Nitrite therapy improves survival postexposure to chlorine gas

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Nitrite therapy improves survival postexposure to chlorine gas. Am J Physiol Lung Cell Mol Physiol 307: L888–L894, 2014. First published October 17, 2014; doi:10.1152/ajplung.00079.2014.—Exposure to relatively high levels of chlorine (Cl2) gas can occur in mass-casualty scenarios associated with accidental or intentional release. Recent studies have shown a significant postexposure injury phase to the airways, pulmonary, and systemic vasculatures mediated in part by oxidative stress, inflammation, and dysfunction in endogenous nitric oxide homeostasis pathways. However, there is a need for therapeutics that are amenable to rapid and easy administration in the field and that display efficacy toward toxicity after chlorine exposure. In this study, we tested whether nitric oxide repletion using nitrite, by intramuscular injection after Cl2 exposure, could prevent Cl2 gas toxicity. C57Bl/6 male mice were exposed to 600 parts per million Cl2 gas for 45 min, and 24-h survival was determined with or without postexposure intramuscular nitrite injection. A single injection of nitrite (10 mg/kg) administered either 30 or 60 min postexposure significantly improved 24-h survival (from ~20% to 50%). Survival was associated with decreased neutrophil accumulation in the airways. Rendering mice neutropenic before Cl2 exposure improved survival and resulted in loss of nitrite-dependent survival protection. Interestingly, female mice were more sensitive to Cl2-induced toxicity compared with males and were also less responsive to postexposure nitrite therapy. These data provide evidence for efficacy and define therapeutic parameters for a single intramuscular injection of nitrite as a therapeutic after Cl2 gas exposure that is amenable to administration in mass-casualty scenarios.

EXPOSURE TO CHLORINE GAS (Cl2) and resultant toxicity are concerns in civilian and military settings. Several examples pertaining to the use of Cl2 as a chemical weapon and large-scale accidental exposures attributable to the derailment of trains transporting Cl2 through populated areas have been documented (3, 8, 21, 31, 34). The potential for future incidents remains because of the demand for Cl2 in multiple industrial processes (8). Several studies have provided insight into the pathogenesis of Cl2 toxicity, which is characterized by injury to the airways during the initial halogen insult. This is followed by a postexposure injury phase occurring over a time span ranging from days to months, which affects the airways and pulmonary and systemic vasculature (9, 11, 20, 25, 26, 30, 37). Clinically, this presents as reactive airway syndrome, hypoxemia, noncardiogenic edema leading to acute lung injury, and development of acute respiratory distress syndrome, which chronically leads to reactive airway disease, increased sensitivity to pulmonary infections, fibrosis, and bronchiolitis obliterans, as well as dermal injury (9, 10, 12, 17, 24, 26, 28, 30). Exciting recent studies are providing insights into the postexposure mechanisms that include increased oxidative stress, neurogenic and airway inflammation, fibrosis, loss of nitric oxide (NO) signaling homeostasis, and loss of endogenous airway repair mechanisms (2, 9–11, 15, 20, 22, 25, 26, 36). This information has led to the testing and demonstration of efficacy for antioxidant therapy (9, 16, 22, 23, 37), anti-inflammatory (using corticosteroids) treatment (5), β2-agonists (29), phosphodiesterase inhibitors (4), transient receptor potential channel inhibitors (1, 2), and strategies that improve NO bioavailability (28, 36).

An important consideration for Cl2 toxicity is that potential therapies are effective when administered postexposure. They must also be amenable to stockpiling with a rapid and easy method of administration. These are all key features for mass-casualty scenarios. In this context, intramuscular (IM) injection is a preferred modality. Given the multitude of mechanisms involved in postexposure toxicity, it is unlikely that one therapeutic will prevent all aspects of pathogenesis of Cl2 toxicity. Moreover, in some cases, therapeutics targeted to specific aspects of Cl2 toxicity may not be amenable to rapid administration in a mass-casualty scenario. For example, inhaled therapies, although being the most efficient means of delivering large amount of therapeutic agents to lung epithelia, are logistically more challenging as a first-line therapeutic in a mass-casualty scenario but more feasible if given in a hospital setting. Thus therapeutics that improve immediate/short-term survival after Cl2 exposure are also critical to develop, the goal being that this will allow for administration of other targeted therapies that can be administered in a more controlled and clinical environment.

NO is a critical mediator of homeostasis in all organ systems. In addition to its generation by one of three nitric oxide synthases, multiple studies over the last decade have shown that enzymatic production of NO is complemented by nonenzymatic mechanisms for NO generation from nitrite (18). A functional role for the latter pathway has been demonstrated in both physiological and pathophysiological contexts, and perhaps the most evidence is in therapeutics where the goal is to improve or replete NO bioactivity in ischemic and inflammatory diseases (14, 19, 33). Consistent with this, our recent studies showed that nitrite administered by intraperitoneal (IP) or IM injections, after Cl2 exposure prevented acute lung injury and development of reactive airways in rats (28, 36). Importantly, nitrite also meets the therapeutic criteria outlined above for mass-casualty scenarios and has an established safety profile for use in humans in the context of cyanide antidotes, which is further supported by more recent phase 1/2 studies. In this study, using a model of lethal Cl2 exposure, we demonstrate the therapeutic potential for nitrite administered postex-
posure to improve survival and provide insights into underlying mechanisms of protection.

MATERIALS AND METHODS

Materials. Unless stated otherwise all reagents were purchased from Sigma (St. Louis, MO). Quantitative chemokine (CXCL2 and CXCL1) ELISA kits were purchased from R&D Systems (Minneapolis, MN). Bio-Rad Protein Assay Reagent Kits were purchased from Bio-Rad (Hercules, CA). Male (25–27 g, 8–11 wk of age) and female (18–20 g, 8–11 wk of age) C57Bl/6 mice were purchased from Harlan (Indianapolis, IN) and kept on 12-h:12-h light/dark cycles with access to standard chow and water ad libitum before and after chlorine gas exposure.

Mouse exposure to chlorine gas. Whole body exposures of male and female mice to Cl₂ gas were performed as previously described (16, 37). Exposures were performed with either two or five mice in the same chamber at any one time, and all exposures were performed between 8:00 AM and 12:00 PM. Exposure conditions were either 400 parts per million (ppm) Cl₂ in air for 30 min or 600 ppm for 45 min using 400-ppm and 600-ppm Cl₂ tanks, respectively. Tanks were replaced when the pressure in the tanks reached 500 psi. In each case, immediately following exposure, mice were returned to room air. The mice were monitored hourly for 12 h and every 6 h thereafter for 24 h. All experiments involving animals were conducted according to protocols approved by the UAB IACUC.

IM nitrite administration. Mice received a single injection of sodium nitrite in PBS (0.1–100 mg/kg final concentration) in the gluteus maximus region at either 30 min or 60 min after cessation of Cl₂ exposure. In addition, a multiple-dose nitrite therapy protocol (10 mg/kg at 60 min, 180 min, and 300 min) was also tested. Nitrite stocks were prepared daily in sterile PBS with injection volumes of 25 μl.

Acute lung injury assessment. Mice were euthanized with intraperitoneal ketamine and xylazine (100 and 10 mg/kg body wt, respectively). An incision was made at the neck to expose the trachea, and a 3-mm endotracheal cannula inserted. Lungs were lavaged with 3 ml PBS and counted using a Neubauer hemocytometer. Cells were then placed on slides using a cellspin (Tharmac, Drosselweg, Germany) and stained using a two-stain set consisting of eosin Y and a solution of thiazine dyes (Quik-Stain; Siemens, Washington, DC). Differential counts (specifically monocytes, neutrophils, and lymphocytes) were then performed on slides via light microscopy.

Chemokine measurement. Mouse CXCL1 (keratinocyte-derived chemokine, KC) and CXCL2 (macrophage inflammatory protein 2, MIP-2) were measured in the plasma and bronchoalveolar lavage fluid (BALF) by ELISA per manufacturer protocols.

Inducing neutropenia. C57Bl/6 mice were rendered neutropenic as described previously (35) by IP injection with 200 μg of either anti-Ly-6G (clone IA8) (cat. no. BE0075-1; Bxcel, London, UK) or IgG2a Isotype control (cat. no. BE0089, Bxcel) 24 h before Cl₂ gas exposure. Mice were exposed to Cl₂ gas and received nitrite (10 mg/kg, 30 min after Cl₂ exposure) therapy as described above.

Survival analysis. Survival was assessed using the Log rank (Mantel-Cox) test. For each exposure, mice were preassigned to either be exposed to Cl₂ only or Cl₂ followed by nitrite therapy. In some cases, mice assigned to a nitrite therapy group died before receiving nitrite, i.e., during or within 30 min or 60 min postexposure. These animals were excluded from the study because they did not receive therapy and constituted n = 2 for 1 mg/kg nitrite, n = 5 for 10 mg/kg nitrite at 30 min postexposure; n = 4 for 10 mg/kg nitrite, n = 1 for 100 mg/kg nitrite at 60 min postexposure; n = 1 for 10 mg/kg nitrite at 60 min, 180 min, and 300 min postexposure. The numbers of replicates are indicated in the figure legends. All analyses were performed using GraphPad Prism (La Jolla, CA). Significance was set at P < 0.05.

RESULTS

Nitrite administration after chlorine exposure improves 24-h survival. The goals of this study were to test whether nitrite administered by IM injection postexposure is an effective postexposure therapy in a lethal model of Cl₂ exposure. To

Fig. 1. Nitrite-dependent protection against chlorine-induced mortality in mice. A: male mice were exposed 2 at a time to Cl₂ 600 parts per million (ppm), 45 min and then brought back to room air, 30–60 min thereafter, mice received either saline or nitrite (Nit) at 0.1 mg/kg, 1.0 mg/kg, or 10.0 mg/kg at 30 min after Cl₂ exposure by intramuscular injection. Data show Kaplan-Meier survival curves. *P < 0.006 between 10 mg/kg nitrite 30 min and saline by the Log-rank test. No significant difference between 0.1 mg/kg nitrite (P = 0.33) or 1.0 mg/kg nitrite (P = 0.26) and saline was observed. B: male mice were exposed to Cl₂ at 600 ppm, 45 min and then brought back to room air, and nitrite was administered as indicated in figure by intramuscular (IM) injection. Data show Kaplan-Meier survival curves. P < 0.03 between saline and nitrite (10 mg/kg, 30 min) and P < 0.002 between saline and nitrite (10 mg/kg, 60 min). No significant difference was observed between saline and either multiple nitrite administration (P = 0.15) or nitrite (100 mg/kg, 60 min) (P = 0.75) groups.
date, the beneficial effects of nitrite therapy on sublethal Cl$_2$ injury have been established (28, 36). Using male mice, initial studies determined exposure conditions leading to >75% mortality over 24 h. When five mice at a time were exposed to 600 ppm of Cl$_2$ for 45 min, <10% mortality was observed over 24 h; however, mortality significantly increased (>85% by 24 h) if only two mice at a time were exposed (not shown). Consistent with our previous studies, 400 ppm Cl$_2$ for 30 min did not cause any mortality despite causing significant lung injury (29, 36, 37). We therefore chose exposure conditions of 600 ppm, 45 min with two mice being exposed at any given time.

Figure 1A shows the effects of nitrite (0.1, 1.0, and 10.0 mg/kg) administered by IM injection 30 min after Cl$_2$ exposure. Neither 0.1 mg/kg nor 1.0 mg/kg nitrite affected mortality. However, a single administration of 10.0 mg/kg at 30 min postexposure significantly improved 24-h survival by ~50%. Figure 1B shows that nitrite (10 mg/kg) also afforded protection when administered 60 min after Cl$_2$ exposure, which was no different compared with nitrite administered at 30 min. The protective effect of nitrite injection at 60 min after Cl$_2$ exposure was lost, however, if a higher dose (100 mg/kg) was used, consistent with previous studies showing that nitrite has an optimum therapeutic concentration (7, 28). Because the circulating half-life of nitrite is ~20–40 min, we also tested a multiple-dose regimen to establish whether a single injection of nitrite was sufficient to afford protection or whether multiple doses would provide better protection. Nitrite (10 mg/kg) administered at 1 h, 3 h, and 5 h after Cl$_2$ exposure did not improve survival relative to Cl$_2$ alone although a trend toward protection was noted ($P = 0.15$, Figure 1B). We also noted that, when combined, data from Fig. 1, A and B, indicated that the greatest degree of survival benefit was observed over the first 12–16 h postexposure.

**Role of polymorphonuclear leukocytes in nitrite-dependent protection.** To evaluate the potential mechanisms by which IM nitrite afforded survival benefit, mice were exposed to Cl$_2$ gas

Fig. 2. Effect of nitrite therapy on indices of acute lung injury. Male mice were exposed to Cl$_2$ 600 ppm, 45 min and then brought back to room air and 30 min thereafter received saline or nitrite 10 mg/kg by IM injection. Mice were continually observed over the next 6 h, and, if they died during this time, bronchoalveolar lavage fluid (BALF) and blood were collected immediately. At 6 h, all mice were killed, and BALF and plasma were collected. A and B: BALF levels of protein and total cell accumulation. *$P < 0.01$ relative to air, #$P < 0.05$ relative to Cl$_2$ alone by 1-way ANOVA with Tukey’s posttest. C: differential cell analysis. D: viability of BALF inflammatory cells. *$P < 0.01$ relative to respective + nitrite group by t-test. E: BALF and plasma macrophage inflammatory protein 2 (MIP-2) levels, *$P < 0.01$ relative to air and #$P < 0.05$ relative to Cl$_2$-alone group by 1-way ANOVA with Tukey’s posttest. F: BALF and plasma keratinocyte chemokine (KC) levels, *$P < 0.01$ or #$P < 0.05$ relative to air, by 1-way ANOVA with Tukey’s posttest. All data are means ± SE ($n = 5–9$).
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600 ppm, 45 min and administered saline or IM nitrite (10 mg/kg) 30 min postexposure. Mice were continually observed over the next 6 h, and, if they died during this time, BALF and blood were collected immediately for assessment of MIP-2 or KC and accumulation of protein and inflammatory cells. At 6 h, all mice were killed, and similar measurements of acute lung injury were made (5/8 mice in the Cl2 gas-only group and 9/9 mice in the nitrite-treatment group survived to 6 h). Figure 2A shows that BAL protein increased to similar extents in both Cl2 and Cl2 + nitrite groups. However, BAL levels of inflammatory cells were markedly lower in nitrite-treated mice compared with Cl2-alone-exposed mice (Fig. 2B); this effect was specific for polymorphonuclear leukocytes (PMN) (Fig. 2C). Figure 2D shows that, in addition to decreasing PMN infiltration, nitrite therapy also improved viability of cells collected from the BALF. To gain insights into how nitrite prevented PMN trafficking into the lungs, BAL and plasma levels of the proinflammatory chemokines MIP-2 (CXCL2) and KC (CXCL1) were measured (Fig. 2, E and F, respectively). Surprisingly, highest chemokine levels were observed in the nitrite group relative to air, with trends toward increased levels relative to Cl2 also noted.

Effects of neutropenia on Cl2 toxicity and nitrite therapy. Figure 2 suggests an association between preventing PMN accumulation in the BAL and short-term survival benefit after Cl2 gas exposure. To directly test whether PMN are involved, mice were rendered neutropenic first, then exposed to Cl2 gas, and mortality followed over 12 h. Neutropenia was confirmed by no change in blood macrophage counts and >90% depletion of blood PMN 24 h after treatment with anti-Ly-6 antibody compared with isotype control (not shown). Figure 3 shows that mortality was no different between mice exposed to Cl2 only vs. mice exposed to Cl2 after treatment with isotype control antibody. However, mortality was significantly lower in neutropenic mice. Moreover, nitrite therapy still afforded protection in mice treated with isotype control antibody but had no effect in neutropenic mice.

Effects of nitrite therapy on nitrite and nitrate concentrations. Similar to our previous studies (11), circulating nitrite levels decreased in Cl2-exposed mice (Fig. 4A) with no significant changes in nitrate (Fig. 4B). We have also shown previously that IM injection of nitrite maximally increased circulating levels ~5 min after administration and with a half-life of ~20 min. (28). Consistent with this short plasma half-life, nitrite therapy had no effect on circulating nitrite at 6 h postadministration. Although not significant, trends toward increased plasma nitrate formation were evident, however, consistent with nitrite oxidation. More interestingly, trends (P between 0.08 and 0.1) toward increased lung tissue levels of nitrite and nitrate were observed in Cl2-exposed animals. Most striking, however, were the significant increases in nitrate in nitrite surgery groups. Figure 4 shows that female mice are more sensitive to Cl2-induced injury compared with males. Nitrite therapy afforded protection in males similar to that observed in Fig. 1. However, nitrite did not statistically improve survival in females although a trend (P = 0.09) was noted.

DISCUSSION

In this study, we provide evidence that nitrite therapy improves 24-h survival in mice exposed to Cl2. Importantly, nitrite was efficacious when administered at 30 min or 60 min after Cl2 exposure, providing the first example, to our knowledge, of a postexposure IM injectable therapeutic that was effective in improving mortality in a model of Cl2 toxicity. Together with the potential ability of nitrite to be stockpiled, these data underscore the potential for nitrite therapy as a front line therapeutic for inhaled toxicants in mass-casualty situations.

The last decade has seen many studies document the potential for nitrite to replete NO signaling in acute and chronic diseases affecting all major organ systems, which are characterized by loss of endogenous NO function (14, 18, 33). We chose to test nitrite therapy on the basis that endothelial NO synthase-dependent signaling is inhibited, and nitrite levels are decreased during the initial stages (hours) after Cl2 exposure (Fig. 4) (11). Also, nitrite afforded protection against acute lung injury in a model of sublethal Cl2 exposure, and similar results have been shown in a model of mechanical ventilator-induced injury (27, 28, 36). This suggests that the therapeutic mechanisms of action of nitrite may target aspects of lung injury common to distinct initiators of acute lung injury. Further studies testing nitrite therapy for lung injury induced by other infectious and noninfectious/mechanical stimuli are warranted.

Less clear is how nitrite improves survival after Cl2 exposure. We have not characterized precisely how mice were

![Fig. 3. Effects of neutropenia on Cl2-induced mortality and nitrite therapy. Male mice were untreated (no pretreatment) or injected intraperitoneally with 200 mg of either anti Ly-6G or IgG2a isotype control antibody. 24 h later, all mice were exposed to Cl2 gas (600 ppm, 45 min) and then treated with saline or nitrate (10 mg/kg) 30 min after Cl2 exposure. Data show Kaplan-Meier survival curves. P < 0.01 between anti Ly-6G or IgG2a groups, P < 0.05 between IgG2a and IgG2a + nitrite groups, P = 0.6 between Ly-6G and Ly-6G + nitrite groups.](http://ajplung.physiology.org/)
Dying in these studies with hypoxemic stress and critical-end organ dysfunction likely key. Notably, nitrite was able to improve 24-h survival even when administered 1 h after Cl2 exposure, a time over which much damage to the lungs has already occurred and induction of inflammation likely. Our previous study using a sublethal Cl2 exposure protocol showed that nitrite protected against both increased permeability and inflammation components of Cl2 injury, which was associated with increased airway reepithelialization (28, 36). In the current study, we demonstrate a more prominent role for PMN, which may be attributable to a different model being used here (mice and lethal exposure protocol), testing of only one nitrite dose, and assessment of acute lung injury at one time point. Neutropenia alone improved survival and resulted in a loss of nitrite-dependent protection, suggesting that prevention of PMN-dependent injury is one mechanism by which nitrite elicits protective effects. A recent study also noted attenuated nitrogen mustard-induced skin injury in mice lacking the predominantly neutrophilic enzyme myeloperoxidase (13), suggesting a central role for PMN in mediating postexposure toxicities to diverse chemical threat agents. The anti-PMN effects of nitrite were not mediated by altering MIP-2 or KC, two principal chemokines responsible for PMN homing to the lungs in acute lung injury. In fact, nitrite therapy increased levels of these chemokines in the BALF and plasma. At first glance, such increases in MIP-2 and KC are expected to further stimulate PMN egress into the lungs; however, it is also possible that nitrite increased plasma chemokines more so than BALF levels, which would dissipate the chemokine gradient into the lung, lowering the driving force for PMN infiltration from the circulation into the pulmonary compartment. Other potential mechanisms by which nitrite could affect PMN infiltration include modulation of endothelial adhesion molecules and PMN activation, and future studies need to evaluate whether nitrite modulates PMN-dependent clearance of pathogens, which we have shown are inhibited after Cl2 exposure (10).

Other potential protective mechanisms of nitrite include improving blood flow, protecting against cell death, and allowing endogenous repair mechanisms to function. In the latter context, macrophage viability in the BAL was also improved by nitrite therapy. The dose of nitrite that was effective in the

Fig. 4. Nitrite and nitrate levels after nitrite therapy. Male mice were exposed to Cl2 600 ppm, 45 min and then brought back to room air and 30 min thereafter received saline or nitrite 10 mg/kg by IM injection. Plasma nitrite (A) and nitrate (B), lung nitrite (C) and nitrate (D), and BALF nitrite (E) and nitrate (F) were measured after 6 h. Data shown are means ± SE, n = 5–9. *P < 0.05 relative to air by for A and *P < 0.05 or **P < 0.01 relative to air and Cl2 alone for E and F by 1-way ANOVA with Tukey’s posttest.
data show that nitrite was less effective at preventing Cl₂-hypoxia less effective in female human volunteers (32). Similarly, our female mice also. Recent studies have shown that nitrite-based therapeutic dose and time window exist. Thus the precise dose of nitrite required appears to depend in part on the exposure and the end point being assessed. In the current study, use of 10-fold higher or lower dose of nitrite resulted in a loss of protection. Similar “U”-shaped dependence for nitrite therapy has been observed previously for ischemia-reperfusion injury (7, 27). That said, even at the highest dose tested here, nitrite did not increase Cl₂ toxicity (see Fig. 1), suggesting that a relatively large therapeutic dose and time window exist.

Interestingly, female mice were more sensitive to Cl₂-induced mortality compared with males. Whether this is a consequence of estrogen remains to be tested. We used age-matched males and females, and therefore females were lighter compared with males; whether this is an effect for different responses also requires further investigation. Our primary goals were to test whether nitrite therapy was effective in female mice also. Recent studies have shown that nitrite-based anticoagulation therapy (via nitrate supplementation) may be less effective in female human volunteers (32). Similarly, our data show that nitrite was less effective at preventing Cl₂-induced mortality in female mice compared with male mice. This could be a consequence for greater injury in females vs. males and/or less sensitivity for nitrite-based protection in females. That said, a trend toward significance was still noted, however, suggesting the general utility for nitrite therapy for males and females, although we note that this requires much further study.

We also observed that the degree of Cl₂-induced toxicity can vary significantly depending on the exposure protocol. We utilized whole body exposure protocols, in contrast to nose-only exposure. The pros and cons of these approaches have been compared previously (6). Cl₂ toxicity was significantly lower if five mice were exposed at the same time compared with two. We speculate that “huddling of mice” may have limited exposure of Cl₂ to individual mice in the former setting as well as potentially introducing uncontrolled variables. For these reasons, we opted for a protocol with two animals per exposure. Moreover, variances in Cl₂ toxicity responses were observed between different experiments. For example, for all data shown in this study, the time for 50% mortality in Cl₂-gas-only groups varied from 3–12 h. The precise mechanisms for such variance is not clear but underscores the importance of ensuring that exposures are performed with a pairwise manner when comparing different experimental/therapeutic groups.

The purpose of this study was to test whether IM injection of nitrite may be an effective postexposure therapeutic in a lethal model of Cl₂ exposure. The extent of Cl₂-induced injury depends on the dose and exposure time. Although these variables can be controlled experimentally, testing of therapeutics across all potential Cl₂ exposure scenarios that likely occur in an accident or military setting is not feasible. We therefore opted to use 12–24-h mortality as the end point, with the rationale being that a postexposure therapeutic that can be administered within 60 min of exposure, to model first-responder times, and which also improves short-term survival will be key in allowing victims to reach a primary care facility where targeted therapeutics can be initiated. A limitation of this study is the lack of testing of this concept and assessing mortality effects beyond 24 h. Future studies evaluating long-term outcomes after postexposure nitrite therapy are required.

Fig. 5. Effect of sex on chlorine toxicity and nitrite therapy. Male or female mice were exposed to Cl₂ at 600 ppm, 45 min and then brought back to room air, and nitrite was administered as indicated in figure by IM injection 30 min postexposure. Data show Kaplan-Meier survival curves. P < 0.03 between male and female Cl₂-alone groups; P < 0.02 for nitrite therapy in males and P = 0.09 for nitrite therapy in females.
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