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ON THE BENEFIT OF WHEY-CULTURED *LACTOBACILLUS CASEI* IN MURINE COLITIS

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The objective of this study was to examine the prophylactic and therapeutic effect of whey-cultured *Lactobacillus casei* (*L. casei*) in a murine model of colitis. Colitis was induced by intracolonic administration of a mixture of 2,4,6-trinitrobenzenesulphonic acid (TNBS)/absolute ethanol in male Wistar rats. Animals were divided into 5 groups including sham (normal group), control (vehicle-treated), positive control (dexamethasone 1 mg/kg/day, orally), prevention (10^8 cfu *L. casei*/day, orally, 14 days before induction of colitis), and treatment (10^8 cfu *L. casei*/day, orally, 14 days after induction of colitis). After 14-days treatment, the animals were sacrificed on the day 15. Distal colons were removed for examining histological and biochemical assays. Biomarkers including TNF-α, myeloperoxidase (MPO), and lipid peroxidation (LPO) were measured in the homogenate of colon. Results indicated an apparent improvement in colon histopathology scores, TNF-α, MPO, and LPO in the treatment group, whereas prevention group did not demonstrate positive efficacy in prevention of colonic damage. It is concluded that *L. casei* grown in whey culture is very effective in ameliorating both biochemical and histopathological markers of colitis if used post induction of colitis but not if used before induction of colitis. The difference between effects of *L. casei* when used pre-colitis and post-colitis confirms its mechanism of action as an anti toxic stress agent. Further studies should be made in IBD patients.

Key words: *colitis, inflammatory bowel disease, Lactobacillus casei, probiotics, tumor necrosis factor-α, colonic myeloperoxidase, lipid peroxidation*

INTRODUCTION

Inflammatory bowel disease (IBD) is a severe form of intestinal inflammation including two related conditions, namely ulcerative colitis (UC) and Crohn's disease (CD) with an unknown etiology (1, 2). Genetic, irregular immune and inflammatory response, the enteric microbial flora, environmental factors and childhood infections considered to be involved in IBD (2-7). Medical management of IBD still remains challenging. Although, aminosalicylates, corticosteroids, and immunosuppressants are used to treat IBD with modest results, they are not without adverse effect. Furthermore, probiotics and N-acetylcysteine (NAC) have been recommended to be used in treatment of IBD (8-11). Recently, the possible effect of herbal medicines has been reviewed in management of IBD (12).

Probiotics are defined as living organisms which upon ingestion in certain numbers are beneficial for human health due to improvement of intestinal microbial balance (13, 14). Previous studies have shown that alteration in concentration of bacterial flora in the colon is helpful in treatment of IBD (15, 16). Probiotics enhance the normal homeostatic mechanisms in the intestine and improve the enteric flora which is effective in treatment of colitis. A number of probiotics have been recognized such as *Lactobacilli*, *Bifidobacteria*, *Streptoccus*, *Entrococcus*, nonpathogenic *E. coli*, and *Saccaromyces boulardi*.

In previous studies, *Lactobacillu*s species have been shown to reduce secretion of proinflammatory cytokines such as tumor necrosis factor (TNF-α) in lipopolysaccharide (LPS)-stimulated lamina propriamononuclear cells in CD patients (17-19). Also *L. casei* modulates the expression of cytokines and decreases neutrophilic infiltration of dexteran-induced colitis in Toll-like receptor 4 (TLR-4) mice (20). Previous studies have also demonstrated that *L. casei* have intestinal anti-inflammatory activity and on the other hand several studies have shown that colonization of *L. casei* reduces the lesions induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) in rats and significantly decrease translocation of bacteries to the mesenteric lymph nodes, liver, and spleen. Another study showed that fructooligosaccharides decrease intestinal inflammatory activity predominantely by increasing intestinal *Lactobacillus* counts in TNBS colitis rats (21, 22).

Therefore, we aimed to evaluate the effect of whey-cultured *L. casei* in experimental colitis *via* examining (TNF-α), myeloperoxidase (MPO) activity, thiobarbituric acid-reactive substances (TBARS) as measure of cellular lipid peroxidation (LPO), and the macroscopic and histological damage scores in the colonic tissue.

MATERIALS AND METHODS

Chemicals

TNBS from Sigma-Aldrich Chemie (Gmbh Munich, Germany), TBA, trichloroacetic acid (TCA), n-butanol, hexadecyl trimethyl ammonium bromide (HETAB), hydrochloric acid diamine tetra acetic acid (EDTA), *o*-dianisidine hydrochloride, acetic acid, sodium acetate, Coomassie reagent, bovine serum albumin (BSA), sodium sulphate, phosphoric acid, H_2O_2, potassium dihydrogen phosphate, sodium carbonate, Na-k-tartarate, cupric sulphate from Merck (Tehran-Iran), rat-specific tumor necrosis factor-α (TNF-α) kit from (Bander Med System GmbH, Austria), whey powder from Pooyan-Milk Co. (Tehran, Iran), and *Lactobacillus casei* (PTCC1608) were used in this study.

Preparation of probiotics

Whey was reconstituted (2-4% w/v) with water to prepare liquid whey having lactose concentration of (70-72% w/v). Whey contained protein (10-12% w/v) and ash (8-8.5% w/v), and was supplemented with yeast extract (<50 w/v) and lipid (2% w/v). The whey culture (10% w/v) was sterilized at 121°C for 20 minutes. After sterilization, it was inoculated with 10^6 cfu/100 ml of *L. casei* in whey culture and stored at 37°C for 48 hours under stationary conditions. Eventually whey culture contained 10^8 cfu/100 ml of *L. casei*.

Animals

Male Wistar rats (220-250 g) were maintained under standard conditions of temperature (23°C±1°C), relative humidity (55%±10%), and 12/12 hours light/dark cycle, and fed with a standard pellet diet and water *ad libitum*. They were housed individually in standard polypropylene cages. All ethical themes of studies on animals were considered carefully.

Experimental design

Five groups of male rats containing six in each group were used in this study. Colitis was induced by rectal administration of TNBS. One group of animals received normal saline instead of TNBS (Sham). Other four groups that received TNBS were: control (no treatment), dexa that treated with dexamethasone (as a positive standard at 1 mg/kg), treatment group that treated with whey (2 ml/day) for 14 days after induction of colitis, and prevention group which received 14 days whey (2 ml/day) before induction of colitis. Whey and dexamethasone were dissolved in water and administered to rats by gavage.

Induction of colitis

For induction of colitis, 36-hours-fasted rats were anesthetized with administration of 50 mg/kg pentobarbital sodium intrapreritoneally (23) and were positioned on their right side and then 0.3 ml of a mixture containing six volumes of 5% TNBS plus 4 volume of 99% ethanol was instilled through anus using a rubber cannula (8 cm long). When TNBS was instilled, the rats were maintained in a supine Trendelenburg position in order to prevent anal leakage of TNBS (24).

Sample preparation

Treatments took 14 days long. On the 15th day, animals were sacrificed by an overdose of ether inhalation. Abdomen was immediately opened and the colon was removed. Then colon was cut in pieces and cleaned with saline, and examined for macroscopic changes and scored as described later. Then samples were divided into two pieces, one piece for histopathology assessment (maintained in 10 ml formalin 10% as fixator) and the other for measuring biomarkers. The first one was weighed and maintained in -20°C for 24 hours. The latter, were homogenized in 10 volume ice cold potassium phosphate buffer (50 mM, pH 7.4), then sonicated and centrifuged for 30 min at 3500 g. The supernatants were transformed into several microtubes for biochemical assays and all were kept at -80°C until analyses.

Determination of tumor necrosis factor-α

TNF-α was quantified by enzyme linked immunosorbent assay rat-specific (ELISA) kit. The absorbance of the final colored product was measured in 450 nm as the primary wave length and 620 nm as the reference wave length. The results were expressed as pg/mg protein (11).

Lipid peroxidation assay

Cellular LPO react with TBA and produce a complex with a maximum absorbance at 532 nm. Results were expressed as μg/mg protein (25).

Myeloperoxidase activity

As mentioned earlier, the colonic samples were homogenized in 10 volumes of ice cold potassium phosphate buffer (50 mM, pH 7.4), sonicated and centrifuged at 3500 *g* for 30 min. The supernatant was centrifuged at 12,000 *g* for 20 min and then 0.1 ml of supernatant was added to 50 ml of phosphate buffer containing 0.167 mg/ml *o*-dianisidine and 0.0005% H_2O_2 that resulted in an orange complex. The absorbance was measured for 3 min spectrophtometrically in 460 nm. One unit of MPO activity was described as the change in absorbance per min at room temperature in the final reaction and expressed as unit per mg protein of colon tissue (26).

Total protein of colon tissue

The concentration of protein in the colon homogenate was measured by the Bradford method using BSA as the standard. Results were reported as mg/ml of the homogenized tissue.

Macroscopic and microscopic recognition of colonic damage

The severity of colonic tissue damage was measured using colon macroscopic and microscopic examination. For microscopic examination, colon samples were fixed in formalin 10% and were embedded in paraffin and stained with hematoxylin and eosin. The severity of colonic damage in macroscopic examination was evaluated using the following scoring system: 0 (normal appearance with no damage); 1 (localized hyperemia without ulceration); 2 (localized hyperemia with ulceration); 3 (linear ulceration with inflammation at one site); 4 (two or more sites of ulceration and extending more than 1 cm along the length of colon); and 5-8 (damage extending more than 2 cm along the length of colon and the score was enhanced by 1 for each increased cm of involvement). The microscopic scoring was performed by an observer blind to the treated groups. Microscopic scores were determined as follow: 0 (no damage); 1 (focal epithelial edema and necrosis); 2 (disperse swelling and necrosis of the villi); 3 (necrosis with neutrophil infiltration in submocusa); and 4 (wide spread necrosis with massive neutrophil infiltration and hemorrhage).

Statistical analysis

One-way ANOVA followed by post hoc Tukey were used to evaluate changes between groups. P-values less than 0.05 were considered significant.

RESULTS

Macroscopic and microscopic evaluation of the colonic damage

Histological examinations of macroscopic and microscopic colonic damage are shown in *Table 1*. Colitis induced by TNBS/ethanol in the control group resulted in severe ulceration, necrosis, adhesions, wall thickening and inflammation in comparison to sham group that were normal ($p<0.01$). Dexamethasone improved macroscopic scores in colitis rats in comparison to control group ($p<0.01$). Control, treatment, and prevention groups were significantly different from sham group ($P<0.01$).

Microscopic study of control group showed sever ulcer, necrosis, mucosal and submucosal polymorphonuclear leucocytes (PMN) infiltration with crypt abscess, whereas in sham group features of colons were within normal limits ($p<0.01$).

Histological examination of treatment group showed improvement in the ulcer and a reduction in macrophages and lymphocytes in ulcer region and submucosa. Also no necrosis was observed in crypts of treatment group but in the prevention group, ulcer and necrosis developed within mucosa and number of macrophages and lymphocytes increased in submocosa. In Dexa group, the extent of ulcer, necrosis, and infiltration of mucosal/submucosal PMN was improved (*Table 1*).

Colonic tumor necrosis factor-α level comparison

TNF-α level was elevated in control group as compared to sham group ($p<0.001$). Administration of whey that contained *L. casei* lowered TNF-α in treatment group in comparison to control group but it was not significant. Also, TNF-α was significantly decreased in Dexa group in comparison to control group ($p<0.001$). TNF-α was significantly different in prevention group in comparison to that of sham and Dexa groups ($p<0.001$).

Colonic lipid peroxidation

Lipid peroxidation (LPO) was increased in control group in comparison to sham group ($p<0.001$). A significant decrease in LPO was shown in treatment group in compression to control group ($p<0.05$). LPO was significantly different in Dexa group in comparison to control group ($p<0.001$). LPO significantly increased in prevention group in comparison to Dexa group ($p<0.05$). LPO in prevention and treatment groups was significantly higher than that of sham group ($p<0.05$, *Fig. 3*).

Colonic myeloperoxidase

Colonic myeloperoxidase (MPO) activity in control group was markedly higher than that of sham group ($p<0.001$). A significant reduction of MPO activity was observed in treatment group in comparison to control group ($p<0.01$). MPO activity was significantly lower in Dexa group in comparison to control group ($p<0.001$), but in prevention group, MPO activity was higher than that of Dexa group ($p<0.001$). *L. casei* reduced MPO activity in treatment group in comparison to prevention group ($p<0.001$). Data showed remarkably difference between sham and prevention groups ($p<0.001$, *Fig. 4*).

DISCUSSION

The present study for the first time demonstrates the beneficial effects of *L. casei* in an animal model of TNBS-induced colitis. *L. casei* ameliorated histopathological scores of

Table 1. Extent of colonic damage according to macroscopic and microscopic scores.

Groups	Macroscopic Score (Mean±S.E.M.) Median (Min-Max)	Microscopic Score (Mean±S.E.M.) Median (Min-Max)
Sham	(0.0±0.0) 0(0.0-0.0)	(0.0±0.0) 0 (0.0-0.0)
Control	(6.28±0.28)† 6(5.0-7.0)	(3.5±0.34)† 4(2.0-4.0)
Dexa	(1.5±0.34)‡ 2(0.0-2.0)	(2.1±0.3)†,‡ 2(1.0-3.0)
Prevention	(5±0.856)† 5.5(2.0-7.0)	(3.3±0.33)† 3(2.0-4.0)
Treatment	(2.57±0.29)† 2(2.0-4.0)	(1.71±0.28)†,‡ 2(1.0-3.0)

†Significantly different from Sham group at $p<0.01$.
‡Significantly different from control group at $p<0.01$.

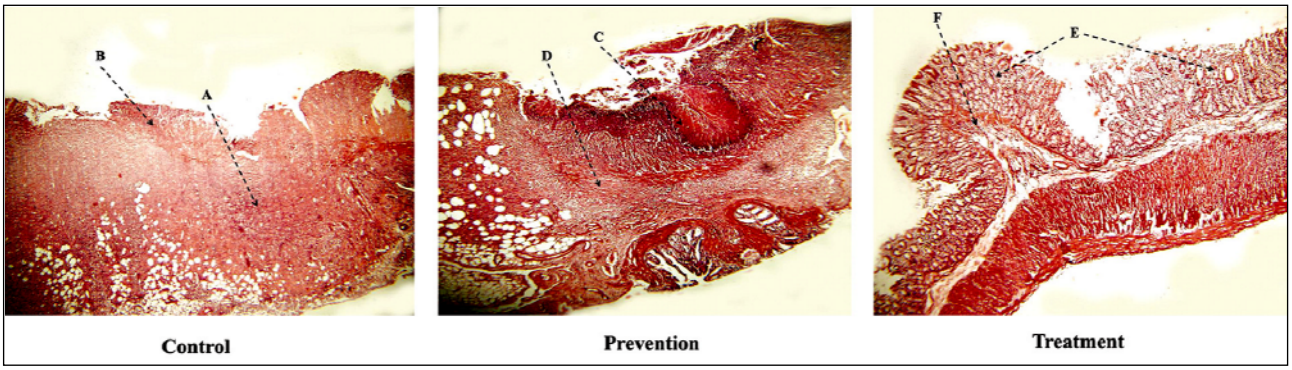

Fig. 1. Histology images of colon tissues obtained from different groups. Microscopic evaluation of control group showed highly severe, intense transmural inflammation and/or diffuses necrosis (A) and severe crypt destruction (B). In prevention group, necrosis of crypts, hemorrhage (C) and mucosal and submucosal inflammation were seen (D). Histological examination of treatment group showed no necrosis in crypts (E) and mild inflammation of mucosa were seen (F).

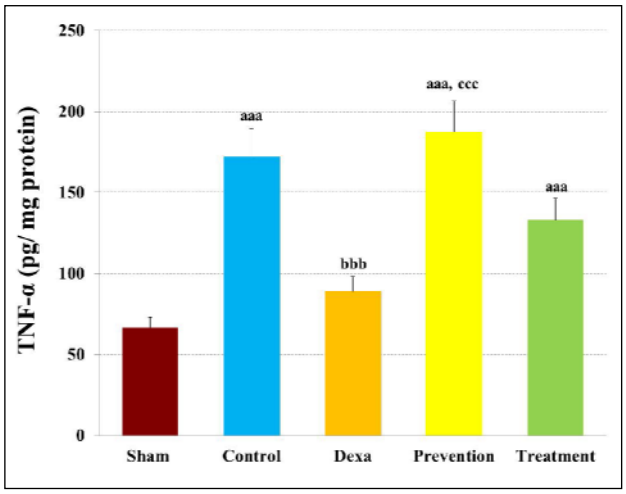

Fig. 2. Tumor necrosis factor-alpha (TNF-α) level in colon. Values are mean±S.E.M.
[a] Significantly different from sham group at p<0.05.
[b] Significantly different from control group at p<0.05.
[c] Significantly different from Dexa group at p<0.05.
[aa] Significantly different from sham group at p<0.01.
[bb] Significantly different from control group at p<0.01.
[cc] Significantly different from Dexa group at p<0.01.
[aaa] Significantly different from sham group at p<0.001.
[bbb] Significantly different from control group at p<0.001.
[ccc] Significantly different from Dexa group at p<0.001.

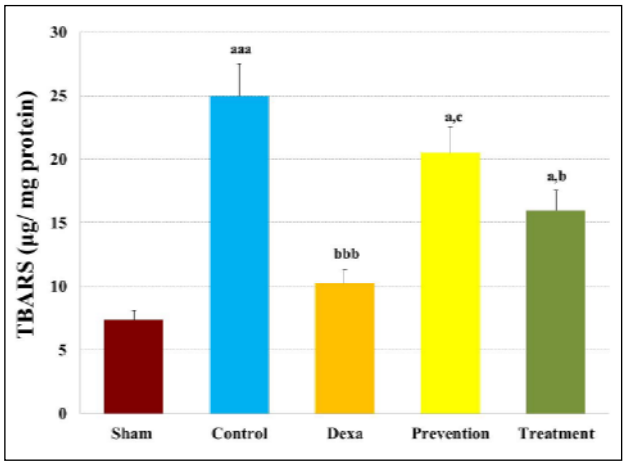

Fig. 3. Lipid peroxidation as TBARS in colon. Values are mean±S.E.M.
[a] Significantly different from sham group at p<0.05.
[b] Significantly different from control group at p<0.05.
[c] Significantly different from Dexa group at p<0.05.
[aa] Significantly different from sham group at p<0.01.
[bb] Significantly different from control group atp<0.01.
[cc] Significantly different from Dexa group at p<0.01.
[aaa] Significantly different from sham group at p<0.001.
[bbb] Significantly different from control group at p<0.001.
[ccc] Significantly different from Dexa group at p<0.001.

colitis and improved biochemical markers of inflammation and toxic stress including TNF-α, MPO activity and LPO.

Recently, probiotics have been shown effective in the treatment of IBD in several models of colitis (27). Probiotics stimulate the secretion of antimicrobial peptides from intestinal cells (28), but their definite molecular mechanism for this

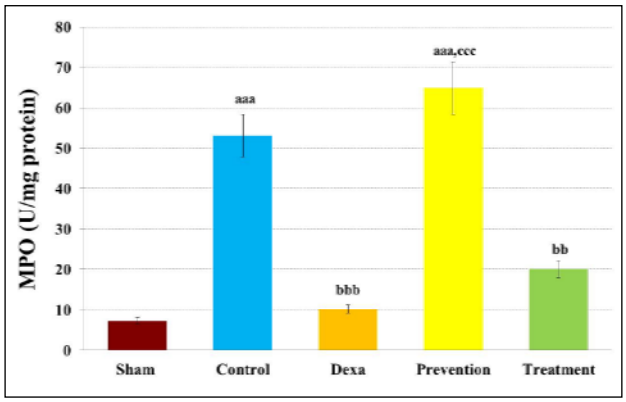

Fig. 4. Myeloperoxidase (MPO) activity in colon. Values are mean±SEM.
[a] Significantly different from sham group at p<0.05.
[b] Significantly different from control group at p<0.05.
[c] Significantly different from Dexa group at p<0.05.
[aa] Significantly different from sham group at p<0.01.
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[aaa] Significantly different from sham group at p<0.001.
[bbb] Significantly different from control group at p<0.001.
[ccc] Significantly different from Dexa group at p<0.001.

therapeutic action is unclear yet (18). In this study we examined potential beneficial effects of *L. casei* in whey culture for the first time in a colitis model induced by TNBS/ethanol. In this method, ethanol devastates the intestinal mucosal barrier and TNBS acts as a hapten and induces such a delayed-type of hypersensitivity resulting in colitis similar to that of human IBD (29). Some investigations demonstrated that *L. casei* can stop or delay mucosal damage and inflammation induced by TNBS. Also, probiotics have been useful in treatment of bowel diseases (16, 28, 30, 31).

Previous studies have shown overexpression of several pro-inflammatory cells such as monocytes, macrophages, B cells, NK cells and mast cells that participate in secretion of TNF-α during inflammation that is the main marker of toxic colitis in both experimental models and human IBD (12, 32, 33). Our results showed that repetitive use of *L. casei,* growing in the whey, could decrease TNF-α in the treatment group, by modifying intestinal anti inflammatory activity. Matsumoto *et al*. reported that cellular components of *L. casei* down-regulates the nuclear translocation of NF-κB and host immune responses *via* affecting cytokines secretion such as TNF-α and IL-6.

Effect of some kind of probiotics is related to immune responses that promote a shift from a T_H-1 mediated immune response toward a T_H2/T_H3 profile that similarly occur with *Lactobacillus* GG (34). Rezaie *et al*. demonstrated the role of oxidative stress as an important factor in initiation of IBD (7). In this regard, MPO activity is believed as a marker of neutrophil aggregation relevant to IBD (35). In the present study, we observed a marked reduction in MPO in the treatment group that is supported by previous studies (36-38). *L. casei* reduced MPO activity and neutrophilic infiltration and increased expression of anti-inflammatory cytokines (36), and production of gut-secreted immunoglobulins like IgA (37, 38). These results are in agreement with study of Hegazy *et al*. who reported that *Lactobacillus* could reduce MPO activity (39).

Previous reports indicated that LPO is increased in inflamed mucosa in patients suffering from IBD (40). Moreover increase of LPO was shown in animal models of colitis. Also, Bay *et al*.

reported that *L. casei* grown in milk culture can reduce LPO in rat brain (41).

Interestingly, there is evidence that bacteria in fermented milk may alter host immune responses or in other words, the fermented products may alter the intestinal microflora (18). It seems that lactic acid bacteria might stick to mucosal surfaces in intestinal or lumen to prevent from adherence of aerobic gram negative bacteria. Furthermore, *Lactobacillus* strains GR-1 and RC-14 can inhibit the adhesion and growth and colonization of *Escherichia coli* (42-43). Thus, they could develop nonspecific instigation of immune system leading to cell proliferation and increased phagocytic activity (44). Also Lam *et al*. showed that *Lactobacillus* can increase production of MUC6 and basal mucosal PGE_2 resulting in thickness of mucosal layer of stomach (45). Treatment with *L. casei* can be useful in healing of colitis. Previous studies have shown that lactic acid bacteria decrease pH in culture, and high concentration of them induce erosions of the epithelium and inflammation in the colon when the pH is <4.5. But pH>4.5, can stimulate mucosal repair and causes proliferation of epithelial cells in rat cecum. In the present study, the pH of whey culture that *L. casei* was grown in it was 5. According to previous studies, it can be useful in treatment of IBD (46-48).

Anti-inflammatory effect of bacteria on the gastrointestinal epithelium (GI) is *via* regulation of mucosal T cells or dendritic cells that observed in colitis. Probiotics increase their effects on stabilization of gut barrier by modifying function of epithelial cells. Similarly, the exchange of regulatory signals between the commensal floras, the epithelial and subepithelial components of the mucosa can be proposed. Indeed, certain nonpathogenic organisms have been shown to counterbalance epithelial responses to invasive bacteria *via* regulation of cytokine transcription factors (49). *Lactobacilli* have an advantage as a carrier to deliver drug to mucosa, because of the potential to make consistency in the gastrointestinal tract or modulating immune response that are important elements in IBD (50-51).

In conclusion, the present finding support the idea that *L. casei* grown in whey culture is very effective in ameliorating both biochemical and histopathological markers of colitis if used post induction of colitis. The present findings do not support benefit of *L. casei* grown in whey culture is used before induction of colitis. The difference between effects of *L. casei* when used pre-colitis and post-colitis confirms its mechanism of action as an anti toxic stress agent. Further studies should be trialed in IBD patients.

Conflict of interests: None decared.

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