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Lonomia obliqua venom action on fibrinolytic system

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Abstract

Accidental skin contact with the *Lonomia* caterpillar bristles causes a severe hemorrhagic syndrome. While fibrinolytic activation is considered to be the main cause of hemorrhage in *Lonomia achelous* envenomation, a consumptive coagulopathy was found to be a major component involved in the bleeding complications observed in patients envenomed by contact with *Lonomia obliqua*. Although we have previously observed that in *L. obliqua* envenomations, fibrinolysis activation appeared to be secondary to coagulation system activation, there are no reports regarding the ability of *L. obliqua* venom to activate directly fibrinolytic pathways. We examined the action of *L. obliqua* crude bristles extract (LOCBE) on several fibrinolytic system components. We demonstrated that LOCBE degraded the A-alpha fibrinogen chain only at high concentrations and after long incubation times. Under these conditions, LOCBE also induced prolongation of the fibrinogen clotting time, but no clot lysis was observed before 24 h. LOCBE did not contain t-PA- or u-PA-like activities. Gel filtration and SDS-PAGE showed that LOCBE did not induce FXIII digestion. In addition, no FXIII activity inhibition was detected by dansylcadaverin method. FXIII levels remained unchanged when FXIII was measured in fibrinogen-depleted LOCBE-treated rat plasma, suggesting that the observed 50% FXIII reduction in rats was related to consumption. In conclusion, our results clearly demonstrated that LOCBE did not display either FXIII inhibition or degradation nor fibrinolytic activity. Furthermore, although proteolytic activity on Aα fibrinogen chain was observed, cross-linked fibrin was not affected by LOCBE.

Keywords: Caterpillar; Factor XIII; Fibrinogen; Envenomation; Fibrinolysis

1. Introduction

Lonomia caterpillar bristles, especially those from *Lonomia obliqua* and *Lonomia achelous* caterpillars, contain toxic substances that in contact with human skin cause urticarial dermatitis, respiratory allergy and a hemorrhagic syndrome as the most important clinical complications. In Southern Brazil, caterpillar accidents are caused by *L. obliqua* [1]. The *L. obliqua* tegument contains several cuticular specializations, and these bristles, the venom store, can be easily broken when touched, releasing venom [2]. We have recently reported that patients envenomed by *L. obliqua* present with a consumptive coagulopathy, due to severe depletion of coagulant factors, as well as secondary fibrinolysis activation [3]. In vitro studies have shown that *L. obliqua* crude bristles extract (LOCBE) induces clot formation by triggering activation of both prothrombin and Factor X [4]. Moreover, we have purified and characterized a prothrombin activator named Lopap from LOCBE that in rats induces a consumptive coagulopathy similar to that observed in human patients [5].

L. achelous is another *Lonomia* found in Venezuela. Patients envenomed by *L. achelous* also present with bleeding complications but the cause of the hemorrhages has been related to increased fibrinolysis. In fact, plasma FXIII levels were found to be markedly reduced in *L. achelous* patients. This decrease has been attributed to FXIII degradation by Lonomin, a protein present in *L. achelous* hemolymph [6]. In addition, it has been demonstrated that *L. achelous*

Abbreviations: LOCBE, *Lonomia obliqua* crude bristles extract; L, *Lonomia*; SOFIA, Solid fibrin assay.

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contains plasminogen activator activity [7]. Since the major biological activity of *L. achelous* venom is activation of the fibrinolytic system, antifibrinolytic agents such as aprotinin is used for treatment of *L. achelous* envenomed patients [8].

Although we found that the increased fibrinolysis in *L. obliqua* envenomed patients appeared to be secondary to a consumptive coagulopathy, the presence of potential fibrinolytic activities in *L. obliqua* venom has yet to be addressed. In the present study, we analyzed the action of LOCBE on components of the fibrinolytic system.

2. Materials and methods

2.1. Materials

Bovine thrombin, human prothrombin, human Factor X, BSA, dansyl cadaverine, alpha-Casein and orthophenanthroline were purchased from Sigma (St. Louis, MO,USA). The Coomassie reagent, protein assay and beta-mercaptoethanol were from Bio-Rad (CA, USA). Sodium iodoacetate was from Aldrich (St. Louis, MO, USA). BERICHROM FXIII and Fibrogrammin (FXIII concentrate) kits were from Aventis Behring (Marburg, Germany). Imidazole and HEPES from USB (OH, USA), PEG 6000, human fibrinogen, *H*-D-phenylalanyl-L-pipecolyl-L-arginin-*p*-nitroanilide dihydrochloride (S-2238), *N*-alpha-benzyl-oxicarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroanilide (S-2765), human plasminogen, urokinase and t-PA were from Chromogenix (Mölndal, Sweden). The Superdex 200 HR column was purchased from Pharmacia (Uppsala, Sweden).

2.2. L. obliqua crude bristles' extract

LOCBE was prepared as previously described [9]. Briefly, insects were anesthetized under CO_2 atmosphere, their bristles cut off, and a 10% (w/v) homogenate was prepared with cold PBS (pH 7.4). The homogenate was centrifuged at 1000 rpm for 20 min at 4 °C, and the supernatant was aliquoted and stored at − 70 °C until use. Protein concentration was determined by Bradford [10].

2.3. LOCBE activity on purified fibrinogen

Specific cleavage of fibrinogen was shown on 10% SDS-PAGE gel. Fibrinogen (30 μg) was incubated with LOCBE (1.6 μg) at 37 °C for 2–4 h. Samples were analyzed under reducing conditions in the presence of beta-mercaptoethanol. The homogeneous gel was stained by Coomassie Brilliant Blue R-250 [11]. Controls were performed under similar conditions but without LOCBE. An inhibitory test was realized by first preincubating LOCBE with ortho-phenanthroline (5 mM) or aprotinin (250 U/ml) during 1 h at 37 °C. After fibrinogen addition, the suspension was further incubated for 4 h.

2.4. Coagulation of purified fibrinogen after LOCBE incubation

Purified fibrinogen (200 μg) was incubated at 37 °C with increasing LOCBE concentrations for different time intervals (5, 15, 30, 60 and 120 min). Then, thrombin (1 U/ml) was added and coagulation time was determined. Fibrinogen clot lysis time was observed during 24 h after clot formation.

2.5. LOCBE activity on purified FXIII

Either Factor XIII purification or its interaction with LOCBE were analyzed by gel filtration chromatography performed in a Superdex 200 HR column using a FPLC system and by SDS-PAGE. The FXIII activity was assayed by two different methods to evaluate possible interactions between FXIII and LOCBE: the dansyl cadaverine incorporation [12] and a commercial kit named Berichrom.

2.6. LOCBE activity on FXIII levels in vivo

Rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and either a LOCBE infusion (25 μg/500 μl) or saline was administered during 30 min into the cannulated right jugular vein of animals [13]. Blood samples were taken 5 h after the infusion (from the carotid artery with a siliconized needle) in sodium citrate

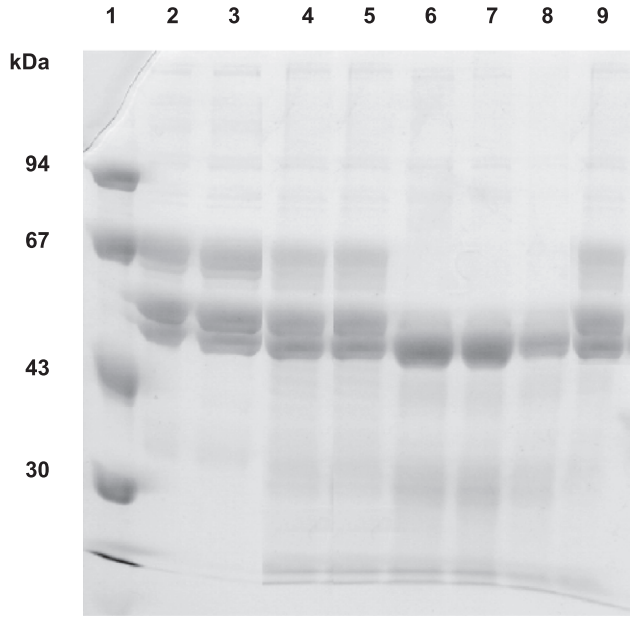

Fig. 1. Hydrolysis of fibrinogen Aα alpha chain by LOCBE. SDS-PAGE (polyacrylamide gel 10%) Coomassie blue stained. Molecular weight marker (1); reduced fibrinogen at 0, 2 and 4 h. (2–4) ; fibrinogen incubated with LOCBE for 0, 2 and 4 h (5–7); Preincubation of LOCBE for 1 h with ortho-phenanthroline (5 mM) (8) or aprotinin (250 U/ml) (9), then the mixture was further incubated during 4 h with fibrinogen.

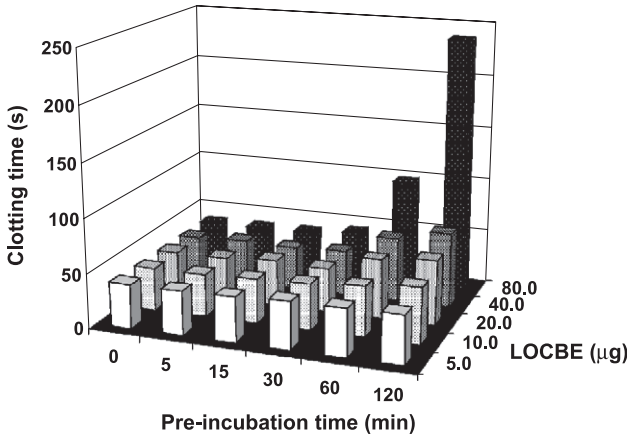

Fig. 2. Fibrinogen (200 μg) was preincubated with increasing LOCBE concentrations (5, 10, 20, 40 and 80 μg) for 0, 5, 15, 30, 60 and 120 min before thrombin addition (20 U/ml). Clotting time was measured in seconds.

3.8% (1:10 v/v), and plasma was obtained by centrifugation for 15 min at 3500 rpm at 4 °C. FXIII levels were measured by the Dansyl cadaverin incorporation method [12]. Bleeding time, prothrombin time and fibrinogen were determined [13].

2.7. LOCBE activity on FXIII levels determined in defibrinated rat plasma

Normal rat plasma was defibrinated according to Sheltawy et al. [12], and FXIII levels were determined by the Dansyl cadaverine method in defibrinated plasma incubated (30 min at 37 °C) or not with LOCBE (100 μg).

2.8. LOCBE direct fibrinolytic activity

2.8.1. Fibrin plates

Human fibrinogen solutions (1.0 mg/ml) were prepared in sodium barbital buffer (50 mM), $CaCl_2$ (1.66 mM), NaCl (92 mM), $MgCl_2$ (0.7 mM), pH 7.75. Ten milliliters of fibrinogen solution and $CaCl_2$ (13.1 mM) were added to a petri dish (14 × 1 cm). After bovine thrombin addition (20 U NIH/ml), plates were maintained at RT during 1 h. Then, 10 μl of LOCBE (10 μg), Urokinase (0.25 U/ml) or Tris–HCl (20 mM), NaCl (100 mM), pH 8.0 were placed on the fibrin layer. Fibrin plates were incubated for 18 h at 37 °C in a humid chamber, and the lysis areas were measured.

2.8.2. Plasminogen activation-related fibrinolysis (u-PA and t-PA like)

Solid fibrin assay (SOFIA) plates were prepared from purified fibrinogen according to Angles-Cano et al. [14]. Briefly, microtiter plates were treated with polyglutaraldehyde 2.5% in $NaHCO_3$ and washed in distilled water. Plates were then incubated with fibrinogen in Na_2PO_3 (0.1 M), $CaCl_2$ (1 M), pH 7.4. After fibrinogen removal, plates were treated with ethanolamine (0.3 M) pH 7.4. Following washes with washing buffer [Na_2PO_3 (0.05 M), NaCl (0.08 M), BSA (2 mg/ml), Tween 20 (0.01%) and sodium

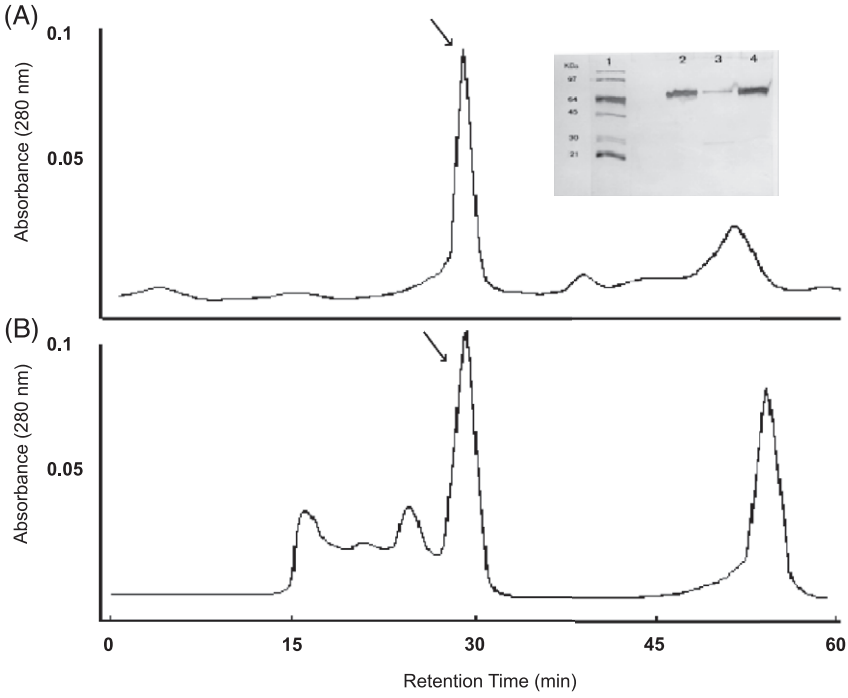

Fig. 3. Analysis of FXIII and LOCBE interaction by gel filtration. Column: Superdex 200HR. Samples: (A) FXIII purification; (B) purified FXIII incubated with LOCBE. Insert: SDS-PAGE (10%) stained with Coomassie. Molecular weight marker (1), FXIII 40 μg (2), LOCBE 40 μg (3), FXIII incubated with LOCBE (4). The arrows indicate the active peak.

Table 1
Effect of LOCBE on Purified FXIII activity

	Dansyl cadaverine (%)	Berichrom (%)
Factor XIII	100	100
Factor XIII + LOCBE	98.65	95.60

azide (0.01%) pH 6.8] bound fibrinogen was transformed into fibrin by addition of human thrombin (15 U/ml) containing $CaCl_2$ (1 mM). Thrombin was removed by washings with NaCl (0.5 M), $CaCl_2$ (8 mM) containing Tween 20 (0.05%), followed by washings in Na_2PO_3 (5 mM) containing Tween 20 (0.01%). Degraded fibrin was obtained by pretreatment of each well with plasmin (25 nM) during 30 min at 37 °C. Plasmin was then removed by washings with washing buffer in the presence of inhibitors such as VPL (10 μM), *trans*-4-aminomethylcyclohexane-1-carboxylic acid (0.2 M) and benzamidine (1 nM).

2.8.3. u-PA like activity

A mixture (200 μl) containing Tris–HCl (50 mM) NaCl (100 mM) pH 8.0, plasminogen (800 nM), LOCBE (10 μg) and plasmin-specific chromogenic substrate S-2251 (125 μM) was incubated in fibrin-coated wells. Plasmin generation was monitored at 405 nm during 120 min at 37 °C.

2.8.4. t-PA like activity

A plasminogen solution (200 μl) was first incubated during 1 h in fibrin-degraded coated wells followed by LOCBE (10 μg) addition and further incubation for 1 h. Then, S-2251 was added and the amidolytic activity measured. Positive control was t-PA (2 μg).

3. Results

3.1. Fibrinogenolytic activity mediated by LOCBE

LOCBE fibrinogenolytic activity was examined on purified fibrinogen. Fig. 1 shows that at a molar ratio of 20:1 fibrinogen/LOCBE, alpha chain of fibrinogen was degraded after 2 h incubation. Control sample showed no significant fibrinogenolytic activity after 2 h at 37 °C. Fibrinogen cleavage was prevented by preincubation of LOCBE with aprotinin. Rupture of the Aα fibrinogen chain resulted in a reduction of its coagulant capacity as demonstrated by the prolonged clotting time induced by incubation of purified fibrinogen in the presence of LOCBE. Fig. 2 shows that high LOCBE concentrations (1:2 LOCBE/fibrinogen) and a minimum of 1 h were required to observe prolongation of the clotting time. Once the clot was formed, no lysis was observed before 24 h incubation, indicating that LOCBE did not contain enzymes capable of degrading cross-linked fibrin.

3.2. Effect of LOCBE on FXIII

Purification of FXIII from a commercial concentrate was performed by gel filtration chromatography. This purification procedure yielded four protein peaks, but FXIII activity (assessed by dansyl cadaverin and Berichrom kit) was just found in one of the peaks (Fig. 3). Potential interactions between LOCBE and FXIII might include either complex formation or degradation. To evaluate any of these interactions, fraction containing FXIII was incubated with LOCBE and gel filtered. The elution profile as well as the activity of FXIII alone were similar to that of the mixture between FXIII and LOCBE, indicating that FXIII molecule was not altered by LOCBE (Fig. 3) (Table 1). The possibility of FXIII alteration was also examined by SDS-PAGE. It would be expected that if LOCBE binds or degrades FXIII, changes on molecular weight of this protein would have been noticed in the electrophoresis. However, no differences were found in the FXIII migration pattern (Fig. 3).

3.3. Effect of LOCBE on FXIII levels in vivo and using defibrinated plasma

Five hours after LOCBE infusion, PT was markedly prolonged, the BT was four times increased, fibrinogen was undetectable and FXIII was 50% reduced (Table 2).

To address the question whether FXIII activity was reduced by consumption or LOCBE inhibition, in another set of in vitro experiments, FXIII levels were measured by the dansyl cadaverin method using rat defibrinated plasma preincubated or not with LOCBE. We found that FXIII levels were similar (100%, $n=4$), regardless whether the plasma was treated or not with LOCBE, suggesting that the bristles extract did not induce FXIII inhibition or degradation.

3.4. LOCBE direct fibrinolytic activity

Fibrinolytic activity was measured by the lysis area (mm^2) in a fibrin plate. While urokinase induced 450-mm^2 lysis area after 2 h incubation, no lysis mediated by LOCBE

Table 2
Blood clotting parameters after LOCBE infusion

	PT (s)	BT (s)	Fg (mg %)	FXIII (MI)	FXIII (DC %)	FXIII (Berichrom) (μg/ml)
Control	12.9 ± 0.9	124.1 ± 13.2	556.6 ± 52.1	insoluble	100	19.8 ± 8.2
5 h after LOCBE infusion	α	494.1 ± 68.7	ND	ND	49.5 ± 8.0	9.2 ± 0.8

Prothrombin time (PT), bleeding time (BT), fibrinogen (Fg), monoiodoacetate (MI), dansyl cadaverine (DC), unclottable (α), Not detectable (ND).

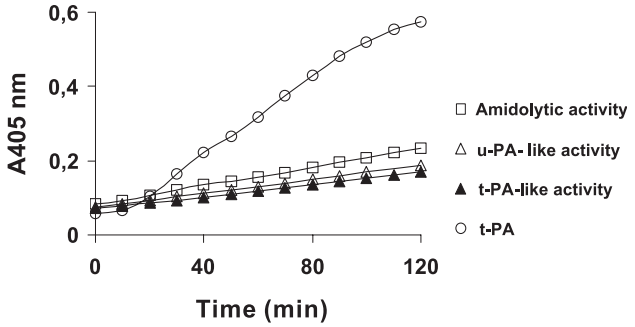

Fig. 4. SOFIA plates prepared with or without plasmin degradation were incubated with 200 μl/well of a mixture containing plasminogen (800 nM) and LOCBE (10 μg). Then, the generated plasmin was monitored by the hydrolysis of S-2251 (125 μM) during 120 min, at 405 nm and at 37 °C.

($n=3$) was observed after 18 h incubation, indicating that it had no fibrinolytic activity.

3.5. Plasminogen activator activities

To examine whether LOCBE is able to activate plasminogen (like t-PA or u-PA plasminogen activators), it was co-incubated with plasminogen in a degraded or undegraded fibrin matrix (SOFIA plate), and the formed plasmin was measured using the chromogenic substrate S-2251. Fig. 4 shows that while t-PA efficiently induced plasmin formation, LOCBE did not display either t-PA- or u-PA-like activities.

4. Discussion

In this study, we examined the *L. obliqua* crude bristles extract (LOCBE) for fibrin(ogen)lytic activity. Our data showed that high concentrations of and long incubation time with LOCBE were required to induce Aα chain fibrinogen degradation. Although a prolongation of the clotting time was observed, it occurred only at 1:2 stoichiometry, a situation not normally achieved during envenomation. In addition, this fibrinogen hydrolysis did not affect cross-linked fibrin (fibrin-plate, fibrinogen clot by thrombin). A direct plasminogen activator activity (u-PA or t-PA like) was also not found in LOCBE. Since the effectiveness of fibrinolysis in vivo is critically regulated by the endothelial cell surface [15], it could be argue that the in vivo situation could be somehow different from our in vitro results. However, we have recently shown that patients envenomed by *L. obliqua* had high levels of D-dimer, moderate decrease in t-PA, plasminogen and alpha2-antiplasmin levels [3]. All together, these data suggested that fibrinolysis was activated secondary to fibrin formation. Our present results further support the notion that *L. obliqua* venom does not directly activate the fibrinolytic system.

In *L. achelous* envenomations, the hemorrhagic syndrome is ascribed to intense fibrinolysis secondary to the plasminogen activation and FXIII degradation [6]; thus, we also studied the effect of LOCBE on FXIII. SDS-PAGE, gel filtration chromatography and three different methods for FXIII measurement demonstrated that LOCBE neither induced hydrolysis nor inhibition of purified FXIII. Although experimental rat envenoming by LOCBE caused complete fibrinogen depletion and a 50% decrease of FXIII levels, this FXIII reduction appeared to be related to a consumption and not degradation since the in vitro studies showed that FXIII levels were unchanged irrespective of whether they were measured in LOCBE-treated or -untreated fibrinogen rat-depleted plasma.

In a recent study of 106 patients envenomed by contact with *L. obliqua*, patients presented with a consumptive coagulopathy with significant reduction of factors V, XIII, VIII and prekallikrein and increase in thrombin–antithrombin complexes and in prothrombin fragments F1+2 [3]. FXIII reduction was detected in patients with fibrinogen levels lower than 0.5 g/l. However, in patients with fibrinogen levels higher than 0.5 g/l, FXIII levels were normal. D-Dimer levels were extremely high in all groups (fibrinogen $<0.5-1.5$ g/l), pointing out that fibrinolysis had been activated, possibly secondary to fibrin production [3]. Thus, our in vivo studies (patients and rats) clearly show that FXIII levels correlate with fibrinogen levels, further reinforcing the view that decreases in FXIII levels in envenomed rats were associated with the consumption coagulopathy induced by *L. obliqua* venom.

In summary, our data suggest that direct LOCBE fibrinolytic activity is not relevant and support previous findings showing that *L. obliqua* venom mainly exerts procoagulant activity. In fact, Donato et al. [4] have described that the spicules of *L. obliqua* contain both a factor X activator and a calcium-dependent prothrombin activator. In experimental thrombosis studies, we have shown that while streptokinase infusion induced local thrombolysis, LOCBE promoted fibrinogen depletion and decrease in thrombus size without thrombolysis [13]. We have also demonstrated that infusion of Lopap, a prothrombin activator isolated from LOCBE, triggered a condition similar to that in human poisoning in mice [5], indicating that procoagulant activities are the major biological effects of the bristle extract.

Our present results strengthen the notion that the pathogenic mechanisms involved in the hemorrhagic syndrome observed in patients envenomed either by *L. obliqua* or by *L. achelous* involve venom proteins with different specific activities in each species. While in *L. achelous* envenomed patients, hemorrhage is due to FXIII degrading enzymes and fibrinolytic activating proteins, in *L. obliqua* envenomed patients, the bleeding manifestations seems to be related to a consumptive coagulopathy induced by the presence of procoagulant proteins. Therefore, therapy should be addressed towards the main mechanism involved in each case. Antifibrinolytic agents should not be recommended in *L. obliqua* accidents and treatment of patients is largely based on the administration of antivenom serum, which is

an effective way to reverse haemostatic disturbances and bleeding.

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