

Thalidomide Attenuates Airway Hyperresponsiveness and Eosinophilic Inflammation in a Murine Model of Allergic Asthma

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Received December 26, 2009; accepted March 8, 2010; published online March 11, 2010

Asthma is characterized by chronic eosinophilic inflammation and hyperresponsiveness of the airways. We hypothesized that thalidomide, which has numerous immunomodulatory properties, may have anti-inflammatory effects in allergic asthma. BALB/c mice sensitized and challenged with ovalbumin (OVA) were treated orally with thalidomide (30, 100, or 300 mg/kg) or a vehicle. When thalidomide was administered to OVA-challenged mice, the number of eosinophils in bronchoalveolar lavage fluid (BALF) was significantly decreased. The numbers of inflammatory cells other than eosinophils were not reduced by thalidomide. Thalidomide inhibited the elevated levels of interleukin-5 (IL-5) and tumor necrosis factor- α (TNF- α) in BALF by OVA challenges. Histological analysis of the lung revealed that both the infiltration of inflammatory cells and the hyperplasia of goblet cells were significantly suppressed by thalidomide treatment. Furthermore, thalidomide significantly inhibited the response to methacholine induced by OVA challenges. Taken together, thalidomide treatment decreased airway inflammation and hyperresponsiveness in a murine model of allergic asthma. These results might provide an opportunity for the development of novel therapeutics to treat severe asthma.

Key words thalidomide; asthma; eosinophilic infiltration; airway hyperresponsiveness; cytokine; ovalbumin

Bronchial asthma has clinical manifestations, which include variable degrees of airflow obstruction, airway hyperresponsiveness, and airway inflammation.¹⁾ The inflammatory process in asthma is dominated by T helper-2 (Th2) cells which produce interleukin-4 (IL-4), IL-5, and IL-13.²⁾

Thalidomide was synthesized in 1954 by CIBA Pharmaceutical Company, and prescribed as a sedative, tranquiliser, and antiemetic for morning sickness. Because the highest risk for teratogenicity arose when the drug was taken between weeks 3 and 8 after conception, it was withdrawn from many countries including Japan. However, the drug was revived as an effective treatment for erythema nodosum leprosum lesions in 1965. The U.S.A. Food and Drug Administration approved it for this indication in 1998.³⁾

Thalidomide, which is metabolized by cytochrome P450,^{3,4)} has a wide range of pharmaceutical and biological activities. Currently, clinical investigations of thalidomide have been conducted in patients with diverse diseases, including multiple myeloma, rheumatoid arthritis, and human immunodeficiency virus infection.^{5,6)} The beneficial pharmacological effects elicited by thalidomide include anti-angiogenic, anti-viral, anti-inflammatory, and immunomodulatory activities,^{5,7)} although the precise mechanisms are unknown.⁸⁾ It has been reported that thalidomide administered with albendazole suppressed eosinophilic infiltration in cerebrospinal fluid in a murine vermination model.⁹⁾ However, little is known about the effect of thalidomide on the airway hyperresponsiveness and eosinophilic inflammation in allergic asthma.

The purpose of this study was to investigate the effects of thalidomide on airway inflammation and hyperresponsiveness in a murine model of allergic asthma. We hypothesized that thalidomide would have anti-inflammatory effects in allergic asthma.

MATERIALS AND METHODS

Sensitisation and Challenge with Ovalbumin Seven-week-old female BALB/c mice (SLC, Shizuoka) were purchased and maintained in our specific pathogen-free animal facility. The mice were maintained at room temperature ($23 \pm 2^\circ\text{C}$) and humidity ($55 \pm 10\%$) in an air-conditioned room. They were fed a standard laboratory diet and given water *ad libitum*. They were sensitised by an intraperitoneal injection with 100 μg ovalbumin (OVA) and 1 mg alum in 200 μl of phosphate-buffered saline (PBS) on day 0, and then by a hypodermic injection with 10 μg OVA in 20 μl of PBS on day 14. They were then placed in an acrylic chamber and challenged with 1% OVA for 10 min daily by a nebulizer (IS-2; Paris, Starnberg, Germany) between days 21 and 25.¹⁰⁾ Saline was used in place of OVA during the sensitisation and challenge stages of the protocol as a negative control. All protocols described in this study were approved by the Animal Ethics Committee of Nagoya University Graduate School of Medicine, Nagoya, Japan.

Treatment Protocols For *in vivo* animal experiments, thalidomide (30, 100, or 300 mg/kg) was suspended in 0.5% carboxymethylcellulose, vortexed for 5 min, and administered orally *via* a stainless steel needle 1 h before each of the OVA challenges at a volume of 0.1 ml during this period. The dose was referred to previous studies.^{11,12)} Control mice received 0.1 ml of the vehicle orally. All drugs were prepared immediately before use.

Bronchoalveolar Lavage Bronchoalveolar lavage (BAL) was performed 24 h after the final OVA challenge. Mice were then killed by an intraperitoneal injection of sodium pentobarbital (100 mg/kg). The lungs were lavaged *in situ* *via* tracheal cannula with ice-cold PBS (1.0 ml $\times$ 3), and the BAL fluid (BALF) was centrifuged at 1500 *g* for 10 min. The supernatant of BALF was stored at -80°C until the assay of cytokines. Total cell counts were counted with a he-

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mocytometer (KX-21; Sysmex, Kobe). For cytological examination, differential cell counts were performed on cytopsin preparations with rapid Giemsa staining (Diff-Quik; International Reagent Corp., Kobe).¹⁰⁾

Lung Histology Lungs were isolated from the mice 24 h after the final OVA challenge. The lungs were fixed in 10% neutral formalin, paraffinised, cut into 4- μ m sections, and stained with hematoxylin and eosin (HE) for examining cell infiltration and with periodic acid-Schiff (PAS) for measuring mucus production. Total lung inflammation was defined as the sum of the peribronchial and perivascular scores.^{13,14)} The scoring system was as follows: 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe. An increment of 0.5 was used when the inflammation fell between two levels. To determine the extent of mucus production, goblet cell hyperplasia in the airway epithelium was quantified with a five-point grading system.^{15,16)} The adopted grading system was as follows: 0, no goblet cells; 1, <25%; 2, 25–50%; 3, 50–75%, and 4, >75%. The blinded quantitative analysis was performed with the described method previously.¹⁰⁾

Enzyme-Linked Immunosorbent Assay (ELISA) To determine the levels of cytokines *in vivo*, BALF samples were collected 24 h after the final OVA challenge. IL-4, -5, -13, and tumor necrosis factor- α (TNF- α) in BALF were assayed with commercially available ELISA kits (R&D, Minneapolis, MN, U.S.A.). The detection limit of each kit is 2 pg/ml for IL-4, 7 pg/ml for IL-5, 1.5 pg/ml for IL-13, and 5.1 pg/ml for TNF- α .

Measurement of Airway Hyperresponsiveness Airway hyperresponsiveness was assessed 24 h after the final OVA challenge. Mice were anaesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally. The tracheas were exposed and cannulated for mechanical ventilation. To abolish spontaneous respiration, pancuronium bromide (0.1 mg/kg) was injected intraperitoneally. Mice were mechanically ventilated with a small animal ventilator (Model 687; Harvard Apparatus, Holliston, MA, U.S.A.) at a tidal volume of 10 ml/kg, and a frequency of 120 breaths/min. To examine the development of airway hyperresponsiveness, total respiratory resistance in response to increasing concentrations of methacholine (3.125, 12.5, and 50 mg/ml) every 3 min were recorded (Max 1320; Buxco, Wilmington, NC, U.S.A.). The values were expressed as a percentage of the respective basal values in response to PBS.¹⁰⁾

Materials Reagents and drugs were as follows: OVA, pancuronium bromide, methacholine, thalidomide (Sigma, St. Louis, MO, U.S.A.), alum (Pierce, Rockford, IL, U.S.A.), and sodium pentobarbital (Abbott Laboratories, Chicago, IL, U.S.A.).

Statistical Analysis All data were expressed as means $\pm$ standard deviation (S.D.). Total cell counts, differential cell counts, and cytokines in BALF were compared by one-way analysis of variance (ANOVA). Histological scores were compared by the Kruskal–Wallis test, and airway hyperresponsiveness was tested by two-way repeated measures ANOVA. Each test was followed by the Tukey multiple comparison. *p* values less than 0.05 (*p* < 0.05) were considered statistically significant.

RESULTS

Thalidomide Suppresses Eosinophil Recruitment in BALF OVA challenges significantly increased the numbers of total cells, macrophages, lymphocytes, neutrophils, and eosinophils in BALF. In OVA-challenged mice, a large number of inflammatory cells were eosinophils. When thalidomide (300 mg/kg) was administered orally, the number of eosinophils were significantly decreased by thalidomide (300 mg/kg) treatment (*n* = 7, *p* < 0.05). In contrast, other cells were not affected by treatment with thalidomide (Fig. 1).

Thalidomide Reduces IL-5 and TNF- α Levels in BALF The levels of all four cytokines (IL-4, -5, -13, and TNF- α) in BALF obtained from OVA-challenged mice were significantly higher than those from control. Thalidomide significantly decreased the levels of IL-5 in a concentration-dependent manner (*n* = 7, *p* < 0.01) and TNF- α (*n* = 7, *p* < 0.05) in BALF as compared with those from OVA-challenged mice (Fig. 2). On the other hand, thalidomide did not significantly decrease the IL-4 and IL-13 levels in BALF (Fig. 2).

Thalidomide Inhibits Infiltration of Inflammatory Cells and Mucus Production Control mice had no detectable inflammatory response in the lung, whereas OVA challenged mice showed extensive infiltration of inflammatory cells around airways and blood vessels (Figs. 3A, B). The majority of the infiltrated inflammatory cells were eosinophils. The administration of thalidomide (300 mg/kg) significantly reduced the infiltration of inflammatory cells in the peribronchial and perivascular areas as compared with the OVA-challenged mice (Fig. 3C). The Inflammation scores in OVA-challenged mice and thalidomide-treated mice were 3.3 ± 1.0 and 1.9 ± 1.0 , respectively (*n* = 4, *p* < 0.01) (Fig. 4A). OVA-challenged mice, but not control mice, had the hyperplasia of goblet cells within the bronchi in the lung (Figs. 3D, E). Thalidomide (300 mg/kg) significantly reduced the hyperplasia of goblet cells (Fig. 3F). The mucus scores in OVA-challenged mice and thalidomide treatment were 3.3 ± 0.9 and 1.7 ± 1.0 , respectively (*n* = 4, *p* < 0.01) (Fig. 4B).

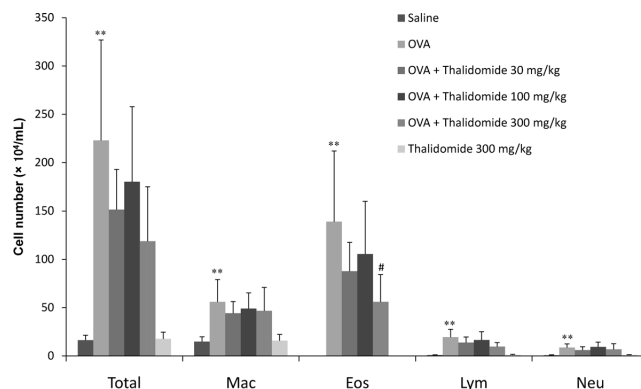


Fig. 1. Effects of Thalidomide on Eosinophilic Infiltration to the Airways by Allergen Challenges

The numbers of total cells, macrophages (Mac), eosinophils (Eos), lymphocytes (Lym), and neutrophils (Neu) in bronchoalveolar lavage fluid (BALF) collected from ovalbumin-challenged (OVA-challenged) mice (OVA), OVA-challenged mice treated with thalidomide by oral administration (30, 100, or 300 mg/kg), saline-challenged mice (Saline), and saline-challenged mice treated with thalidomide by oral administration (300 mg/kg) (Thalidomide) were counted. *n* = 7, ***p* < 0.01 vs. Saline, #*p* < 0.05 vs. OVA.

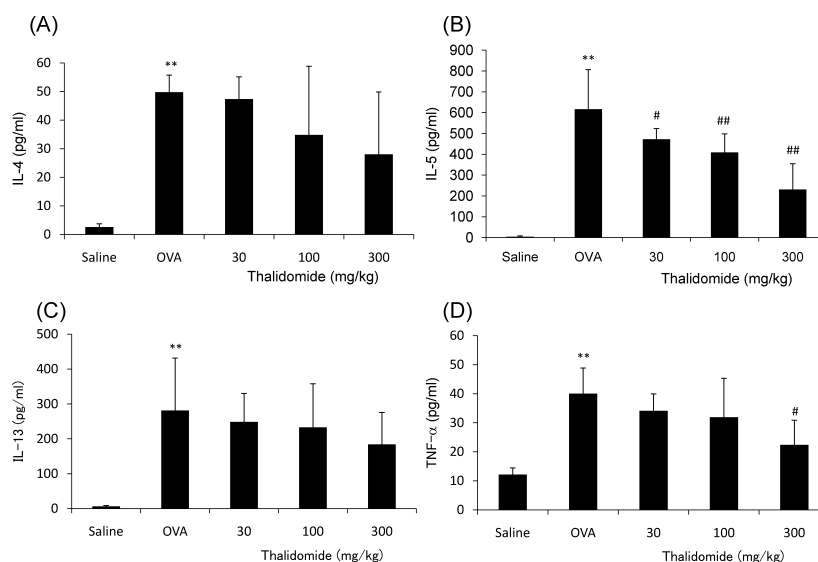


Fig. 2. Effects of Thalidomide on Cytokine Levels in BALF

The levels of IL-4 (A), IL-5 (B), IL-13 (C), and TNF-α (D) in BALF collected under the same experimental condition as shown in Fig. 1 were analysed with enzyme-linked immunosorbent assay (ELISA). $n=7$, ** $p<0.01$ vs. Saline, # $p<0.05$ vs. OVA, ## $p<0.01$ vs. OVA.

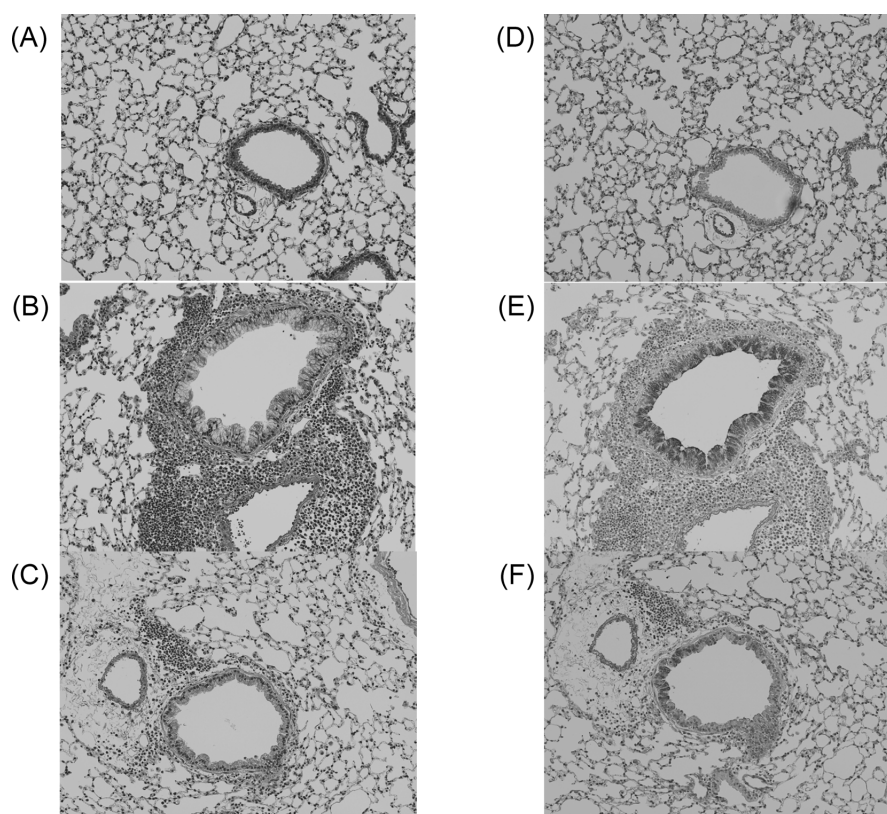


Fig. 3. Effects of Thalidomide on Inflammatory Cell Infiltration and Mucus Production in the Lung by Allergen Challenges

Histological examinations of lung tissue were performed for the inflammatory cell infiltration (A–C, magnification $\times 400$) and the mucus secretion (D–F, magnification $\times 400$) in saline-administrated mice (A, D), OVA-challenged mice (B, E), and OVA-challenged mice treated with thalidomide by oral administration (300 mg/kg) (C, F).

Thalidomide Suppresses Airway Hyperresponsiveness

Next, the effects of thalidomide on OVA-induced airway hyperresponsiveness were examined. Total respiratory resistance in response to methacholine was significantly increased in OVA-challenged mice as compared with control mice ($n=6$, $p<0.05$). At 50 mg/ml methacholine, the values of the percentage of total respiratory resistance in control mice and

OVA-challenged mice were $113\pm 6\%$ and $134\pm 8\%$, respectively ($n=6$, $p<0.01$). Thalidomide (300 mg/kg) significantly inhibited the response to methacholine induced by OVA challenges. The value of the percentage of total respiratory resistance at 50 mg/ml methacholine was significantly decreased from $134\pm 8\%$ to $116\pm 8\%$ by 300 mg/kg thalidomide treatment ($n=6$, $p<0.01$) (Fig. 5).

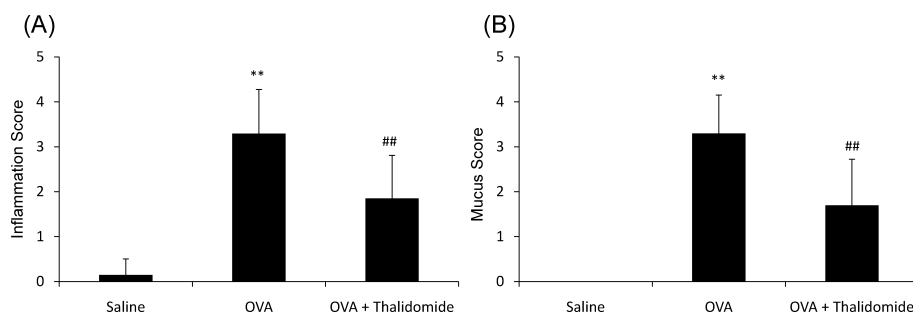


Fig. 4. Effects of Thalidomide on Inflammatory Cell Infiltration and Mucus Production in the Lung by Allergen Challenges

Quantitative analyses of inflammatory cell infiltration (A) and mucus production (B) in saline-administrated mice (Saline), OVA-challenged mice (OVA), and OVA-challenged mice treated with thalidomide by oral administration (300 mg/kg) (OVA + Thalidomide) were evaluated in lung sections with a scoring method described in Materials and Methods. $n=4$, ** $p<0.01$ vs. Saline, ## $p<0.01$ vs. OVA.

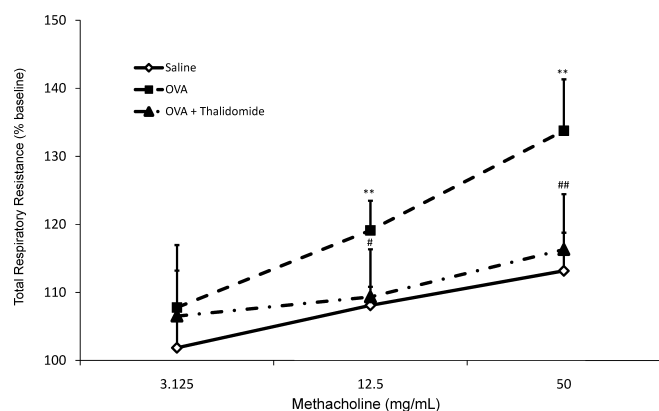


Fig. 5. Effects of Thalidomide on Airway Hyperresponsiveness by Allergen Challenges

Dose-response relationship for inhaled methacholine (3.125, 12.5, 50 mg/ml) on total respiratory resistance was measured in saline-administrated mice (Saline), OVA-challenged mice (OVA), and OVA-challenged mice treated with thalidomide by oral administration (300 mg/kg) (OVA + Thalidomide). The values were expressed as a percentage of the respective basal values in response to PBS. $n=6$, ** $p<0.01$ vs. Saline, # $p<0.05$ vs. OVA, ## $p<0.01$ vs. OVA.

DISCUSSION

The main findings of the present study are that in OVA-challenged mice (1) thalidomide attenuated the increased number of eosinophils, the elevated levels of IL-5 and TNF- α in BALF, (2) suppressed both the infiltration of inflammatory cells and hyperplasia of goblet cells to the airways, and (3) reduced airway responsiveness to methacholine. To our knowledge, this study is the first to demonstrate that thalidomide inhibited eosinophilic inflammation in the airways and airway hyperresponsiveness in a murine model of asthma.

Thalidomide significantly suppressed the levels of IL-5 and TNF- α in BALF in OVA-challenged mice, but it did not affect the IL-4 and IL-13 levels (Fig. 2). IL-5 is involved in the development and maturation of eosinophils.^{1,17} TNF- α is a key regulator of other pro-inflammatory cytokines and priming activator of inflammatory cells.¹⁸ The inhibitory mechanisms by thalidomide may be implicated in Ras, which is associated with signalling of IL-5,¹⁹ and nuclear factor- κ B (NF- κ B), which regulates the production of TNF- α ,²⁰ since thalidomide reportedly inhibits Ras and NF- κ B signalling.^{21,22} Therefore, we consider that thalidomide might modulate cytokines according to diverse signal pathways, and might have an effect on monocytes, because it inhibits the levels of IL-5 and TNF- α produced by human monocytes *in*

vitro.^{23,24}

Thalidomide significantly reduced the numbers of eosinophil in BALF (Fig. 1). Moreover, histological analysis of the airways revealed that both the infiltration of inflammatory cells and hyperplasia of goblet cells were significantly suppressed by thalidomide treatment (Figs. 3, 4). Until now, it is reported that the decrease of eosinophil numbers in BALF was correlated with attenuation of asthma symptoms in animal models.^{15,25} Many clinical studies have also documented a correlation between pulmonary eosinophilia and asthma, and the degree of eosinophils in BALF correlates with disease severity.²⁶ Thus, pulmonary eosinophilia has been closely related to asthma symptoms. The suppression of pulmonary eosinophilia by thalidomide could be explained by the inhibition of both differentiation and migration of eosinophils. IL-5 critically regulates expression of genes involved in proliferation, cell survival and maturation of eosinophils.¹⁹ TNF- α may also function as a pro-inflammatory cytokine that causes the recruitment of eosinophils.²⁷ Both cytokines play an important role in differentiation and migration of eosinophils. We speculate that thalidomide caused the decrease of eosinophil numbers in BALF through suppression of these cytokines. In addition, our findings may result from blocking NF- κ B, which is associated with the perpetuation of chronic airway inflammation,^{28,29} through a mechanism involving the inhibition of activity of inhibitor κ B kinase by thalidomide.²¹

Thalidomide significantly suppressed airway hyperresponsiveness (Fig. 5). There are many reports of a relationship between the number of inflammatory cells, particularly eosinophils, and the level of airway hyperresponsiveness.³⁰ Consequently, it is likely that anti-inflammatory therapy improves both airway inflammation and airway hyperresponsiveness.³⁰ Moreover, inhibition of the IL-5 and TNF- α can improve airway hyperresponsiveness.^{31,32} Thus, anti-inflammatory effects of thalidomide treatment may have been in part responsible for the observed results.

Rodrigues *et al.* reported that thalidomide, given in a relative low dose orally, caused a dose-dependent inhibition of TNF- α production in a lipopolysaccharide (LPS) model of uveitis in rats,³³ whereas we observed that thalidomide mildly reduced the level of TNF- α in BALF. This difference is presumed to be due to timing for sample recovery, because TNF- α production in lysed BAL cells reportedly peaks at 1–2 h by LPS stimulation.³⁴ Through the earlier sample recovery, thalidomide may well have caused more inhibition of

the TNF- α level.

In summary, thalidomide serves to control the airway inflammation and hyperresponsiveness characteristic of bronchial asthma in a murine model of allergic asthma. Although thalidomide may be difficult to apply to all patients with asthma, some patients suffering from asthma often fail to respond to conventional therapy.³⁵ More research on thalidomide or its derivatives may yield opportunities for the development of novel therapeutics to treat severe asthma.

Acknowledgements We thank Ms. Keiko Shimamoto for her valuable technical assistance.

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