Mercaptoethylguanidine Inhibits the Inflammatory Response in a Murine Model of Chronic Infection with *Pseudomonas aeruginosa*

ROBERT W. WILMOTT, JOSEPH A. KITZMILLER, CSABA SZABÓ, GARRY J. SOUTHAN, and ANDREW L. SALZMAN

Division of Pulmonary Medicine, The Children’s Hospital Research Foundation, Cincinnati, Ohio (R.W.W., J.A.K., A.L.S.); and Inotek Corporation, Beverly, Massachusetts (C.S., G.J.S., A.L.S.)

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ABSTRACT

Chronic airway inflammation induced by *Pseudomonas aeruginosa* is the eventual cause of respiratory failure in most people affected by cystic fibrosis. Recent evidence implicates the involvement of free radical and oxidant stress in the pathogenesis of the inflammatory injury. Here we report the efficacy of a novel experimental therapeutic, mercaptoethylguanidine (MEG), which has combined actions as a selective inhibitor of the inducible nitric oxide synthase and as a scavenger of peroxynitrite, a potent oxidant formed in the reaction of nitric oxide and superoxide radical. Chronic pulmonary infection was established in FVB/N mice by intratracheal administration of $10^5$ colony-forming units of *P. aeruginosa* in agar beads. Treatment with MEG (10 mg/kg/dose every 8 h i.p.) inhibited weight loss in the first 3 days and reduced histologic injury at 8 days postinfection. MEG also reduced myeloperoxidase activity, a marker of neutrophil infiltration, at 8 days and concentrations of the proinflammatory cytokines interleukin-1β, tumor necrosis factor-α, and macrophage inflammatory protein 2 in whole lung homogenates. MEG-treated animals and controls had similar perioperative mortality and comparable colony counts of *P. aeruginosa* at 8 days, indicating that MEG did not exacerbate infection. Our data suggest that MEG may be an effective immunomodulatory therapy of pulmonary inflammation induced by chronic infection.

Cystic fibrosis (CF) is a complex, systemic, autosomal recessive condition caused by mutations in the CF transmembrane conductance regulator (CFTR) gene on chromosome 7 (Riordan et al., 1989; Rommens et al., 1989). CF is characterized by recurrent respiratory infections with progressive obstructive lung disease, pancreatic insufficiency, and increased sweat electrolyte concentrations. Initial pulmonary infections in CF are usually caused by *Staphylococcus aureus*, and eventually CF patients become colonized with *Pseudomonas aeruginosa*, especially mucoid strains. Patients with such colonization have lower survival rates than non-colonized patients.

Inflammation is a major component of the airways disease in CF. There is a marked pulmonary neutrophilia, and neutrophil products such as elastase, cathepsin G, and eicosanoids contribute to the inflammatory process (Berger, 1991). Extremely elevated concentrations of interleukin-1 and interleukin-8 are present in airway surface liquid and appear to contribute to the recruitment of neutrophils to the lung (Berger, 1991; Wilmott, 1999).

The inflammatory nature of the pulmonary response to chronic infection has indicated that the host response, rather than the infection per se, may be the principal determinant of parenchymal injury. Accordingly, there have been a variety of immunomodulatory approaches proposed to alleviate lung injury. Children with CF who received prednisone (2 mg/kg) on alternate days had a decreased severity of pulmonary disease, demonstrated by improved lung function, decreased hospital admission rate, and improved weight and height compared with controls, after four years (Auerbach et al., 1985). The beneficial effects of systemic steroids on pulmonary function were less impressive in a more recent double-blind, placebo-controlled, multicenter study of 285 patients and were offset by high rates of diabetes and cataract formation (Eigen et al., 1995). Other approaches using anti-inflammatory drugs have focused on inhibiting the immune response to chronic bacterial infection. Such approaches have included the use of inhaled corticosteroids, high-dose oral ibuprofen, and systemic pentoxifylline, a methylxanthine

**ABBREVIATIONS:** CF, cystic fibrosis; cfu, colony-forming units; NO, nitric oxide; iNOS, inducible nitric oxide synthase; NOD, nitric oxide dioxygenase; MEG, mercaptoethylguanidine; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; MIP-2, macrophage inflammatory protein 2; ELISA, enzyme-linked immunosorbent assay; EtOH, ethanol; mod, mill optical density.
that inhibits expression of tumor necrosis factor-α (Wilmott, 1999).

Accumulating evidence points to an important role for nitric oxide (NO), a nitrogen-centered free radical derived from inducible nitric oxide synthase (iNOS), in various forms of pulmonary inflammation and injury (Szabo et al., 1993). In contrast to the constitutive isoforms of NO synthase (ecNOS and bNOS), iNOS produces abundant NO for longer periods of time (days) and at rates that are several orders of magnitude greater (Nicolson et al., 1993). Under quiescent conditions, little iNOS expression is present in the lung. In contrast, iNOS expression is strongly up-regulated in alveolar macrophages in acute clinical bronchopneumonia (Tracey et al., 1994). iNOS is also greatly increased in rodent models of systemic and pulmonary inflammation involving the lung (Yeadon and Price, 1995). In response to intact pathogenic microbes, iNOS expression is rapidly up-regulated (Szabo et al., 1998), suggesting that iNOS-derived NO may play an important role in host defense. Excess quantities of NO or peroxynitrite, the reaction product of NO and superoxide radical, may have deleterious consequences, including DNA single strand breakage and activation of poly(ADP-ribose) synthetase (Szabo et al., 1996), and a loss of epithelial energetics and barrier function (Kennedy et al., 1998).

Recently, Southan and Szabo (1996) discovered that mercaptoethylguanidine (MEG) has unique anti-inflammatory features, acting as a selective inhibitor of iNOS (Southan and Szabo, 1996), a scavenger of peroxynitrite (Szabo et al., 1997a), and a nonselective inhibitor of cyclooxygenases (Zingarelli et al., 1997). The administration of MEG has been

![Fig. 1. Total bacterial cfu at day 7. The numbers in parentheses represent the number of animals used in the experiment. Vertical bars represent the S.E.M.](image)

![Fig. 2. Total body weights over the 8-day course of experimental P. aeruginosa infection. * statistical differences between MEG-treated animals (broken line) and vehicle-treated animals (solid line). P < .02 by repeated measures ANOVA.](image)
shown to have a dramatic protective effect in many experimental models of inflammation, including periodontitis (Lohinai et al., 1998), hemorrhagic shock (Szabo et al., 1999), inflammatory bowel disease (Zingarelli et al., 1998), collagen-induced arthritis (Brahn et al., 1998), carrageenan-induced paw edema and pleuritis (Cuzzocrea et al., 1998), and endotoxic and septic shock (Szabo et al., 1997).

Given the pathologic role of iNOS-derived NO in various forms of pulmonary injury (Szabo and Salzman, 1997), we hypothesized that treatment with MEG would have a salutary effect in a murine model of CF lung disease produced by intratracheal instillation of Pseudomonas-infected agar beads. Our data suggest that MEG may be a novel candidate for therapy of CF-associated pseudomonal pneumonitis.

**Materials and Methods**

**Animals.** Female FVB/N mice were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Animals were maintained in a conventional animal room before and after administration of P. aeruginosa.

**Reagents.** Mercaptoethylguanidine was synthesized and kindly provided by Dr. G. Southan (Inotek Corporation, Beverly, MA).

**Experimental Model of Infection.** Entrapment of a clinical isolate of P. aeruginosa in agar beads was achieved using a modification of a standard method (Cash et al., 1979). A 500-ml suspension [10⁸ colony-forming units (cfu)/ml] P. aeruginosa was grown in tryptic soy broth for 18 to 20 h until mid-log phase, followed by centrifugation (5,000g) and resuspension in PBS (pH 7.4). Bacteria were washed three times in PBS, resuspended in 5 ml of PBS, diluted 1:6 to 10⁶ cfu/ml, and sonicated to disrupt cell walls. Agar beads (ANTA grade, Sigma-Aldrich, St. Louis, MO) were prepared according to a published method (Cash et al., 1979). beads were air-dried on a petri dish overnight and used immediately for experimental infection.

**Experimental Protocol.** Female FVB/N mice (6-8 weeks old) were anesthetized with isoflurane and intubated. An intratracheal bolus of 10⁷ P. aeruginosa entrapped in agar beads was administered to each mouse. Mice were randomly assigned to four groups: control (vehicle), MEG (10 mg/kg/dose, i.p.), MEG (10 mg/kg/dose, i.v.), and MEG (10 mg/kg/dose, i.v., with 10 mg/kg/dose, i.p., every 8 h for 8 days). Mice were monitored daily for signs of infection.

**Histologic Analysis.** At 8 days post-infection, mice were sacrificed and lungs were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 5-μm thickness. Sections were stained with hematoxylin and eosin and analyzed using a light microscope.

**Results.** MEG treatment significantly reduced the severity of lung inflammation as compared to the vehicle-treated control group. Mice treated with MEG showed a marked decrease in the number of infiltrating neutrophils and macrophages. These findings are supported by the histologic analysis of lung tissue.

**Discussion.** The results of this study suggest that MEG may be a promising therapeutic agent for the treatment of CF-associated pneumonitis. Further research is needed to evaluate the long-term effects of MEG and its potential for use in clinical settings.
in warm 2% agarose (50°C), and then pipetted forcefully into warm (50°C) heavy mineral oil. The bacteria-agarose-oil solution was spun rapidly at room temperature for 6 min, followed by rapid cooling on ice for 10 min. The agar beads, which formed in the oil, were washed with 0.5% sodium deoxycholate, followed by a wash with 0.25% sodium deoxycholate and three washes in PBS. The agar beads were finally resuspended in 2 volumes of PBS. Total bacterial counts of the beads were performed by 10-fold, serial dilutions of a hand-homog-

Fig. 4. Nitrotyrosine staining of slides from vehicle-treated animals and animals treated with MEG. A, control staining of vehicle-treated animal. B, nitrotyrosine staining of vehicle-treated animal. C, control staining of animal treated with MEG. D, nitrotyrosine staining of MEG-treated animal. Original magnification of all slides, 300×.

Fig. 5. Measurement of whole lung myeloperoxidase activity 8 days after Pseudomonas infection. *, statistical significance compared with vehicle-treated animals. Numbers in parentheses represent the number of animals used in each experiment.
Intratracheal Instillation of Agar Beads. Mice were anesthetized with isoflurane. Intratracheal injections were performed with a 27-gauge needle after blunt dissection of the soft tissues of the neck to expose the trachea. One-hundred microliters of agar beads without P. aeruginosa or 100 μl of agar beads containing the bacteria at a dose of 10^5 cfu were instilled intratracheally. The mice were allowed to recover and then were sacrificed at various time points.

Processing and Staining of Tissues for Histopathology. Lungs were inflation-fixed, as described previously (Buckingham and Wyder, 1981). H&E and nitrotyrosine stains were used for histological analysis of 5-μm paraffin sections.

Immunostaining for Nitrotyrosine. Slides were prepared on paraffin-embedded mouse lungs. Slides were baked at 60°C for 2 h. The tissues were then deparaffinized in three changes of xylene for 10 min, two changes in 100% ethanol (EtOH) for 5 min each, two changes in 95% EtOH for 5 min each, and two changes in 70% EtOH for 5 min each. Slides were washed five times with PBS (pH 7.2) with 1% Triton X-100 for 3 min each. The slides were then blocked in PBS (pH 7.2), 1% Triton X-100, and 2% goat serum for 2 h at room temperature. A 1:200 dilution of monoclonal anti-nitrotyrosine (Upstate Biotechnology, Waltham, MA) was incubated with the slides for 30 min at room temperature. A 1:2000 dilution of polyclonal anti-nitrotyrosine (Upstate Biotechnology, Waltham, MA) was incubated with the slides for 30 min at room temperature. A 1:200 dilution of goat anti-rabbit IgG (Vector, Burlington, CA) was incubated with the slides for 30 min. Endogenous peroxidase was removed by incubating slides with 0.5% H2O2 in methanol for 15 min. Slides were washed five times with PBS/0.1% Triton. Slides were developed with an avidin/biotin kit (Vector) according to the manufacturer's directions. Equal amounts of color solution A and B were mixed in 30 ml of H2O and incubated with the slides for 30 min at room temperature. Slides were washed five times with PBS/0.1% Triton. Slides were incubated for 1 min in acetate buffer (pH 6.0). Slides were then incubated for 4 min in acetate buffer with diaminobenzidine. Slides were then washed in Tris/cobalt solution for 4 min and washed in distilled H2O. Slides were then counterstained with 0.1% Nuclear Fast Red in 5% aluminum sulfate for 2 min. Slides were washed in running H2O then dehydrated in 70, 95, and 100% EtOH, and xylene, and then coverslips were applied.

Quantification of Cytokines by Enzyme Immunoassay. The presence of cytokines was detected in whole lung homogenate by enzyme immunoassay as previously described (Wilmott et al., 1998). Murine tumor necrosis factor-α (TNF-α), murine interleukin-1β (IL-1β), and macrophage inflammatory protein 2 (MIP-2) were measured using a commercially available enzyme immunoassay, according to the manufacturer's recommended protocols (R&D Systems, Minneapolis, MN). The limits of detection were 23 pg/ml for murine TNF-α and 8 pg/ml for murine IL-1β and MIP-2. Lung homogenates were assayed in duplicate.

Measurement of Lung Myeloperoxidase Activity. Lung neutrophil content was assessed using myeloperoxidase (MPO) activity as an indirect measurement of neutrophil content. Lung MPO content was measured colorimetrically using a modification of the microtiter MPO assay system, previously described (Remick et al., 1990; Stark et al., 1992). Whole lungs were removed and washed in sterile saline, blotted dry, and weighed to determine a wet weight. The lungs were then homogenized in 3 ml of 100 mM sodium acetate (pH 6.0), 0.5% hexadecyltrimethylammonium bromide, and 5 mM EDTA. The homogenate was sonicated and then centrifuged at 13,000g for 15 min. The supernatant was mixed 1:30 in assay buffer (3.2 mM tetramethylbenzidine and 1.0 mM H2O2 in a microtiter plate). The plate was read immediately at 650 nm over a period of 4 min. MPO units were calculated as the change in absorbance/min/whole lung wet weight.

Experimental Plan. Agar beads containing a total dose of 10^5 P. aeruginosa were administered to both the control group and the actively treated group of mice. MEG at a dose of 10 mg/kg/dose or a vehicle control was administered every 8 h by i.p. injection until the animals were sacrificed at 8 days after injection of the beads. The animals were weighed every day throughout the experiment using a Mettler balance (Mettler Toledo, Toledo, OH).

Results

The experimental and control groups of mice tolerated the experimental infection model well, with the only deaths occurring between the time of injection and 24 h after surgery: 4 of 20 animals died in the control group and 2 of 20 in the MEG-treated group. Quantitative bacterial cultures performed on whole lung homogenates at day 8 revealed no statistically significant difference in the concentrations of P. aeruginosa [MEG 3.5 × 10^4 ± 1.1 × 10^4 (S.E.) cfu versus vehicle control 4.7 × 10^4 ± 1.5 × 10^4; Fig. 1]. Both groups of animals became lethargic and anorexic. There was a significant weight loss in both the experimental and control groups, most notably during the first 3 days, but the weight loss was significantly reduced in the MEG-treated animals (P < .02) by repeated measures analysis of variance (Fig. 2).

Histological examination showed that there were marked focal areas of neutrophilic peribronchial inflammation in the controls (Fig. 3a) and less evidence of inflammation in the MEG-treated group (Fig. 3b). To investigate the mechanism of action of MEG, histological sections were stained for nitrotyrosine. There was no significant difference between the MEG-treated group and the sham-treated controls (Fig. 4). As the figure demonstrates, there was only faint staining for nitrotyrosine in the control animals after agar beads instillation, indicating little nitration of structural proteins by peroxynitrite in this model.

Mice treated with MEG had a significantly reduced whole lung myeloperoxidase activity, an index of neutrophil infiltration, at day 8, compared with vehicle-treated controls (16.2 × 10^3 ± 6.3 × 10^3 mod/min/lung wet weight versus 41.2 × 10^3 ± 11.8 × 10^3), with the levels in the MEG-treated group approaching those in sham-treated control mice (10.3 × 10^3 ± 0.74 × 10^3) (Fig. 5).

Concentrations of the pro-inflammatory cytokines IL-1β, TNF-α, and MIP-2 were measured in the whole lung homogenate obtained at day 8, by enzyme immunoassay. There were significantly reduced concentrations of all three cytokines in the animals treated with MEG compared with the vehicle-treated controls (Fig. 6–8). There was no evidence of toxicity from MEG in this series of experiments, and the body weights of both groups of animals returned to baseline by the end of the study.

Discussion

Chronic pseudomonal infection in CF induces an extensive inflammatory response, which contributes to respiratory failure and progressive pulmonary injury. Clinical trials of various anti-inflammatory agents, however, have been disappointing (Wilmott, 1999). Given the increasing role assigned to free radical and oxidant injury in various forms of pulmonary inflammation, we examined the effect of a novel therapeutic agent, MEG, which has a unique mode of action. It acts as both an iNOS-selective inhibitor and a scavenger of peroxynitrite, the reaction product of nitric oxide and superoxide anion (Szabo et al., 1997b). We observed that MEG blocked the inflammatory response in a model of subacute
murine pseudomonal pneumonitis, and the chronicity and inflammatory nature of this model closely resembles the clinical features of infection in CF. Accordingly, the results of this study may have important implications for the use of MEG as a potential therapy in this disease. Female FVB/N mice challenged with $10^5$ *P. aeruginosa* in agar beads were followed for 8 days. Although the final bacterial concentrations in the lung were similar in treated and untreated animals, there was a significant reduction in weight loss and pulmonary inflammation in the MEG-treated group as shown by histological analysis, whole lung myeloperoxidase activity, and concentrations of pro-inflammatory cytokines.

The therapeutic effects of MEG are thought to relate to its inhibition of NO generation and scavenging of peroxynitrite (Szabo et al., 1997b). Altered expression of iNOS is thought to underlie the pathophysiology of a broad range of inflammatory conditions, including rheumatoid arthritis, hemorrhagic shock, asthma, chronic inflammatory bowel disease, and septic shock (Szabo et al., 1995). MEG has been shown in these and other inflammatory conditions to reduce the production of NO and the formation of peroxynitrite, as judged by the reduction in the formation of nitrotyrosine, a useful but non-specific marker of peroxynitrite-mediated nitration. However, the low level of staining and the lack of difference in nitrotyrosine staining of histological sections obtained at 8 days in MEG-treated and control mice suggest that forma-
tion of peroxynitrite is not a major aspect of the inflammatory response to infection in this model and that the activity of MEG was more related to inhibition of iNOS and down-regulation of cytokine expression.

The actions of MEG included suppression of neutrophil infiltration, in agreement with previous reports on the use of MEG in experimental models of arthritis (Brahn et al., 1998), colitis (Zingarelli et al., 1998), and carrageenan-induced models of inflammation (Cuzzocrea et al., 1998). Our results are also analogous to those from a model of neutrophil influx into carrageenan-soaked sponge implants in which there was inhibition by the selective iNOS inhibitor S-methylisothiourea, although not by the nonselective iNOS inhibitor N\(^\text{\rom{3}}\)-nitro-L-arginine methyl ester (Iuvone et al., 1992). The mechanism by which MEG inhibits neutrophil recruitment is unclear, but may reflect its effect on chemokine expression. MEG profoundly suppressed the up-regulated expression of MiP-2 as well as the proinflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\). This result is consistent with earlier studies that showed that nonselective inhibitors of NOS, such as N\(^{\text{\rom{3}}}\)-monomethyl-\(\text{L}\)-arginine, decrease the release of the proinflammatory cytokine MiP-1 by lipopolysaccharide-stimulated monocytes (Muhl and Dinarello, 1997).

It was of considerable interest that MEG did not exacerbate the degree of bacterial infection in the lung. Gardner et al. (1998) reported that bacterial flavohemoglobin serves as a protective agent with a combined mechanism of action. The beneficial results of using MEG in this infection model are encouraging for the development of anti-inflammatory therapy for chronic pulmonary infection with \(P.\ aeruginosa\) and will form the basis for further studies of dose-response by the oral and aerosol routes.

The reduced weight loss observed in the MEG-treated mice with comparable degrees of infection could be the result of decreased pulmonary inflammation, decreased airway obstruction, or possibly the result of reduced systemic TNF-\(\alpha\) release and cachexia.

The beneficial effects of MEG in this infection model are encouraging for the development of anti-inflammatory therapy for chronic pulmonary infection with \(P.\ aeruginosa\) and will form the basis for further studies of dose-response by the oral and aerosol routes.

References


Inhibition of Inflammatory Response to P. aeruginosa


Send reprint requests to: Robert W. Wilmott, M.D., Director, Pulmonary Medicine, Allergy, and Clinical Immunology, Children’s Hospital Medical Center, 3333 Burnet Ave., Cincinnati, Ohio. E-mail: wilmot@chmc.org