Increased Efficacy of Breast Cancer Chemotherapy in Thrombocytopenic Mice

Mélanie Demers1,2,3, Benoit Ho-Tin-Noé1,2,3, Daphne Schatzberg1,2, Janie J. Yang1,2, and Denisa D. Wagner1,2,3

Abstract
Platelets contribute to homeostasis of the tumor vasculature by helping prevent hemorrhage. Thus, we hypothesized that inducing thrombocytopenia would increase tumor vascular leakiness and facilitate the effective delivery of chemotherapeutic agents to tumors. In a mammary carcinoma murine model, platelet depletion induced bleeding specifically at the tumor site, favoring the accumulation of fluorescently labeled microspheres only in the tumor. Moreover, induction of thrombocytopenia in tumor-bearing mice before injection of paclitaxel increased its intratumoral accumulation and reduced growth of both slow- and fast-growing tumors, compared with mice with normal platelet counts that were treated only with paclitaxel. Histologic analysis confirmed the expectation of an increase in tumor apoptosis and a reduction in tumor proliferation in thrombocytopenic mice receiving chemotherapy. No increased toxicity was seen in other organs or blood cells. Taken together, our results indicate that low platelet count selectively induces leakiness of tumor vessels and favors the delivery of chemotherapy to tumor sites, enhancing its tumoricidal effects. Cancer Res; 71(5): 1540–9. ©2011 AACR.

Introduction
Cancer therapy is hampered by dose-limiting side effects that reduce the efficacy of cancer treatments. Conventional drugs used for treatment do not selectively accumulate in the tumor (1). Thus, important research goals include delivering high doses of drug to tumor sites for maximum treatment efficacy while minimizing side effects to healthy tissues (2). In the search for more effective treatments, efforts are made to develop drugs that selectively target tumor antigens to preferentially deliver the therapeutic agent to the tumor site (1, 3). Antigen heterogeneity among diverse types of tumors, and among tumor cells of the same tumor, renders this approach effective only in specific cases.

Other strategies target tumoral neovascularature. Angiogenesis is a hallmark feature of cancer growth, and tumor vessels in distinct tumors may also express markers that are different from normal vasculature (4). The targeting of angiogenesis is therefore applicable to a wide range of different tumors. Antibodies targeting VEGF, platelet-derived growth factor, and integrins have been described and used to alter tumor vessels in animal and clinical studies (5–8). Unfortunately, antiangiogenic therapies in cancer have until now shown only limited or transient effects (9–11).

Platelets are a rich source of pro- and antiangiogenic factors that are released in the circulation upon platelet activation (12). Platelets also maintain the integrity of angiogenic and inflamed microvessels (13, 14). Furthermore, we recently showed that platelets support tumor vascular homeostasis by preventing intratumoral hemorrhaging (15). The tumor bleeding induced by thrombocytopenia cannot be prevented by degranulated platelets, suggesting that the local release of soluble factors protects the tumor endothelium against injuries produced by tumor-associated inflammatory cells (15, 16).

Because thrombocytopenia renders vessels in the tumor leaky, we hypothesized that this leakiness would enhance the access of chemotherapy to the tumor. Paclitaxel is a well-known therapeutic drug used to treat breast cancer. It acts as a mitotic inhibitor that stabilizes microtubules, interfering with their normal breakdown during cell division, inducing apoptosis (17–19). Here we show that thrombocytopenia promotes the accumulation of inert particles at the tumor site, reflecting an increase in porosity of the tumor vessels. Using mammary and lung carcinoma mouse models, we show that thrombocytopenia enhances the inhibitory effect of paclitaxel on tumor growth without increasing its toxicity to other organs.

Materials and Methods

Cell lines and reagents
The 4T1 and Lewis lung carcinoma (LLC) cell lines were maintained in high glucose Dulbecco’s Modified Eagle Medium + l-GluataMAX-1 [supplemented with 10% (v/v) fetal calf
serum, 10 mmol/L HEPES buffer, 10 mmol/L sodium carbonate (4T1 cells)]. The 4T1 cell line was originally isolated from a spontaneously arising mammary tumor in a BALB/c mouse (20) whereas LLC cells originated from C57BL/6 mice. The 4T1 and LLC cells were injected in syngeneic mice after less than 4 and 10 serial passages in vitro, respectively. For the fast-growing 4T1 model, the cells were cultivated for more than 25 passages. All cell lines were obtained from and characterized by ATCC according to the cell line authentification testing (growth curve analysis; mycoplasma, bacteria, and fungi contamination; DNA profiling; and species confirmation) and were used within 6 months after resuscitation. All cell culture products were purchased from Life Technologies/Invitrogen.

Animals
All animal procedures described in this study were carried out using 6- to 8-week-old BALB/c female mice or C57BL/6 male mice (purchased from The Jackson Laboratory), except in experiments using dorsal skinfold chamber for which 10-week-old mice were used. All experimental procedures involving the use of mice were approved by the Animal Care and Use Committee of the Immune Disease Institute.

Induction of thrombocytopenia
Thrombocytopenia was induced by an intravenous (i.v.) injection of 2.5 μg/g of a platelet-depleting antibody (polyclonal rat IgG anti-mouse GPIbα; emfret Analytics). Control mice were injected with a nonimmune rat polyclonal IgG (emfret Analytics). Thrombocytopenia was verified by flow cytometry and resulted in less than 5% of normal platelet count.

Determination of hemoglobin content
Organs and tumors of sacrificed animals were excised, weighed, homogenized in Drabkin’s reagent (Sigma), and centrifuged (2,000 × g, 10 minutes). Hemoglobin content of supernatants was measured by absorbance at 540 nm.

Immunohistology
Tumors and organs were harvested from sacrificed animals and fixed in zinc fixative (100 mmol/L Tris-HCl containing 37 mmol/L zinc chloride, 23 mmol/L zinc acetate, and 3.2 mmol/L calcium acetate). Paraflin-embedded sections were deparaffinized in xylene and rehydrated through a graded alcohol series. For Perl’s Prussian blue staining, the sections were transferred to a mixture of equal parts of 2% potassium ferrocyanide and 2% hydrochloric acid. Sections were counterstained with nuclear fast red. Tissues were dehydrated, mounted in DPX mountant (Fluka BioChemika) and observed by light microscopy. For quantification of apoptosis, the sections were permeabilized and stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Roche Applied Science) according to the manufacturer’s instructions. For quantification of proliferation, the sections were stained with Ki-67-Alexa 555–conjugated antibody (BD Biosciences). For quantification of apoptotic endothelial cells, the sections were stained with TUNEL as described earlier, incubated with a rat anti-CD31 antibody (eBioscience) and anti-rat Alexa-594 (Invitrogen) as secondary antibody. The percentage of apoptotic cells was determined as the ratio of TUNEL-positive nuclei of CD31-positive cells to the total CD31-positive cells. Pericytes were stained using a rabbit anti-NG2 antibody (Chemicon) followed by incubation with an anti-rabbit Alexa-488 (Invitrogen). Sections were counterstained with Hoechst to visualize all nuclei, mounted with Fluoro-gel (Electron Microscopy Sciences), and observed under an epifluorescent microscope.

Imaging of fluorescently labeled microspheres
Dorsal skinfold chambers and surgical preparation were performed as described (21). Two days after recovery, 1 × 10^6 4T1 or LLC cells were implanted in the conjunctive tissue below the striated skin muscle layer and allowed to grow for 5 days. Thrombocytopenia was induced and mice were then injected i.v. with 100 × 10^6 1-μm FluoroSpheres, red fluorescent carboxylate-modified microspheres (Invitrogen) 5 min later. Tumors were photographed 24 hours after injection of the platelet-depleting or control antibody. Microscopy imaging was done using an upright microscope (Axioplan; Zeiss) with ×2.5 or ×4 magnification objectives and recorded by attached digital camera (AxioCam HRC).

Induction of tumors and chemotherapeutic treatments
4T1 cells (4 × 10^5) were inoculated in the mammary fat-pad of 6- to 8-week-old BALB/c mice. For the LLC model, 1.5 × 10^6 cells were inoculated subcutaneously in the right flank of 6- to 8-week-old C57BL/6 mice. Animals were monitored twice a week, carefully measuring the tumors using calipers. The tumor volume was calculated using the formula: V = lw^2 × 0.4, where l is the length and w the width (22). For chemotherapeutic treatment, tumor-bearing mice were randomly distributed into 4 groups and injected i.v. with either the platelet-depleting or control antibody. Three hours later, mice were injected intraperitoneally (i.p.) with paclitaxel (30 mg/kg; Sigma; Cremophor EL/Ethanol, 1:1 diluted in PBS) or vehicle. For treatment characterization 72 hours posttreatment, blood was collected and analyzed using the Hemavet blood cell counter (Drew Scientific, Inc.). Tumor, spleen, liver, and kidneys were examined and collected at necropsy.

Quantification of radiolabeled paclitaxel in the mammary tumor
At day 10 after injection of 4T1 cells, the mice were randomly distributed into 2 groups and injected i.v. with either the platelet-depleting or control antibody. Three hours later, mice were injected i.v. with 3H-paclitaxel (5 μCi/20 g mouse; Moravek Biochemicals Inc.) and sacrificed after 2 hours. The tumors were collected, weighed, and solubilized in Solvable (Perkin Elmer) overnight at 60°C, treated with hydroxyperoxide 30% (Sigma), and analyzed for radioactivity by liquid scintillation counting in a TRI-CARB liquid scintillation analyzer (Perkin Elmer).

LDH activity of tumor homogenate
For quantification of necrosis, the tumors were harvested and assessed for lactate dehydrogenase (LDH)
activity using the Quantichrom Lactate Dehydrogenase Kit (BioAssay Systems) according to the manufacturer’s instructions.

Statistical analysis
Data are represented as mean ± SEM and were analyzed by a 2-sided Kruskal–Wallis test to compare more than 3 groups. Two-sided Mann-Whitney test was conducted between groups at each time point. All P values were considered significant at the 0.05 level.

Results

Acute thrombocytopenia induces bleeding only at the tumor site
We previously showed that thrombocytopenia induces tumor hemorrhage without any macroscopic signs of bleeding in other organs (15). To address this more precisely, we determined the hemoglobin content, reflecting the level of red blood cell extravasation, of the major organs in thrombocytopenic versus control tumor-bearing mice. For this purpose, 4T1 mammary carcinoma cells were implanted in the mammary fat pad of BALB/c syngeneic mice and allowed to grow. At day 8, platelet depletion was induced for 24 hours; subsequently, mice were sacrificed and the hemoglobin content of the organs and tumor was quantified. The hemoglobin content in the tumors of mice with normal platelet count was similar to that of the skin and less than that of highly vascularized organs such as lung, liver, and heart as expected (23). No significant difference in the hemoglobin content of major organs was found between thrombocytopenic mice and controls (Fig. 1A). In contrast, a significant increase in hemoglobin content and the presence of extravasated red blood cells (Fig. 1B) were observed in mammary carcinomas of thrombocytopenic mice compared with mice with normal platelet count. Furthermore, histopathologic analysis of paraffin-embedded tissues revealed neither extravasated red blood cells (yellow/brown) nor positive staining for ferric iron/hemosiderin (blue) in the major organs except in the spleen of both control and thrombocytopenic mice (Supplementary Fig. S1). These results show that platelet depletion in tumor-bearing mice induces red blood cell leakage specifically in the tumoral vasculature, whereas intact vasculature is maintained in other organs.

To determine the status of tumor vessels 24 hours after induction of thrombocytopenia, we evaluated the percentage of apoptotic endothelial cells in the tumors of thrombocytopenic mice compared with control mice. Immunostaining analysis revealed a similar frequency of apoptotic endothelial cells in the tumors (Fig. 1C). No obvious differences in the mean diameter of the tumor vessels (Control 8.0 ± 1.1 μm versus Depleted 8.0 ± 0.2 μm; P > 0.05, n = 3) and in the number of NG2-positive pericytes within the tumor sections (Control 419.9 ± 30.1 pericytes/mm² versus Depleted 473.8 ± 22.4 pericytes/mm²; P > 0.05, n = 4) were observed. These results indicate that the overall structure of tumor vessels is maintained during acute thrombocytopenia and that the thrombocytopenia-induced hemorrhage is not a result of apoptosis of endothelial cells or loss of pericytes around tumor vessels.
Thrombocytopenia induces tumor vascular porosity that allows accumulation of fluorescently labeled microspheres

Knowing that the absence of platelets induces tumor hemorrhage, we hypothesized that thrombocytopenia results in the formation of breaches in the tumor vasculature through which circulating particles could pass. To investigate whether tumor hemorrhage induces accumulation of circulating particles at the tumor site, we implanted 4T1 cells in dorsal skinfold window chambers placed on the backs of BALB/c mice. Two days later, 4T1 tumor cells were injected within skinfold chamber and allowed to grow for 5 days. A, photographs of the tumors were taken 24 hours after mice were injected with either a control antibody (Control) or a platelet-depleting antibody (Depleted). Five minutes after injection of either control or platelet-depleting antibody, $100 \times 10^6$ 1-μm fluorescent microspheres were injected into all mice. B and C, images of a control and a thrombocytopenic tumor were taken at ×25 (B) and ×40 (C) magnifications. Results are representative of 5 different hemorrhagic tumors and 4 nonhemorrhagic tumors. Bars, 2 mm (A), 400 μm (B), 200μm (C); $\star$, center of the tumor; T, tumor delineated by a white line.

Figure 2. Tumor hemorrhage caused by acute thrombocytopenia favors the accumulation of fluorescently labeled microspheres. Dorsal skinfold chambers were surgically implanted on the backs of mice. Two days later, 4T1 tumor cells were injected within skinfold chamber and allowed to grow for 5 days. A, photographs of the tumors were taken 24 hours after mice were injected with either a control antibody (Control) or a platelet-depleting antibody (Depleted). Five minutes after injection of either control or platelet-depleting antibody, $100 \times 10^6$ 1-μm fluorescent microspheres were injected into all mice. B and C, images of a control and a thrombocytopenic tumor were taken at ×25 (B) and ×40 (C) magnifications. Results are representative of 5 different hemorrhagic tumors and 4 nonhemorrhagic tumors. Bars, 2 mm (A), 400 μm (B), 200μm (C); $\star$, center of the tumor; T, tumor delineated by a white line.
accumulation of circulating particles to the tumor site, likely through openings in the vasculature that also allow the red blood cell extravasation.

Porosity of tumor vessels induced by thrombocytopenia enhances the effect of paclitaxel on mammary tumors

The capacity of acute thrombocytopenia to favor the intratumoral accumulation of microbeads suggests that short-term platelet depletion could improve the inhibitory effect of paclitaxel on tumor growth. Therefore, we evaluated the effect of paclitaxel treatment combined with acute thrombocytopenia on the growth of slow- and fast-growing tumors. Mammary tumor 4T1 cells, cultivated for less than 4 in vitro passages (Fig. 3A; slow-growing tumors) or for more than 25 in vitro passages (Fig. 3B; fast-growing tumors), were allowed to grow in BALB/c mice. When the tumors reached 50 to 100 mm³, the mice were infused i.v. with the platelet-depleting or control antibody and 2 to 3 hours later were injected i.p. with paclitaxel or vehicle and tumor growth was measured after 3 and 8 days (Fig. 3). At day 11, 3 days after the injections, a significant reduction in tumor growth of both the slow- and the fast-growing tumors was observed in the group of mice that received both treatments compared with paclitaxel alone, platelet depletion alone, or control mice. At day 16, 8 days after the injections, an average 20% reduction in growth of the slow-growing tumors (2.78 ± 0.1873 versus 3.46 ± 0.051; P ≤ 0.01; **, P ≤ 0.001).
0.3789) and 27% reduction in growth of the fast-growing tumors (4.069 ± 0.3702 versus 5.585 ± 0.2579) were observed in mice that received both treatments compared with control mice. This reduction was significant only in the fast-growing tumors, in which paclitaxel, affecting dividing cells, may have had access to more targets. These results suggest that thrombocytopenia enhances the delivery of paclitaxel to the tumor site and improves its tumoricidal effects.

Acute thrombocytopenia increases delivery of paclitaxel to tumors
To evaluate whether the reduction in tumor growth observed in combination treatments was the result of an increase in the delivery of drug to the hemorrhagic tumors, we quantified the presence of radiolabeled-paclitaxel in mammary tumors of thrombocytopenic mice compared with mice with normal platelet count. Mice bearing 10-day-old tumors were treated with either the platelet-depleting or control antibody and injected 3 hours later with $^3$H-paclitaxel. Quantification of the radioactivity in the hemorrhagic tumors revealed a significant increase of paclitaxel in the tumors of thrombocytopenic mice compared with controls. This result shows that by inducing tumor-bleeding acute thrombocytopenia allows the accumulation of circulating chemotherapeutic drug in tumors.

Increased apoptosis and reduced proliferation in mammary tumors of thrombocytopenic mice treated with paclitaxel
To determine whether the observed reduction in tumor growth was characterized by the known actions of paclitaxel,
we compared the effect of the drug on tumor cell apoptosis and proliferation 72 hours posttreatment. Immunofluorescent analysis revealed a significant increase in the number of apoptotic cells (Fig. 4C) and a significant reduction in the number of proliferative cells (Fig. 4D) in the tumors of mice that received both treatments. No significant change in necrosis was seen in the thrombocytopenic tumors as assessed by LDH activity of the tumor homogenate (vehicle treated, 4,246 ± 762 IU/L; paclitaxel treated, 3,761 ± 132 IU/L) compared with their respective normal platelet count controls (vehicle treated 3,747 ± 772 and paclitaxel treated 4,010 ± 288 IU/L). These results indicate that the observed change in rate of tumor growth in thrombocytopenic mice reflects the expected effects of paclitaxel. Taken together, the results indicate that thrombocytopenia, by increasing the leakiness of tumor vessels, improves presentation of the drug to the tumor, allowing it to further affect the tumor.

**Thrombocytopenia does not increase the overall toxicity of chemotherapeutic treatment**

Chemotherapeutic treatments are known to display a wide range of side effects (24). To determine whether the absence of platelets also has an impact on the unspecific toxicity of paclitaxel treatment, we evaluated apoptosis in the liver, kidneys, and spleen in tumor-bearing mice 3 days after treatment. The results showed that whereas injection of either the vehicle or paclitaxel increased apoptosis in the liver and kidney (Fig. 5), no additional significant increase was observed in mice treated with the platelet-depleting antibody. No signs of apoptosis were seen in the spleen in any of the groups (not shown).

Cell counter analysis of blood samples, collected 3 days after the mice received combined treatments, revealed a persistent significant reduction in platelet count but no significant differences in the number of white blood cells and red blood cells compared with the other groups (Fig. 6). Although not significant, thrombocytopenic mice showed a tendency toward reduced number of red blood cells. This reduction could be explained by the tumor hemorrhage occurring in these mice. Altogether, our results indicate that by depleting platelets and rendering leaky only the vessels of the tumor, we enhanced the access of chemotherapy to the tumor site specifically and did not increase its general toxicity.

**Thrombocytopenia allows accumulation of microspheres and enhances the effect of paclitaxel on LLC tumor growth**

We previously showed that thrombocytopenia-induced tumor hemorrhage was independent of the type, age, and localization of the tumor (15). To examine whether increased drug delivery to tumors in thrombocytopenic mice could be generalized to other tumors, we studied LLC inoculated into the flank of syngeneic mice. First, we evaluated whether thrombocytopenia-induced tumor hemorrhage in this model allows the accumulation of circulating particles at the tumor site. Indeed, fluorescently labeled microspheres accumulated preferentially in the hemorrhagic tumors compared with the nonhemorrhagic tumors (Fig. 7A). Moreover, the combined treatment of platelet depletion and paclitaxel injection also resulted in a significant reduction of LLC tumor growth compared with treatment with paclitaxel alone. (Fig. 7B). These results indicate that low platelet counts can be used to increase the delivery and efficacy of a chemotherapeutic agent to different types of tumors.

**Discussion**

Selective induction of tumor hemorrhage by targeting platelet function may facilitate the delivery of chemotherapeutic agents to tumors. This hypothesis is supported by the following observations: (a) thrombocytopenia induces tumor bleeding...
without detectable hemorrhaging elsewhere, (b) the absence of platelets induces porosity of tumor vessels favoring the accumulation of inert particles in the tumor, and (c) the combination of thrombocytopenia with a chemotherapeutic treatment increases the delivery and enhances the effect of the drug on tumor growth and viability without increasing toxicity to other organs. This study follows previous publications from our group showing that platelets prevent bleeding of angiogenic vessels (13) and continuously prevent intratumoral hemorrhage induced by tumor-infiltrating inflammatory cells by the secretion of their granule contents (15, 16). Given that most tumors are both angiogenic and proinflammatory (25, 26), we now show that the thrombocytopenia-induced leakiness of tumor vessels allows better access of a chemotherapeutic agent to the tumor, thus increasing its efficacy.

An important goal in cancer therapy is to deliver high doses of drug to tumor sites while minimizing side effects to healthy organs. This study follows previous publications from our group showing that platelets prevent bleeding of angiogenic vessels (13) and continuously prevent intratumoral hemorrhage induced by tumor-infiltrating inflammatory cells by the secretion of their granule contents (15, 16). Given that most tumors are both angiogenic and proinflammatory (25, 26), we now show that the thrombocytopenia-induced leakiness of tumor vessels allows better access of a chemotherapeutic agent to the tumor, thus increasing its efficacy.

Figure 6. Thrombocytopenia in combination with paclitaxel does not increase side effects on blood cell counts. Seventy-two hours posttreatment (as indicated), blood of tumor-bearing mice was analyzed. Platelet (Plt), white blood cell (WBC), and red blood cell (RBC) counts are shown. In all cases, platelet depletion in combination with paclitaxel treatment did not significantly affect blood cell counts as compared with paclitaxel treatment alone (n = 6–9 mice per group; P > 0.05; NS).
organs. Conventional drugs used for cancer treatment are administered systemically and distribute within the different organs and tissues of the body. The dose that reaches the tumor mass may be as little as 5% to 10% of that which accumulates elsewhere (27). Therapies that target specific tumor antigens are limited by their specificity and may not be possible to develop for cancers that are not well defined. Our model of thrombocytopenia induction favors the accumulation of particles and drug in bleeding tumors without targeting a particular antigen. We also show that thrombocytopenia in combination with the chemotherapeutic treatment significantly reduced growth of 2 different tumor types. The vascular damage caused by thrombocytopenia is because of the tumor’s inflammatory cells: neutrophils and monocytes (16). Because inflammatory cells are present in most tumors (28), inhibition of platelet function to improve drug delivery should be widely applicable.

Mouse models of disease treatment are not always directly applicable to human patients. In fact, the use of thrombocytopenia to improve chemotherapeutic treatment could raise concerns for the well-being of patients. However, we showed here that no signs of systemic hemorrhage were associated with thrombocytopenia. Moreover, low platelet counts needed to be achieved only transiently to be effective in our model. We previously showed that commencement of tumor hemorrhage can be seen as early as 30 minutes after the induction of thrombocytopenia (15). Perhaps thrombocytopenia would only need to occur long enough to induce tumor hemorrhage and allow drug accumulation. It must also be noted that chemotherapeutic agents are characterized by a short half-life. Maintaining thrombocytopenia in patients for more than a few hours would therefore not increase the effect of the drug. Once the drug is no longer in circulation, normal platelet counts should be restored. In addition, chemotherapies are themselves known to induce thrombocytopenia in patients (29, 30). Treatment schedules could be adjusted to take advantage of the already occurring thrombocytopenia-induced injuries of tumor vessels to enhance drug delivery by delaying platelet transfusion.

Platelets are key players in tumor vascular homeostasis. More than 300 proteins and small molecules have been shown to be secreted from activated platelets (31). It is known that bleeding cannot be prevented by degranulated platelets, suggesting that platelet granule contents are important to arrest bleeding in the tumor by the local release of soluble factors that heal or prevent inflammatory injuries (15, 16). Platelet granules are known to contain both pro- and antiangiogenic factors, cytokines, chemokines, growth factors, and many other molecules that could potentially influence vessel injuries. Since the 1990s, platelet releasate has been used to heal wounds, and autologous platelet-rich plasma has been shown to protect against capillary bleeding (32). To our knowledge, the factor(s) responsible for this particular function of platelets are still unknown and are currently under investigation in our laboratory. Identification of these factor(s) responsible for the prevention of bleeding would open a new area of drug development. Potentially, physicians may target platelets’ ability to prevent tumoral hemorrhage specifically without inducing thrombocytopenia. Such antiplatelet treatment combined with chemotherapy to enhance drug delivery to the tumor would represent a significant improvement in our ability to fight cancer with minimal side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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