Inhibition of CXCR2 attenuates bronchial angiogenesis in the ischemic rat lung

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Sukkar A, Jenkins J, Sánchez J, and Wagner EM. Inhibition of CXCR2 attenuates bronchial angiogenesis in the ischemic rat lung. J Appl Physiol 104: 1470–1475, 2008. First published March 6, 2008; doi:10.1152/japplphysiol.00974.2007.—Under conditions of chronic pulmonary ischemia, the bronchial circulation undergoes massive proliferation. However, little is known regarding the mechanisms that promote neovascularization. An expanding body of literature implicates the glutamic acid-leucine-arginine (ELR+) CXC chemokines and their G protein-coupled receptor, CXCR2, as key proangiogenic components in the lung. We used a rat model of chronic pulmonary ischemia induced by left pulmonary artery ligation (LPAL) to study bronchial angiogenesis. Using a methacrylate mixture, we cast the systemic vasculature of the rat lung at weekly intervals after LPAL. Twenty-one days after LPAL, numerous large, tortuous bronchial arteries were observed surrounding the left main bronchus that penetrated the left lung parenchyma. In stark contrast, the right lung was essentially devoid of vessels. We quantified bronchial neovascularization using 15-μm radiolabeled microspheres to measure systemic blood flow to the left lung (n = 12 rats). Results showed that by 21 days after LPAL, bronchial blood flow to the ischemic left lung had increased >10-fold compared with controls 2 days after LPAL (P < 0.01). Focusing on the predominant rat CXC chemokine that signals through CXCR2, we measured increased levels of cytokine-induced neutrophil chemoattractant-3 protein expression in left lung homogenates early (4 and 24 h; n = 10 rats) after LPAL relative to paired right lung controls (P < 0.01). Treatment with a neutralizing antibody to CXCR2 resulted in a significant decrease in neovascularization 21 days after LPAL (n = 9 rats; P < 0.01). Our results confirm the time course of bronchial angiogenesis in the rat and suggest the importance of CXC chemokines in promoting systemic neovascularization in the lung.

bronchial artery; cytokine-induced neutrophil chemoattractant-3; microspheres

The bronchial circulation is the systemic vascular supply to the lung and provides nutrient blood flow to conducting airways down to the level of the terminal bronchioles as well as nerves, lymph nodes, visceral pleura, and walls of large pulmonary vessels. It normally provides less than 1% of cardiac output to the lung but has been shown to increase to as much as 30% of the original pulmonary blood flow after chronic unilateral pulmonary artery obstruction (13). This pathological feature of bronchial angiogenesis occurs during conditions of chronic inflammation such as cystic fibrosis (4), asthma (12), pulmonary fibrosis (27), lung cancer (16), and chronic thromboembolic disease (18). In animal models, pulmonary ischemia resulting from chronic pulmonary artery obstruction has been shown to cause proliferation of the systemic circulation to the lung in sheep (5), pigs (8), dogs (13), rats (28), and mice (14). However, little is known regarding the mechanisms that promote growth of bronchial arteries in chronic ischemia because most studies have focused on acute reperfusion injury or chronic pulmonary hypertension (8, 20).

Angiogenesis is a complex process regulated by numerous proangiogenic and angiostatic forces (25). Our laboratory has begun to determine the proangiogenic growth factors that may contribute to neovascularization in animal models of left pulmonary artery obstruction (24). Unlike systemic vascular beds in which angiogenesis is studied, the ventilated lung after pulmonary artery obstruction is ischemic but likely not hypoxic. Consequently, hypoxia-inducible growth factors appear not to play a role in the process of angiogenesis. Growth factors significantly upregulated are predominantly cytokines associated with inflammation with a growing body of evidence implicating specifically the glutamic acid-leucine-arginine (ELR+) CXC chemokines and their G protein-coupled receptor, CXCR2, as key proangiogenic players within the lung (2, 10, 24). In the rat, these primarily macrophage-derived proteins and functional ELR+ CXC correlates that signal through CXCR2 are referred to as the cytokine-induced neutrophil chemoattractants (CINC)-1, -2, and -3 (22).

Although similar growth factors may be responsible for systemic vascular proliferation and recruitment to meet the needs of the ischemic lung in all mammals, both the process and the physiological consequences likely differ between mice, and mammals with an extensive bronchial circulation. The mouse does not have a well-developed bronchial vasculature because it appears to terminate in the extraparenchymal airways (14). As a result, neovascularization in the mouse model of left pulmonary artery ligation (LPAL) is limited to growth and invasion of intercostal arteries into pulmonary parenchyma and not bronchial vessels. In a rat model of chronic pulmonary artery obstruction, Weibel (28) used a histological approach and documented proliferative changes in bronchial blood vessels with patent new vessels evident by 10–40 days after LPAL. Furthermore, these results suggested that the response to pulmonary ischemia in the rat more closely resembles bronchial neovascularization in human subjects after pulmonary artery obstruction (6, 18). Therefore, the goals of the present study were to confirm the earlier work of Weibel in a rat model of chronic pulmonary ischemia and provide a three-dimensional visualization and functional assessment of the patency of the neovascularure. In addition, we sought to determine the avail-
ability of the growth factor CINC-3 in the lung and the effects of blockade of its receptor CXCR2 in bronchial angiogenesis during conditions of chronic pulmonary ischemia. Our results confirm the essential nature of the interaction between this receptor and ligand in the extensive bronchial vascular proliferation early after the onset of chronic pulmonary ischemia.

METHODS

LPAL. Our protocol was approved by the Johns Hopkins Animal Care and Use Committee. Sprague-Dawley male rats (150–200 g; Harlan, Indianapolis, IN) were studied 4 h to 21 days after LPAL. Rats were anesthetized, intubated, and ventilated (90 breaths/min, 8 ml·kg⁻¹·breath⁻¹) with an anesthetic-gas mixture (2% isoflurane in O₂). A left lateral thoracotomy was performed at the fourth intercostal space to expose the left lung. The left pulmonary artery was isolated from the left bronchus and ligated using prolene suture (6-0). The space to expose the left lung was created by injecting 1.5 ml of the methacrylate mixture (base solution A; Polysciences, Warrington, PA) at a rate of 250 ml/min. Bupivicaine (2 mg/kg) was injected at the incision site for analgesia, and the skin incision was closed with methyl acrylamide adhesive. Buprenorphine (0.05 mg/kg ip) was given for additional analgesia. The animal was removed from the ventilator, extubated, and allowed to recover. No surgical procedures were performed on naive rats. After specified times following LPAL and unless otherwise described, anesthetized rats were euthanized by KCl injection into the left ventricle.

Methacrylate casting. At weekly intervals after LPAL, we injected methacrylate casting material retrograde into the descending aorta according to the following procedure. Rats were anesthetized, intubated, and ventilated as described above. The abdomen was opened and the descending aorta was cannulated (20-gauge intracatheter). The inferior vena cava was cut, and the systemic circulation was flushed (3 ml of heparinized saline). The systemic vasculature of the lungs was cast by injecting 1.5 ml of the methacrylate mixture (mixture 1: 0.25 g red pigment, 5 ml base solution A, 2 ml catalyst and mixture 2: 0.25 g red pigment, 5 ml base solution A, 2 drops promoter C; Polysciences, Warrington, PA) at a rate of 250 µl/min. The bronchial vasculature was cast in naive rats, rats 14 days and 21 days after LPAL, and rats 21 days after LPAL and CXCR2 neutralizing antibody treatment (n = 2 rats/time point). The rats were extubated and the trachea was cannulated using a 16-gauge blunt-ended needle. The airways were cast by injecting 0.7 ml of a silicone-based material (Silastic ERTV Silicone Rubber, Dow Corning, Midland, MI) at a rate of 250 µl/min. Base (10 g) was combined with the curing agent (1 g). After dissection and injections were complete (~2 h), the entire thorax was excised and placed in NaOH (2 M) for tissue degradation. Representative casts were photographed under a dissecting microscope (×10 amplification; Olympus SZX9, Center Valley, PA) and a SPOT Flex camera (model 15.2, Diagnostic Instruments, Sterling Heights, MI).

Neovascularization assessed by blood flow. To determine the extent of neovascularization, systemic blood flow to the left lung was measured after LPAL in rats using radiolabeled microspheres. Diethylaminoethanol pentaacetic acid-coated polystyrene microspheres (14- to 16-µm diameter; Kisker Products, Steinfurt, Germany) were bound to technetium-99m radioligand. Blood flow measurements were made 2 days (n = 3 rats), 14 days (n = 4 rats), and 21 days (n = 5 rats) after LPAL. Rats were anesthetized and ventilated as described above, the carotid artery was cannulated (PE 20), and 750,000 microspheres were infused (0.5 ml at 0.1 ml/min; Harvard Apparatus, Holliston, MA). Rats were euthanized by exsanguination, and the left lung was excised. Gamma emissions from lodged radiolabeled microspheres were infused into the left lung homogenates from naive rats (n = 3 rats) and after LPAL (4 h: n = 4 rats, 24 h: n = 6 rats, 3 days: n = 6 rats, and 5 days: n = 6 rats) and paired control right lungs. Following these time points, rats were euthanized, and the left and right lungs were excised and immediately frozen in liquid nitrogen. In an additional smaller group, left lungs from rats at the 4-h time point after sham surgery were compared with left lungs from rats 4 h after LPAL (n = 6 rats and 4 rats, respectively). Lung samples were homogenized using a Kinematica Polytron (Brinkmann Instruments, Westbury, NY) in tissue protein extraction reagent (25 mM bicine plus 150 mM NaCl, pH 7.6, product no.78510, Pierce, Rockford, IL) plus protease inhibitor (product no. 1169749801, Roche, Mannheim, Germany). Samples were aliquoted for ELISA and bicinchoninic acid assays. Quantitative protein evaluation of lung tissue CINC-3 expression was performed by ELISA (BioSource, Carlsbad, CA). Total protein measurements were made according to the BCA protein assay kit (Pierce, Rockford, IL).

Inhibitor studies. Sprague-Dawley rats were administered a CXCR2 neutralizing antibody kindly provided by Robert Strieter, MD (University of Virginia) and shown to be effective in both mouse and rat models (10-13, 14, 15). In a blinded study series, our laboratory confirmed the effectiveness of this neutralizing antibody in an in vitro endothelial cell chemotaxis assay (15). In primary culture rat arterial endothelial cells, neutralizing antibody to CXCR2 completely blocked chemotaxis to CINC-3 (R and D Systems, Minneapolis, MN; 1 ng/ml and 10 ng/ml; n = 3 experiments), relative to control goat serum (Sigma-Aldrich, St. Louis, MO). The treatment protocol for this group required intraperitoneal delivery of the neutralizing antibody (2.0 ml goat anti-CXCR2 serum ip) 24 h before LPAL, immediately after surgery, and every 48 h after LPAL until blood flow determination at 21 days (n = 5 rats). This treatment protocol was based on previous studies examining angiogenesis in rat models of lung transplant ischemia-reperfusion injury (3). A serum control group of rats was treated with goat serum (Sigma-Aldrich) using the same volume and time points as the treatment group with blood flow determination 21 days after LPAL (n = 4 rats).

Statistics. All data are presented as means ± SE. Changes in blood flow and CINC-3 protein were evaluated by one-way ANOVA. Relevant within group comparisons were made using either Student’s t-test for unequal data (blood flow) or multiple comparisons using Fisher’s test for least significant differences (blood flow and left lung protein). A P value ≤ 0.05 was accepted as significant.

RESULTS

Methacrylate casting. Our first efforts were to establish the rat model of bronchial vascular proliferation after LPAL and determine the time course of neovascularization. Figure 1 shows representative examples of the changes in the systemic circulation to the lung. Figure 1A shows the bronchial vasculature in a naïve animal. Bronchial arteries can be seen coursing along the large airways. Figure 1B, a cast of a rat 14 days after LPAL, is similar to that of the naïve rat with few bronchial vessels observed. This cast illustrates that at 14 days after LPAL, there is no evidence of bronchial neovascularization on a macroscopic level. However, by 21 days after LPAL, the systemic vasculature of the left lung has undergone substantial growth. Figure 1C provides a remarkable visualization of bronchial angiogenesis in the lung. Especially noteworthy are the large, tortuous bronchial vessels of the left lung in stark contrast to the right bronchus, which is essentially devoid of large vessels. Note the tortuous and enlarged left-sided tracheal arteries adhering to the trachea.

Neovascularization assessed by blood flow. Results of the effects of LPAL on systemic neovascularization of the lung are presented in Fig. 2 showing individual data points. Blood flow measurements were made 2 days, 14 days, and 21 days after
We chose the 2-day time point as a means of establishing the control flow at an early time point, because no new vasculature and flow beyond the normal bronchial blood flow would be expected. We measured blood flow at the 14-day time point despite there being no macroscopic evidence by casting of neovascularization in the event that we might detect microscopic vessel growth. Blood flow measurements were also made 21 days after LPAL when by casting, there was evidence of many new vessels. Blood flow was significantly increased in left lungs 21 days after LPAL (2.3 ± 0.5%) compared with animals 2 days (0.2 ± 0.1%) and 14 days (0.4 ± 0.2%) after LPAL (P < 0.01).

CINC-3 protein. To determine CINC-3 protein expression after LPAL, quantitative protein evaluation of lung tissue homogenates was performed by ELISA in naive lungs, in left lungs 4 and 24 h, 3 and 5 days after LPAL, and in paired control right lungs. Because there were no statistical differences in left lung CINC-3 protein between 4 h and 24 h after LPAL, nor 3 days and 5 days, we combined early (4 h and 24 h) and late (3 days and 5 days) protein levels to increase our sample sizes. Figure 3 shows changes in CINC-3 protein expression early (4 and 24 h) and late (3 and 5 days) after LPAL and normalized for total protein (P < 0.001). Within the first 24 h after LPAL, left lung CINC-3 protein was 56% greater than paired right lungs and statistically greater than all other lung groups (P < 0.01). No differences in CINC-3 protein level were observed among naive lungs, right lungs, and left lungs 3–5 days after LPAL (P > 0.05). No change in absolute lung protein was observed in the samples evaluated (P > 0.05). In an additional smaller group, left lungs 4 h after LPAL. **P < 0.01.

Fig. 1. A: representative methacrylate cast of the bronchial vasculature in a naïve rat. Anterior view of cast systemic vasculature (red) to the lung and airways (white). Note the aortic arch (aa), coronary arteries and chamber of the left ventricle filled with methacrylate. B: representative methacrylate cast of systemic vasculature in a rat, 14 days (14d) after left pulmonary artery ligation (LPAL). Note the similar appearance to the naïve animal with no evidence of gross bronchial neovascularization. C: representative methacrylate cast of bronchial neovascularization in a rat, 21 days (21d) after LPAL. Note the proliferation and enlarged, tortuous left (L) lung bronchial vessels and tracheal vessels. R, right.

Fig. 2. Functional assessment of bronchial angiogenesis in the rat lung. Blood flow to the left lung is presented as percent of cardiac output (number of radiolabeled microspheres in the left lung as a percentage of total body spheres). Each point represents a separate animal. Note the substantial increase in left lung perfusion by 21 days after LPAL. **P < 0.01.

Fig. 3. Change in average cytokine-induced neutrophil chemoattractant-3 (CINC-3) protein expression normalized for total protein in left lung homogenates after LPAL compared with paired right lung controls early (4 and 24 h) and later (3 and 5 days) after surgery (P < 0.001). Values are means ± SE; naive rats: n = 3 rats, 4–24 h: n = 10 rats; 3–5 day: n = 12 rats. CINC-3 protein expression in the left lung within the first 24 h after LPAL was significantly greater than in paired right lungs and all other groups (P < 0.01). No difference was observed among naive lungs, right lungs, and 3–5 day LPAL left lungs (P > 0.05). **P < 0.01.
LPAL \((n = 4)\) showed 60\% greater CINC-3 protein compared with left lungs from rats 4 h after sham thoracotomy \((n = 6)\); however, shams showed considerable variability. This average difference was similar to the early paired left lung-right lung differences presented in Fig. 3.

**Inhibitor studies.** Results of the effects of CXCR2 inhibition on systemic neovascularization of the lung after LPAL are presented in Figs. 4 and 5. A methacrylate cast of the bronchial vasculature in a rat treated with neutralizing antibody to CXCR2 21 days after LPAL illustrates an attenuated bronchial neovascularization with fewer, less dilated vessels (Fig. 4). The second rat cast after neutralizing antibody to CXCR2 21 days after LPAL showed no neovascularization. To quantify this attenuation, we measured blood flow in a group of rats treated with CXCR2 and compared it with a serum control group. Figure 5, displaying individual animal data points, shows that blood flow to the left lung 21 days after LPAL was significantly reduced in rats treated with CXCR2 antibody compared with serum treated LPAL animals \((P < 0.01)\). Blood flow to the left lung after LPAL in the CXCR2 antibody group averaged 0.4 \pm 0.2 vs. 1.4 \pm 0.3\% in the serum control group. When comparing all five blood flow groups, the CXCR2 neutralizing antibody group did not differ from the 2-day control group, suggesting that on average, the CXCR2 inhibition completely blocked neovascularization. However, the serum control group was slightly lower than the 21-day LPAL (no treatment) group suggesting a small vehicle effect.

**DISCUSSION**

The purpose of the present study was to confirm the time course of neovascularization in a rat model of chronic pulmonary ischemia, to provide three-dimensional visualization of the new bronchial vasculature, and to quantify the magnitude of perfusion through the remodeled systemic circulation to the lung. Extensive bronchial vascular proliferation and increased perfusion of the neovasculation was verified 21 days after LPAL. We also measured the early time course of the growth factor CINC-3 and confirmed the importance of its receptor CXCR2 in bronchial angiogenesis during conditions of chronic pulmonary ischemia.

Chronic pulmonary ischemia can result from pulmonary embolism, currently the third most common cardiovascular cause of morbidity and mortality in the United States (23). Pulmonary arterial obstruction can lead to abnormalities in gas exchange, to inflammation of lung tissue, and in cases of chronic thromboembolic disease to angiogenesis of systemic bronchial vessels to the ischemic lung (6, 18). Models of chronic pulmonary thromboembolism have been developed in a variety of animals. Weibel (28) first demonstrated in the rat, using a histologic approach, the time course of bronchial neovascularization after LPAL (28). His studies indicate that in the first few days after LPAL, bronchial arteries dilate slightly secondary to hemodynamic factors. By 5 days after LPAL, however, there is evidence of new vessel growth as determined by proliferative changes in bronchial vessel walls with endothelial and smooth muscle cells undergoing mitosis. His work further suggests that by 10–40 days after LPAL, new patent vessels have formed and stabilized. In the present study, we confirmed these findings using methacrylate casts to assess neovascularization after LPAL. Our casts demonstrate marked macroscopic bronchial neovascularization 21 days after LPAL. Especially noteworthy are the large, tortuous upstream bronchial vessels of the left lung in stark contrast to the right bronchus, which is essentially devoid of these large vessels. These vascular proliferative changes are remarkably similar to those seen in sheep that underwent LPAL shortly after birth and were cast 1 yr later (5). However, the casts of rats 14 days after LPAL look similar to those of naive animals, suggesting insufficient neovascularization to be captured by this technique. Although the histological approach likely demonstrates earlier proliferative changes, casting provides a three-dimensional anatomic perspective of the origin, tortuosity, and extent of new stable bronchial vessels. Regarding the terminology used to describe the observed neovascularization in this adult animal model, angiogenesis may be applied generally to the process of new vessel growth. However, strictly speaking, the term “collaterogenesis” is perhaps the more accurate way to describe the large-vessel phenomenon that we have observed by casting. As defined by Epstein and colleagues (7), collat-
erogenesis refers to the “remodeling and growth of collateral arteries from preexisting arterioles that form true conductance vessels with an elastic membrane and smooth muscle media.” Whether new capillaries also develop in this model requires histological delineation of systemic and pulmonary endothelium.

To obtain a functional assessment of the flow through the new vasculature, we used labeled microspheres to determine systemic bronchial perfusion to the ischemic left lung. We used 15-μm spheres, a size typically used to measure organ blood flow, which lodge at a precapillary site. Results showed no change in left lung perfusion 14 days after LPAL; however, a large increase in flow was observed by 21 days after LPAL. This blood flow measurement corroborates the visual assessment of new vessels based on casting. However, given the size, number, and extent of the neovasculature represented by the methacrylate casts, we predicted that we might have seen a greater increase in microsphere perfusion. These observations lead us to question whether vascular smooth muscle proliferation might also limit the functional perfusion of these vessels in vivo. Weibel’s (28) histological work suggested that vascular smooth muscle is also proliferating in this model and raises the possibility that these new vessels may be reactive and limit the extent of perfusion. Shi and colleagues (20) have shown that pulmonary vessels in a similar rat model of LPAL showed enhanced reactivity to endothelin. Further experimentation with vasoconstrictors and vasodilators is required to determine whether the reactivity of the bronchial neovasculature is altered to accommodate significantly increased flow to the ischemic lung.

Despite the long-standing observations that chronic pulmonary artery ischemia leads to systemic neovascular proliferation within the lung and the uniformity in response of all species studied, little is known about the growth factors that promote bronchial vascular angiogenesis. Knowledge of growth factors and the process of new vessel formation during chronic pulmonary ischemia may also provide information concerning bronchial angiogenesis in asthma, chronic obstructive pulmonary disease, cystic fibrosis, lung cancer, and pulmonary atresia. In addition, the bronchial neovascularization in a number of these conditions, contributes to tissue inflammation, which may exacerbate and prolong existing pathology. Thus, identifying the essential growth factors and their role in systemic angiogenesis of the lung may impact the development of therapies for a variety of lung diseases. The ELR+CXC chemokines have been shown to be key proangiogenic factors in several experimental lung models (25). In the rat, these primarily macrophage-derived proteins and functional ELR+CXC correlate that signal through CXCR2 are referred to as CINC-1, -2, and -3 (22). Recently, our laboratory has confirmed that blockade of CXCR2 in the mouse leads to diminished systemic neovascularization after LPAL (19). Unlike the human and mouse CXC chemokines, which have been shown to cause endothelial cell proliferation and migration (15, 26), little is known regarding the CINC and endothelial cell function in general. Therefore, the observation that increased levels of CINC-3 protein are observed early after LPAL in left lung homogenates is suggestive of the importance of this chemokine in early signaling activity. We focused specifically on CINC-3 because it exhibits close homology to macrophage inflammatory protein-2 (89%; Ref. 9), the mouse chemokine shown to be the most highly expressed CXC chemokine after chronic LPAL (24). Additionally, CINC-3, compared with the other CINC proteins, elicits the greatest calcium influx with binding CXCR2, indicative of its signaling potential (17, 21). The observation that treatment with a neutralizing antibody to CXCR2, the CINC-3 receptor was effective in limiting blood flow 21 days after LPAL, provides evidence that this chemokine ligand-receptor plays an important role in the overall process of lung angiogenesis. However, the early increase and subsequent decrease in CINC-3 protein by 3–5 days after ligation prompts one to question what additional growth factors might be important to sustain and promote neovascularization beyond early time points. At this time we can only speculate that CINC-3 is pivotal in setting off a cascade of events leading ultimately to massive vessel proliferation by 21 days after ligation.

Given the early appearance of CINC-3 protein expression and much later visualization and measurement of a functional enlarged systemic vasculature, it is not clear whether it was necessary to treat the rats with the neutralizing antibody for the entire 21-day period. Because inhibition of CXCR2 through which CINC-3 signals attenuated neovascularization, the observation supports the claim that this ligand-receptor relationship is key to the overall process. It is possible that the other CINC proteins (CINC-1, -2), which also signal through CXCR2, play a role over the longer course of functional vessel growth. Regarding the overall specificity of the neutralizing antibody, numerous confirmatory studies have been performed demonstrating its effectiveness in both mice and rat models (1, 3, 11). In addition, we performed our own limited assessment of the effectiveness of this antibody to CINC-3-induced arterial endothelial cell chemotaxis. In primary culture rat arterial endothelial cells, neutralizing antibody to CXCR2 completely blocked chemotaxis to CINC-3 relative to control goat serum. Furthermore, the data presented in this study corroborate our laboratory’s observations in mice (19) and add to a growing body of evidence suggesting that in several lung pathologies, the CXC chemokines are critical to the initiation of lung vascular remodeling (25).

In summary, we have shown that bronchial vessels undergo marked proliferation within 21 days after LPAL as observed by vascular casting. This neovasculature assumes functional patency and markedly increases the level of perfusion by the systemic circulation to the lung. The rat CXC chemokine CINC-3 is upregulated early after LPAL, and inhibition of its receptor CXCR2 limits neovascularization. Overall, the results of this study provide information concerning an essential growth factor in a small animal model that parallels bronchial vascular changes observed in human subjects.

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GRANTS

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