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• BASIC RESEARCH •

Protective effect of *angelica sinensis* polysaccharide on experimental immunological colon injury in rats

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Abstract

AIM: To study the effect of angelica sinensis polysaccharide (ASP) on immunological colon injury and its mechanisms in rats.

METHODS: Immunological colitis model of rats was induced by intracolon enema with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) and ethanol. The experimental animals were randomly divided into normal control, model control, 5-aminosalicylic acid therapy groups and three doses of ASP therapy groups. The 6 groups were treated intracolonically with normal saline, normal saline, 5-aminosalicylic acid (100 mg·kg^{-1}), and ASP daily (8: 00 am) at the doses of 200, 400 and 800 mg·kg^{-1} respectively for 21 days 7 d following induction of colitis. The rat colon mucosa damage index (CMDI), the histopathological score (HS), the score of occult blood test (OBT), and the colonic MPO activity were evaluated. The levels of SOD, MDA, NO, TNF-α, IL-2 and IL-10 in colonic tissues were detected biochemically and immunoradiometrically. The expressions of TGF-β and EGF in colonic tissues were also determined immunochemically.

RESULTS: Enhanced colonic mucosal injury, inflammatory response and oxidative stress were observed in colitis rats, which manifested as significant increases of CMDI, HS, OBT, MPO activity, MDA and NO contents, as well as the levels of TNF-α and IL-2 in colonic tissues, although colonic TGF-β protein expression, SOD activity and IL-10 content were significantly decreased compared with the normal control (P<0.01). However, these parameters were found to be significantly ameliorated in colitis rats treated intracolicly with ASP at the doses of 400 and 800 mg·kg^{-1} (P<0.05-0.01). Meantime, colonic EGF protein expression in colitis rats was remarkably up-regulated.

CONCLUSION: ASP has a protective effect on immunological colon injury induced by TNBS and ethanol enema in rats, which was propably due to the mechanism of antioxidation, immunomodulation and promotion of wound repair.

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INTRODUCTION

Angelica sinensis polysaccharide (ASP) possesses a variety of pharmacological effects including immunoregulation, anti-oxidation, anti-tumor, anti-irradiation injury, promotion of hematopoiesis[1-6]. ASP not only has regulatory effects on cytokines, complements, immunocompetent cells such as lymphocyte, macrophage but also shows manifold immunoregulation according to different organism immunological status, drug dose and drug administration surroundings; In addition, ASP has been recently found to be effective in preventing gastrointestinal injury induced by a neutrophil-dependent lesion model in rats, to promote gastrointestinal wound healing in rats *in vitro* and *vivo*[7-9].

Immunoregulation dysfunction plays a central role in the pathogenesis of inflammatory bowel disease (IBD) including Crohn' s disease and ulcerative colitis, although their etiology and pathophysiology remain to be elucidated. Immunoregulation dysfunction is mainly manifested as increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines, which introduce the generation of "inflammation cascade", result in overproduction of oxygen and nitrogen reactive species, thus leading to intestinal and/or colonic injury[10-15]. Numerous studies have demonstrated that modulation of immunological disorders is an effective target for the treatment of IBD[15-19].

Based on ASP' s immunomodulatory feature with manifold efficacies, anti-oxidation property, promotion of gastrointestinal wound healing and safety in use, we therefore assumed that ASP might contribute to the treatment of IBD. To the best of our knowledge, there has been no report so far concerning the effect of ASP on IBD. The present study was to observe the effect of ASP on immunological colon injury in experimental colitis rats to test the hypothesis.

MATERIALS AND METHODS

Animals and reagents

Healthy Sprague-Dawley rats of both sexes, weighing 250±30 g, and C57BL/6J mice, weighing 20±2 g, supplied by the Animal Center of the Academy of Hubei Preventive Medical Sciences. The rats were allowed to take food and tap water *ad libitum*. ASP was isolated from angelica sinensis from Minxian County of China by water extraction and ethanol precipitation as described in the literature[4,10] and dissolved in normal saline, sanitized by high pressure before use. TNBS (Lot. 51K5001), ConA, MTT were all purchased from Sigma Co (USA). 5-aminosalicylic acid (5-ASA) was provided by Guoyi Pharmaceutical Ltd (Lot. 20029477, China). Occult blood test paper was provided by Tonyar Biotech Co (USA). Bovine serum and RPMI-1640 medium were purchased from Gibco Co (USA). Recombinant human TNF-α was provided by BangDing Biotech Co (China). Actinomycin D was provided by HuaMei Biotech Co (China). L929 cells were provided by Biological Classic Culture Store Centre of Wuhan University in China. IL-10 radioimmunoassay kits were provided by Radio-immunity Institute of PLA General Hospital in Beijing. Polyclonal rabbit anti-rat-TGF-β and EGF were purchased

from Santa Cruz Co. and Zymed Co., respectively. S-P kits were supplied by Zhongshan Biological Technology CO. Ltd in Beijng. MPO, SOD, MDA, NO detection kits were purchased from Nanjing Jiancheng Bioengineering Institute. Other reagents used in the present study were all of analytical grade.

Experimental protocol

According to the references[19,20], the rats were anesthetized with ether, then a flexible plastic rubber catheter with an outside diameter of 2 mm was inserted rectally into the colon, its tip was 8 cm proximal to the anus. TNBS (100 mg· kg^{-1}) dissolved in 50 % ethanol (v/v) was instilled into the colon lumen through the rubber cather (the final volume was 0.25 mL), saline was instilled as control. The experimental animals were randomly divided into 6 groups: normal group, model group, 5-ASA therapy group and three doses of ASP therapy groups. The 6 groups were treated intracolicly with normal saline, normal saline, 5-ASA (100 mg· kg^{-1}), and ASP daily (8:00 am) at the doses of 200, 400 and 800 mg· kg^{-1} respectively for 21 days 7 d following induction of colitis in rats. Then the colon mucosa damage index (CMDI) and the histopathological score (HS) were evaluated and the colonic tissue was sampled for a variety of determinations after the animals were sacrificed by decapitation.

Assessment of CMDI, HS and OBT

The tissue of colon 10 cm proximal to anus of the sacrificed rats was excised, opened longitudinally, washed in saline buffer, and pinned out on a wax block. The colonic tissue samples for HS were prepared according to the reference[19]. CMDI and HS in each colon were evaluated respectively by two independent observers. The assessment criteria of CMDI and HS were reported in previous literature[19]. CMID was as following: 0: normal mucosa, no damage on mucosal surface; 1: mild hyperemia and edema, no erosion or ulcer on mucosal surface; 2: moderate hyperemia and edema with erosion on mucosal surface; 3: severe hyperemia and edema with necrosis and ulcer on mucosal surface, the major ulcerative area extended less than 1 cm; 4: severe hyperemia and edema with necrosis and ulcer on mucosal surface, the major ulcerative area extended more than 1 cm. HS: (1) Infiltration of acute inflammatory cells: 0, without; 1, mild; 2, severe. (2) Infiltration of chronic inflammatory cells: 0, without; 1, mild; 2, severe. (3) Fibrin deposition: 0, negative; 1, positive. (4) Submucosal edema: 0, without; 1, focal; 2, diffuse. (5) Necrosis of epithelial cells: 0, without; 1, focal; 2, diffuse. (6) Mucosal ulcer: 0, negative; 1, positive. At the end of experiment, rat feces was collected for occult blood test (OBT) according to the instructions.

Determination of MPO, SOD activities and MDA and NO contents

The colon tissue samples taken for MPO detection were homogenized (50 g· L^{-1}) in ice-cold potassium phosphate buffer 50 mmol· L^{-1} (pH 6.0) containing 0.5 % hexadecyltrimethyl-ammoninm bromide. The homogenates were frozen and thawed three times, then centrifuged at 4 000 rpm for 20 min at 4 ℃. The level of MPO in supernatant was measured using a commercial kit according to its instructions. Other colon tissue samples were homogenized (50 g· L^{-1}) in ice-cold PBS (pH 7.4). The homogenates were centrifuged at 3 000 rpm for 10 min at 4 ℃, and the supernatant was stored at -20 ℃ until determination for SOD, MDA and NO using corresponding commercial kits according to the instructions.

Measurement of TNF-α, IL-2, IL-10 levels

The colon tissue samples were homogenized (100 g· L^{-1}) in ice-cold phosphate buffer saline (pH 7.4) containing penicillin 100 u· ml^{-1} and streptomycin 100 ug· ml^{-1}. The homogenates were centrifuged at 40 000 rpm for 10 min at 4 ℃, and the supernatant was stored at - 80 ℃ until determination for TNF-α, IL-2 and IL-10. The activity of TNF-α and IL-2 were measured by L929 cell cytotoxicity and C57BL/6J mice splenocytes using MTT colorimetry respectively as described in the literatue[19,21]. The content of IL-10 was detected by using a radioimmunoassay kit following the manufacturer's instructions.

Detection of colonic TGF-β and EGF expression

Immunohistochemical detection was performed using S-P technique. The experiment procedures were performed following the manufacturer's recommendations. Polyclonal rabbit anti-rat-TGF-β and EGF were diluted in PBS to 1:100, 1:150, and used as primary antibodies respectively. The dewaxed sections were incubated with polyclonal primary antibodies overnight at 4 ℃ after antigen repair. Biotinylated goat-anti-rabbit IgG was added as second antibody. Horseradish peroxidase labeled streptomycin-avidin complex was used to detect second antibody. Sections were stained with diaminobenzidine. Negative control sections were prepared by substituting primary antibodies with PBS. The bluely stained nucleus was considered as negative while the brown or dark brown stained cytoplasm and/or cell membrane were considered as positive. Expressions of these target proteins were semiquantitated respectively with an automatic image analyzer (Nikon, Japan) and HPIAS-2000 image analyzing program, in which the average value of positive cell's absorbance (A) in ten randomly selected high power fields (400×) for each section was used to compare the target protein expression.

Statistical analysis

Experimental results were analyzed by ANOVA and *t*-test for multiple comparisons between the groups. Data were finally expressed as mean ±SD. *P* value less than 0.05 was considered statistically significant.

RESULTS

Protective effect of ASP on colon injury

Results are shown in Table 1. CMDI, HS, OBT and MPO activity were regarded as main parameters that reflected the degree of colon injury and inflammation in inflammatory gut tissue. Compared with normal group, these parameters were significantly increased in model group ($P<0.01$). Both ASP (400, 800 mg· kg^{-1}) and 5-ASA (100 mg· kg^{-1}) could remarkably decrease these elevated parameters ($P<0.05$-0.01). Furthermore, the therapeutic effect of 800 mg· kg^{-1} ASP was as effective as that of 5-ASA (100 mg· kg^{-1}).

Table 1 Effect of ASP on CMDI, HS, OBT and MPO activity in colonic tissue of colitis rats (n=8, $\bar{x}\pm s$)

Group	Dose (mg·kg^{-1})	CMDI	HS	OBT	MPO (U·g^{-1})
Control	-	0.0±0.0	0.7±1.1	0.0±0.0	29±18
Model	-	3.1±1.1[d]	5.6±0.8[d]	2.2±0.8[d]	194±32[d]
5-ASA	100	1.7±0.6[b]	4.7±0.7[a]	1.0±0.9[a]	117±15[b]
ASP	200	2.1±0.5[a]	4.5±1.3	1.4±0.9	172±16
ASP	400	1.8±0.7[a]	4.3±1.1[a]	1.1±0.8[a]	158±19[a]
ASP	800	1.5±0.5[b]	4.1±0.9[b]	0.8±0.7[b]	133±17[b]

[a]$P<0.05$, [b]$P<0.01$, *vs* model group; [d]$P<0.01$ *vs* normal group.

Effect of ASP on colonic oxidation of colitis rats

Compared with normal group, the contents of MDA and NO

were significantly elevated while SOD activity was significantly decreased in model group (P<0.01). ASP (400, 800 mg· kg^{-1}) not only obviously decreased MDA content but also evidently increased SOD activity (P<0.05-0.01). Furthermore, ASP (200, 400, 800 mg· kg^{-1}) remarkably reduced the elevated NO content in a dose-dependent manner (P<0.05-0.01) (Table 2).

Table 2 Effect of ASP on activity of SOD and contents of MDA and NO in colonic tissue of colitis rats (n=8, $\bar{x}\pm s$)

Group	Dose (mg·kg^{-1})	SOD (kU·g^{-1})	MDA (nmol·g^{-1})	NO (nmol·g^{-1})
Control	-	23.16±5.13	27.41±9.66	294±73
Model	-	8.41±3.17^{d}	83.47±22.53^{d}	568±65^{d}
5-ASA	100	15.26±2.14^{b}	55.32±8.61^{b}	367±26^{b}
ASP	200	11.95±3.22^{a}	67.31±13.84	496±52^{a}
ASP	400	14.84±2.45^{b}	58.52±14.36^{a}	445±47^{b}
ASP	800	16.27±1.96^{b}	49.14±10.73^{b}	381±34^{b}

^{a}P<0.05, ^{b}P<0.01, *vs* model group; ^{d}P<0.01 *vs* normal group.

Effect of ASP on colonic TNF-α, IL-2 and IL-10 levels

Compared with normal group, the levels of TNF-α, IL-2 were significantly increased while the content of IL-10 was obviously reduced in model group (P<0.01). ASP (400, 800 mg· kg^{-1}) not only obviously decreased the levels of TNF-α, IL-2 but also significantly increased the content of IL-10 in model group (P<0.05-0.01) (Table 3).

Table 3 Effect of ASP on levels of TNF-α, IL-2, IL-10 in colonic tissue of colitis rats (n=8, $\bar{x}\pm s$)

Group	Dose (mg·kg^{-1})	TNF-α (kU·g^{-1})	IL-2 (A)	IL-10 (ng·g^{-1})
Control	-	30.6±12.6	0.176±0.028	43±8
Model	-	82.8±18.6^{d}	0.381±0.069^{d}	15±5^{d}
5-ASA	100	45.3±21.9^{b}	0.264±0.042^{b}	22±10
ASP	200	61.9±25.6	0.318±0.032^{a}	23±11
ASP	400	57.4±20.8^{a}	0.303±0.036^{a}	23±8^{a}
ASP	800	51.7±21.5^{b}	0.285±0.031^{b}	26±10^{a}

^{a}P<0.05, ^{b}P<0.01, *vs* model group; ^{d}P<0.01 *vs* normal group.

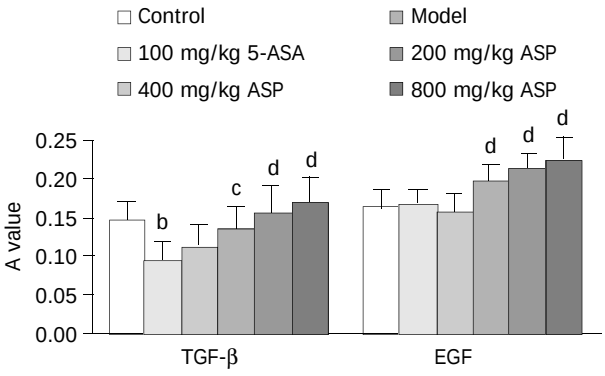

Figure 1 Effect of ASP on expressions of TGF-β and EGF in colonic tissue of colitis rats. n=8, $\bar{x}\pm s$, ^{b}P<0.01, *vs* normal control; ^{c}P<0.05, ^{d}P<0.01, *vs* model control.

Effect of ASP on colonic TGF-b and EGF expressions

As shown in Figures 1, 2 and 3, compared with normal group, TGF-β expression in model group was significantly decreased (0.096±0.021 *vs* 0.145±0.025, P<0.01) and EGF expression was hardly changed. ASP (200, 400, 800 mg· kg^{-1}) remarkably up-regulated the expressions of TGF-β and EGF in a dose - dependent manner (TGF-β: 0.132±0.031, 0.154±0.036, 0.169±0.032 *vs* 0.096±0.021; EGF: 0.197±0.021, 0.212±0.023, 0.225±0.029 *vs* 0.166±0.024, P<0.05-0.01), whereas 5-ASA (100 mg· kg^{-1}) had no obvious effect on the expressions of TGF-β and EGF.

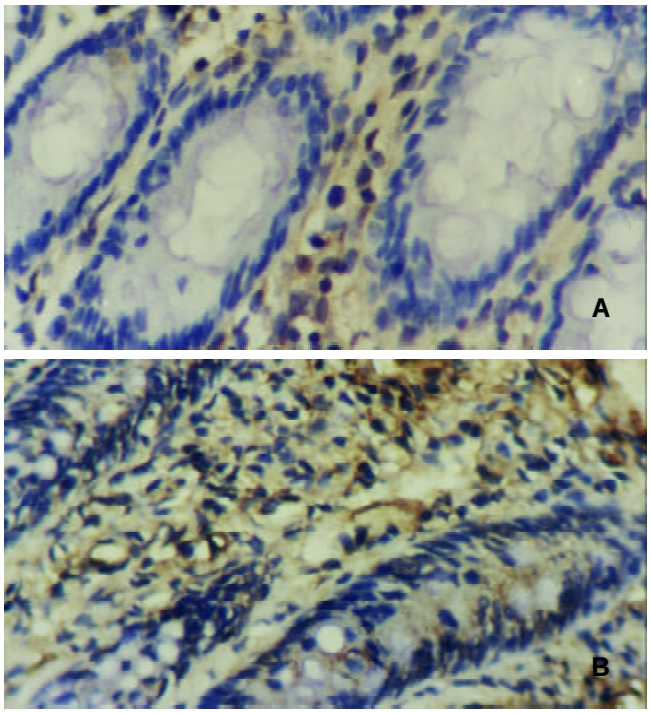

Figure 2 Expressions of TGF-β (A) and EGF (B) in inflammatory areas of colonic tissue from rats with colitis induced by TNBS and ethanol, respectively. Weakly positive signals were found. SP stain ×400.

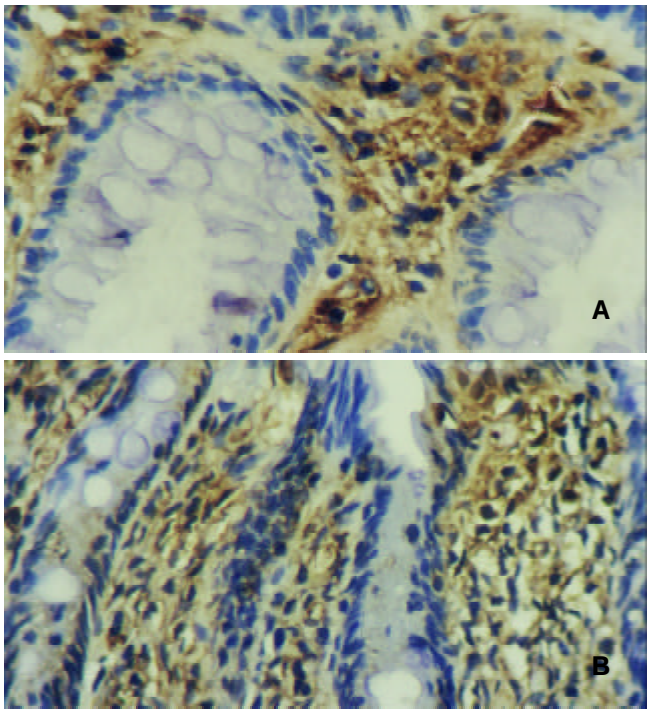

Figure 3 Significantly increaed expressions of TGF-β (A) and EGF (B) were in colonic tissue from rats with colitis induced by TNBS and ethanol after treated with 800 mg·kg^{-1} ASP. Strongly positive staining could be found. SP stain ×400.

DISCUSSION

TNBS-induced rats colitis model established by Morris *et al*

was regarded as a classic model for immunological colon injury which shares many of the histopathological and clinical features and pathogenesis of human IBD. The whole process was summarized as following: after destruction of mucosa integrity by ethanol, hapten TNBS was bound to colon tissue proteins, and changed into a modified protein compound, which was recognized by macrophages as an abnormal antigen and presented quickly to the sensitized T lymphocytes. So a series of immunoresponsiveness and severe colon inflammation were initiated subsequently[19,20]. In this study, an IBD model was successfully established, and intracolon enema with ASP showed obviously protective effects on immunological colon injury, which might be related with ASP's antioxidation property and function of balancing cytokine generation and modulating immune.

Many studies have revealed that reactive metabolites of oxygen and nitrogen were a notable characteristic of IBD, which led to the pathological aggravation of a series of free radicals chain reactions and strongly attack DNA, proteins, enzymes, biological membrances as well as disruption of the integrity and function of intestinal mucosa barrier, activaton of inflammatory mediators[22-25]. Besides direct and severe impairment of the function of intestinal barrier, excessive NO participated in complicated web system between inflammatory cells and immunocytes in IBD[12,15,22-25]. Our study showed that ASP, an anti-oxidant agent which can directly scavenge oxygen-derived free radicals, could not only remarkably decrease the elevated contents of MDA and NO, but also evidently increase the reduced SOD activity in colonic tissues of colitis rats. Reduction of NO by ASP might be related with its inhibition of nitric oxide synthase activity, which was proven by recent researches[3,4].

As the most important cytokine in "inflammation cascade" of IBD, TNF-α could directly impair gut mucosa, promote production of inflammatory mediators and oxygen free radicals, up-regulate the expression of adhesive factors. Moreover, TNF-α is also a main activator for NF-κB, a critical transcription factor, thus up-regulating many genes involved in proinflammatory and immune responses[12,15,16,26]. In this article, ASP, an immunomodulatory reagent, obviously decreased the elevated colonic TNF-α level in colitis rats. As it is known, TNF-α and NO mainly came from activated macrophages in inflammatory gut[12,19,26]. It deserves further investigation that whether inhibition of TNF-α and NO by ASP is related with its regulation of macrophage activation through the polysaccharide receptors on the surface of macrophages such as β-glucan receptor and mannose receptor[27,28].

As it is known, imbalance of type 1 helper T lymphocyte (Th1) and Th2 in IBD could promote excretion of important pro-inflammatory cytokines including TNF-α, INF-γ, and activate macrophages, which mediate inflammatory and immunological injury. Moderate IL-2 level is important for keeping the dynamic balance of Th1 and Th2 in normal gut mucosa, whereas excessive IL-2 could up-regulate the activity of Th1[29-31]. As a major anti-inflammatory cytokine mainly secreted by Th2, IL-10 could suppress inflammation by decreasing Th1 activation, reducing HLA class II expression, and diminishing production of pro-inflammatory cytokines from activated macrophages such as IL-1α, IL-1β, TNF-α and IL-8[12,32-34]. Data obtained in this study indicated that ASP could not only significantly reduce the markedly elevated IL-2 level but also increase the reduced IL-10 level in colonic tissue of colitis rats, thus partly correcting the aberrant immunological status, which might be related with the marked induction of TGF-β by ASP. As a strong immunosuppressant and anti-inflammation cytokine mainly secreted by macrophages, TGF-β plays an important role in gut local immunity in IBD. TGF-β could not only suppress the immunoresponse of Th1 in intestine, depress the activity of activated macrophages but also induce production of IL-10[12,18,34]. Investigations have demonstrated that deficiency of TGF-β in the intestine contributed to the development of IBD, whereas maintenance of TGF-β might be important in regulating immune homeostasis in the intestine[18, 35].

Besides modulating immune response, TGF-β could control cell growth, differentiation and migration, stimulate synthesis of extracellular matrix proteins, and promote angiogenesis, thus promoting wound repair and tissue reconstruction. Its functions are similar to those of cytokine EGF[36-41]. In addition, EGF could induce production of TGF-β and they showed cooperative effects[40-42]. Reseaches have proven that TGF-β and EGF were crucial for maintaining the integrity and functions of intestine mucosa barrier, and could effectively prevent and ameliorate its injury and dysfunction induced by oxidants, thus contributing to the treatment of intestinal inflammation[42-45]. Our study showed that ASP significantly increased the levels of EGF and markedly reduced TGF-β in colon tissue of colitis rats, thus accelerating repair of colon lesions, which agreed with investigations of Ye *et al*[8,9]. The underlying mechanism might be that ASP has pharmacological properties similar to heparin which can interacts with a variety of proinflammatory chemokines and growth factors, thus promoting production of many growth factors[7-9,42,47,48].

To sum up, the results of this study showed that intracolonic treatment with ASP at the doses of 400 mg·kg^{-1} and 800 mg·kg^{-1} could obviously attenuate experimental immunological colon injury in rats, suggesting that ASP in combination with well-established drugs, may contribute to the optimal therapy of IBD.

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