Preservation of Complement-induced Lung Injury in Mice with Deficiency of NADPH Oxidase

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Abstract

Mice with chronic granulomatous disease (X-CGD mice) generated by mutating the X-linked gene for a subunit of NADPH oxidase have been analyzed for their ability to respond to intravenous injection of purified cobra venom factor (CVF). This agent in wild-type mice produces a neutrophil-dependent and catalase-sensitive form of lung injury. Lung injury was evaluated by measuring the accumulation of extravascular albumin. Quite unexpectedly, the lungs of X-CGD mice showed no difference in the increased accumulation of extravascular albumin after injection of CVF when compared to wild-type mice. In both X-CGD and wild-type mice, full development of injury required neutrophils. While catalase was highly protective in wild-type mice, its protective effects were completely lost in the X-CGD mice. Furthermore, a competitive antagonist of L-arginine, N^G-methyl-L-arginine, was protective in X-CGD mice but not in wild-type mice. Allopurinol was protective in both types of mice. Both the basal and the CVF-inducible lung mRNA for inducible nitric oxide synthase and IL-1β was similar in X-CGD and wild-type mice. These data indicate that oxygen radical production and lung injury in response to injection of CVF occurs through alternative pathways in mice with genetic deletion of NADPH oxidase. (*J. Clin. Invest.* 1996. 97:2680–2684). Key words: neutrophils • hydroxyl radical • nitric oxide • chronic granulomatous disease • lung injury

Introduction

Chronic granulomatous disease (CGD)[1] encompasses a group of rare inherited disorders characterized by defects in a phagocyte-specific NADPH-oxidase complex that forms the superoxide radical during the respiratory burst and hydrogen peroxide (1). Mice with chronic granulomatous disease (X-CGD mice) have been generated by mutating the X-linked gene for the 91-kD subunit of NADPH oxidase (2). Neutrophils from these mice lack superoxide ($O_2\cdot$) formation by NADPH oxidase and have an increased susceptibility to *Staphylococcus aureus* and *Aspergillus fumigatus*, two important causes of infection in patients with CGD (1, 2). For example, these mice develop progressive *A. fumigatus* pneumonia after instillation of spores of this opportunistic pathogen into the respiratory tract, which is associated with a high mortality compared to wild-type mice (1, 2).

Intravenous infusion of cobra venom factor (CVF) into rats or mice induces sequestration of neutrophils within the pulmonary capillaries and an increase in vascular permeability (3, 4). This acute lung injury requires sudden, massive activation of complement, based on studies using soluble complement receptor 1 (5). The injury occurs within 30 min of infusion and is self-limited and reversible. Both platelets and neutrophils are also required, as demonstrated by depletion studies (3, 4). Furthermore, neutrophil adhesion to endothelial cells appears to be a key step in this injury, as demonstrated by studies in rats using inhibitors which depress the function of adhesion molecules (6–10). This injury also requires the production of oxygen radicals in mice and rats, particularly H_2O_2 generated from $O_2\cdot$, as determined by the protective effect of catalase. Generation of $O_2\cdot$ may reduce intracytoplasmic iron to its transition state (Fe^{2+}), after which interaction of Fe^{2+} with H_2O_2 generates the hydroxyl radical (HO·). HO· may be important in this injury, as demonstrated by the protective effect of iron chelators and hydroxyl radical scavengers (3, 4, 11).

This study tested the hypothesis that X-CGD mice are resistant to lung injury induced by CVF. The role of neutrophils and the contribution of oxidants was also evaluated. Because the initial studies showed that alternative pathways of oxygen-derived radical production were used in the X-CGD mice, studies investigating the production of murine inducible nitric oxide synthase (iNOS), IL-1β, and ICAM-1 mRNA were also performed.

Methods

Animals. Mice with chronic granulomatous disease (X-CGD mice) were generated by mutating the X-linked gene for the 91-kD subunit of NADPH oxidase (2). These animals are completely deficient in NADPH oxidase activity (2). Control animals were wild-type littermate mice of the same genetic background (C57BL6J × E129).

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; CVF, cobra venom factor; EVA, extravascular albumin; iNOS, inducible nitric oxide synthase; L-NMA, N^G-methyl-L-arginine.

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Lung injury. Lung injury induced by CVF was evaluated by quantitating the accumulation of extravascular albumin (EVA) using ^{125}I-albumin and ^{51}Cr-labeled murine RBC (^{51}Cr-RBC [12]). Five X-CGD mice and five wild-type mice were studied in each group. The mice were anesthetized by intramuscular injection of ketamine hydrochloride (Fort Dodge Laboratories, Inc., Fort Dodge, IA; 10 mg/kg) and acepromazine maleate (Fort Dodge Laboratories, Inc., 0.37 mg/kg). ^{125}I-albumin (IHSAI 125; Mallinckrodt Medical, Inc., St. Louis, MO; 0.20–0.25 μCi/mouse) was injected intravenously. 15 min later, CVF (2.0 U/mouse) was injected intravenously. At 28 min after the CVF injection, ^{51}Cr-RBC (0.25–0.30 μCi/100 μl/mouse) were injected intravenously to measure intravascular pulmonary blood volume. At 30 min, the mice were given an overdose of halothane. A blood sample was collected from the inferior vena cava. The chest was opened, and the base of the heart was rapidly tied off to prevent blood loss from the lungs. The thoracic organs were removed, and the lungs were fixed using intratracheal instillation of 6.0% glutaraldehyde in phosphate buffer. After 1 h, the lungs were separated from the heart and mediastinal tissues. The lungs, reference blood sample, and plasma sample were placed in scintillation vials and counted in a gamma counter (Packard Instrument Co., Meriden, CT) linked to an IBM computer for separation of multiple isotopes and correction for radiodecay. EVA was calculated as (12):

$$EVA = (\text{total } ^{125}\text{I-albumin volume}) - (\text{intravascular } ^{125}\text{I-albumin volume}) \quad (1)$$

where:

$$\text{total } ^{125}\text{I-albumin volume} = (^{125}\text{I-albumin in lung}) / (^{125}\text{I-albumin/g plasma}) \quad (2)$$

and

$$\text{intravascular } ^{125}\text{I-albumin volume} = (^{51}\text{Cr-RBC in lung}) / (^{51}\text{Cr-RBC/g blood}) \times (1 - \text{hematocrit}) . \quad (3)$$

Depletion of neutrophils. Wild-type and X-CGD mice were depleted of neutrophils by intraperitoneal injection of cyclophosphamide (C 7397; Sigma Chemical Co., St. Louis, MO; 200 mg/kg dissolved in 0.4 ml saline/mouse). This procedure was repeated daily for 4 d. The mice were treated with antibiotics, ceftriaxone sodium (Rocephin®; Hoffmann-LaRoche Inc., Nutley, NJ; 75 mg/kg i.m.) twice a day for 4 d to prevent infections. Circulating leukocyte and neutrophil counts were evaluated in blood samples obtained from the retro-orbital plexus using hemacytometer counts and Wright-stained smears.

Lung injury was induced by intravenous injection of CVF in neutrophil-depleted wild-type and X-CGD mice, and the injury was evaluated as described above. Paraffin-embedded sections of lung tissue were obtained to evaluate the presence of inflammatory foci as a complication of neutropenia.

Inhibition of oxidant production. Catalase (C-40; Sigma Chemical Co.; 0.65 mg/kg [273,000 U/mouse] dissolved in 100 μl saline/mouse) was injected intravenously to scavenge H_2O_2 (13). N^G-methyl-L-arginine (L-NMA) (M 7033; Sigma Chemical Co., dissolved in saline [10 mmol/l], 1.0 ml/mouse) was injected intraperitoneally to inhibit nitric oxide synthase (NOS [14]). Allopurinol (A8003; Sigma Chemical Co.; 200 mg/kg [4 mg/mouse] dissolved in a solution containing 25 μl 1 N NaOH, 25 μl H_2O, and 50 μl saline/mouse) was injected intravenously to inhibit xanthine oxidase (15). All agents or saline (100 μl/mouse) were given with ^{125}I-albumin 15 min before the CVF injection. CVF-induced lung injury was evaluated as described above.

Expression of calcium-independent, inducible NOS (iNOS), IL-1β, and ICAM-1 mRNAs in lung tissue. Lungs from uninjured and CVF-treated wild-type and X-CGD mice (n = 3 in each group) were frozen in liquid nitrogen. The tissue was pulverized in RNAzol (Tel-Test, Inc., Friendswood, TX) and the total RNA was extracted following the manufacturer's instructions. Total RNA was run on agarose/formaldehyde gels and transferred to nylon membranes. Northern blots were prepared as previously described after electrophoresis of total RNA on an agarose/formaldehyde gel and transfer to Magnacharge nylon membranes (Micron Separations, Westboro, MA [16]). Conditions for hybridization and stripping were as suggested by the manufacturer. Random-primed radiolabeled cDNA probes were generated using the Prime-a-Gene kit (Promega Corp., Madison, WI), according to the manufacturer's instructions. The murine iNOS cDNA was kindly provided by Drs. Qiao-wen Xie and Carl Nathan (Cornell University Medical College, New York, NY), the murine IL-1β cDNA by Dr. Patrick Gray (Genentech, Inc., South San Francisco, CA), and the murine ICAM-1 cDNA from the American Type Culture Collection (#63111; Rockville, MD). To correct for differences in loading, the Northern blots were probed for β-actin.

Statistics. Data were expressed as the mean±standard error of the mean. Data were compared using analyses of variance (17). When overall differences were identified, multiple contrasts with a Bonferonni adjustment were used to identify which groups were significantly different (18). Statistical significance was defined as $P < 0.05$.

Results

Requirement for NADPH oxidase in CVF-induced lung injury. The lungs of uninjured X-CGD mice that did not receive CVF had similar values of EVA as those in wild-type mice (Fig. 1). Intravenous injection of CVF caused a significant increase in the accumulation of EVA in both wild-type mice and X-CGD mice to values nearly double that found in uninjured mice. Unexpectedly, the increase in EVA was similar in both X-CGD and wild-type mice.

Effect of neutrophil depletion. Daily intraperitoneal instillation of cyclophosphamide for 4 d resulted in virtually nondetectable levels of circulating neutrophils in both wild-type and X-CGD mice ($0.001 \pm 0.002 \times 10^6$/ml in wild-type mice, $0.002 \pm 0.002 \times 10^6$/ml in X-CGD mice). Histological evaluation of lungs from either type of mouse showed no evidence of infection or inflammation. In wild-type mice, the accumulation of EVA was inhibited by 71% in neutropenic animals compared to controls. Similarly, the increase in EVA was reduced by 60% in the neutropenic X-CGD mice compared to X-CGD mice with normal circulating numbers of neutrophils (Fig. 1). These data indicate that neutrophils were required for full expression of CVF-induced injury in both X-CGD and wild-type mice.

Effect of inhibitors of oxidant production on lung injury. When mice were treated with catalase before intravenous infusion of CVF, there was, as expected based on previous studies (3), no increase in the accumulation of EVA in wild-type mice, indicating that treatment with catalase was completely protective (Fig. 1). In striking contrast, no protective effects of catalase were found in X-CGD mice, suggesting that formation of H_2O_2 was no longer required for development of lung injury.

Both X-CGD and wild-type mice were also treated with L-NMA to determine if an L-arginine–dependent pathway was operative. While treatment with L-NMA was not protective in the wild-type mice, this treatment in X-CGD mice completely prevented the increase in the accumulation of EVA after infusion of CVF, implying that a nitric oxide (·NO) generating pathway was engaged in X-CGD mice but not in wild-type mice.

Because previous studies have shown that the xanthine oxidase system, as measured in vivo by the inhibitory effect of al-

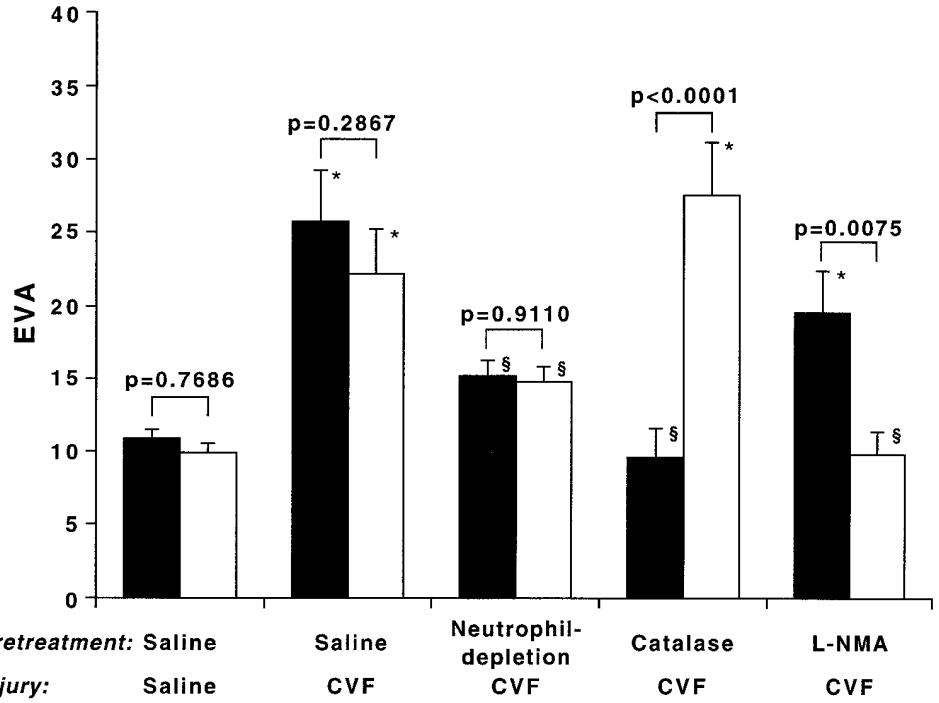

Figure 1. Edema formation in CVF-induced lung injury in wild-type and X-CGD mice. CVF induced a similar lung injury in both wild-type mice (*filled bars*) and X-CGD mice (*open bars*) compared to mice that received saline. Neutrophils were required for lung injury in both wild-type mice and X-CGD mice. Catalase inhibited the injury in wild-type mice, but not X-CGD mice. L-NMA inhibited injury only in X-CGD mice. $n = 5$ mice in each group. *Significantly different from uninjured mice pretreated with saline, $P < 0.01$. §Significantly different from mice pretreated with saline before injection of CVF, $P < 0.01$.

lopurinol, contributes to lung injury in rats (19), wild-type and X-CGD mice were treated with allopurinol before injection of CVF. Allopurinol significantly prevented the accumulation of EVA in both wild-type mice by 78% (14.1±2.1 μl EVA after allopurinol, 25.8±3.5 μl EVA without allopurinol) and in X-CGD mice by 82% (12.1±1.2 μl EVA after allopurinol, 22.2±3.1 μl EVA without allopurinol), suggesting that products of xanthine oxidase may be contributing to the outcome of the injury.

Expression of iNOS, IL-1, and ICAM-1 mRNAs in lung tissue. There was no difference in the basal expression of iNOS mRNA in X-CGD mice compared to wild-type mice, and no changes in the iNOS mRNA level were detected in either X-CGD or wild-type mice 30 min after injection of CVF (Fig. 2). There were also similar levels of expression of IL-1β mRNA in uninjured lungs of X-CGD and wild-type mice. CVF induced a large increase in IL-1β mRNA that was similar in X-CGD (23.0±6.6-fold increase) and wild-type mice (21.8±9.7, Fig. 2). Abundant quantities of ICAM-1 mRNA were observed in uninjured lungs that were similar in X-CGD and wild-type mice, and CVF did not induce a significant increase (Fig. 2).

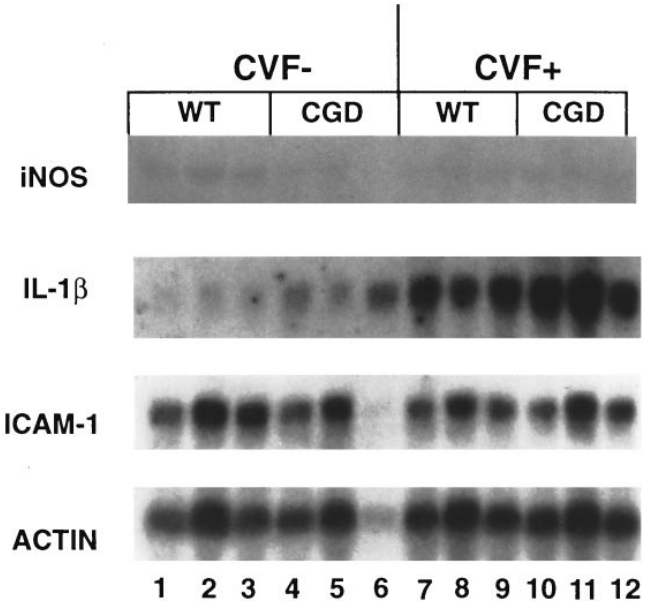

Figure 2. Expression of iNOS, IL-1β, and ICAM-1 mRNA in lung tissue. Wild-type (*WT*) and X-CGD (*CGD*) mice received cobra venom factor (*CVF+*, lanes *7–12*) or saline (*CVF−*, lanes *1–6*) as described in Methods. 10 mg of total RNA was electrophoresed per lane. After transfer to a nylon membrane, the blot was sequentially probed with radiolabeled cDNAs for iNOS, IL-1β, ICAM-1, and β-actin, as shown. Differences in expression were assessed by densitometry, with β-actin expression used to correct for differences in loading. The expression of iNOS mRNA was similar in uninjured lungs of wild-type and X-CGD mice and did not change after injection of CVF. The expression of IL-1β mRNA was similar in uninjured lungs of X-CGD mice compared to wild-type mice and increased similarly in both types of mice after injection of CVF. The expression of ICAM-1 mRNA was also similar in X-CGD and wild-type mice and did not increase after CVF.

Discussion

In rats and mice, the development of lung injury after intravascular injection of CVF is neutrophil and platelet dependent and sensitive to protective effects of catalase and superoxide dismutase (SOD) (3–5, 11). Surprisingly, X-CGD mice were not resistant to CVF-induced injury, and their lungs developed an injury similar to those of wild-type mice in response to CVF. While neutrophils remained vital to the induction of lung injury in the X-CGD mice, catalase was no longer able to cause protective effects. The ability of L-NMA to protect against injury in X-CGD mice but not wild-type mice suggests that X-CGD mice are using alternative pathways of oxidant production, such as the ·NO-generating pathway. The protec-

tive effects of allopurinol both in wild-type mice and in X-CGD mice suggest that xanthine oxidase in either neutrophils, endothelial cells, or both is at least partially responsible for $O_2\cdot$ generation when these cells are activated.

Our studies taken together with those of other investigators, suggest a pathway through which injury occurs in wild-type mice (Fig. 3). C5a generation after injection of CVF causes upregulation of adhesion molecules (including CD11b/CD18 on neutrophils and P-selectin on endothelial cells [6–10]), facilitating adhesive interactions between neutrophils and endothelial cells (6–10). Neutrophils activated by C5a generate $O_2\cdot$ from the NADPH oxidase, which is rapidly converted into H_2O_2 by superoxide dismutase. H_2O_2 is then released rapidly in the vicinity of endothelial cells. The generation of $O_2\cdot$ by either activated neutrophils or endothelial cells itself contributes to the full development of endothelial cell injury, as demonstrated by the partial protective effects of superoxide dismutase (3, 4). $O_2\cdot$ may react with intracellular endothelial ferritin, resulting in reduction of Fe^{3+} to its transition state, Fe^{2+}. H_2O_2 provided by activated neutrophils would then react with Fe^{2+} within endothelial cells to generate hydroxyl radical (HO·), resulting in endothelial cell injury. This pathway has been demonstrated in vitro to explain killing of rat pulmonary artery endothelial cells by activated neutrophils or by H_2O_2, the cytotoxic outcome being diminished by preloading endothelial cells with allopurinol, superoxide dismutase, or iron chelators (20–22). CVF-induced lung injury in rats is prevented by these same interventions (11).

In X-CGD mice, these studies suggest that alternative pathways resulting in lung vascular injury do not require oxidants generated by NADPH oxidase. $O_2\cdot$ may be the product of xanthine oxidase contained in endothelial cells. As reflected by the protective effects of L-NMA in X-CGD mice, neutrophils may be using an alternative oxygen-generating pathway involving nitric oxide synthase which generates ·NO. ·NO would then gain entry into endothelial cells, where it would react with endothelial $O_2\cdot$ generated by xanthine oxidase to form the peroxynitrite (ONOO−), a highly reactive radical species (23–25). Under conditions of protonation, HONOO would then undergo homolytic cleavage to form HO· in endothelial cells in the absence of requirement for a transition metal such as Fe^{2+}.

Why this alternative pathway is not used in wild-type mice given catalase is not readily apparent. The inability to detect differences in iNOS mRNA obtained from lungs of X-CGD compared to wild-type mice may reflect the insensitivity of this technique and the possibility that 30 min may be insufficient time for transcriptional and translational expression of iNOS. However, the basal levels of iNOS present in neutrophils may be sufficient for production of ·NO and lung injury by CVF in X-CGD mice. Il-1β is known to cause enhanced production of ·NO by pulmonary artery smooth muscle cells and by alveolar macrophages and rat type II pneumocytes (26, 27). Although no differences in the upregulation of IL-1β mRNA were observed in X-CGD mice when compared to wild-type mice, the large increase suggests that this pathway of ·NO upregulation was available in X-CGD mice.

While an alternative pathway may exist in X-CGD mice for the production of $O_2\cdot$ after infusion of CVF, this pathway does not provide a fully intact antimicrobial defense mechanism for the lung in response to infecting agents. In particular, this pathway is insufficient for containment of *Aspergillus* in X-CGD mice (2). In addition, this ·NO-generating pathway can not compensate in the killing of coagulase negative *Staphylococcus aureus*, since this organism has increased virulence in X-CGD mice and patients with CGD (1, 2, and unpublished data).

In summary, these studies show that mice deficient in NADPH oxidase use alternative pathways to mediate CVF-induced lung injury. The use of these pathways demonstrates the redundancies in the mechanisms through which protective and injurious oxidants are made. The production of ·NO by human neutrophils is controversial (19, 28, 29), and whether this particular pathway involving production of ·NO is impor-

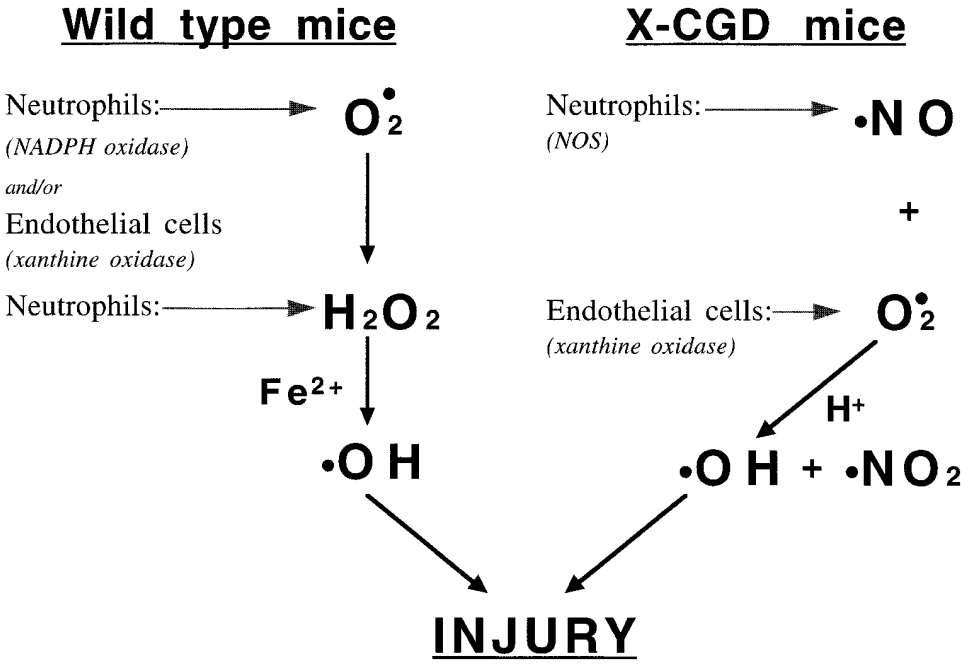

Figure 3. Diagram describing postulated alternative pathways of oxygen-derived radical production in X-CGD compared to wild-type mice. Please see text for details.

tant in patients with CGD remains to be determined. However, the data presented in this manuscript provide evidence that biological systems can use alternative pathways to compensate for the loss of an important molecule.

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