulation of cell activation rather than post hoc attempts at minimizing PMN-mediated injury to target organs, a strategy that has achieved only modest success in man (41).

References