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Research Paper

Protective effects of water-soluble low-molecular-weight β-(1,3-1,6)D-glucan purified from *Aureobasidium pullulans* GM-NH-1A1 against UFT toxicity in mice

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Abstract

Objectives 5-Fluorouracil and its derivatives are widely used in the treatment of a variety of tumours. However, their use is associated with gastrointestinal toxicity, myelotoxicity and immune toxicity. In this study, we examined the protective effects of low-molecular-weight β-glucan isolated from *Aureobasidium pullulans* GM-NH-1A1 against toxicity of UFT (combination of tegafur (1-(2-tetrahydrofuryl)-5-fluorouracil) and uracil) in mice bearing colon 26 tumours.

Methods UFT was administered orally at 50 mg/kg once daily for 14 days alone or with orally administered low-molecular-weight β-glucan, 25, 50 and 100 mg/kg twice daily.

Key findings Tumour growth was inhibited equally in all treatment groups. Onset of diarrhoea, which started on day 9 of UFT administration, was delayed by concomitant administration of the β-glucan (50 and 100 mg/kg twice daily). Histological analysis showed that damage to small-intestine villi by UFT was inhibited by the orally administered β-glucan.

Conclusions Oral administration of low-molecular-weight β-glucan prevents gastrointestinal mucositis associated with UFT therapy without interfering with its anti-tumour activity.

Keywords adverse reaction; anti-tumour activity; gastrointestinal toxicity; UFT; water-soluble low-molecular-weight β-glucan

Introduction

Polysaccharide (β-glucan) fractions prepared from many Basidiomycetes mushrooms, such as *Ganoderma lucidum*,[1] *Phellinus lintenus*,[2] *Agaricus blazei*,[3,4] *Grifola frondosa*,[5,6] *Sparassis crispa*[7,8] and *Lentinus edodes*[9] have been the subject of several studies. For example, it is well documented that when administered in medicines and health foods, β-glucan has anticancer activity through a biological response modifier effect.[1,6,7] Lentinan from *L. edodes*,[10] schizophyllan (SPG) from *Schizophyllum commune*[11] and Krestin (PSK) from *Cotolus versicolor*[12] have been used in Japan as anticancer drugs and/or adjuvants.

Based on the anti-tumour activities of polysaccharides isolated from Basidiomycetes mushrooms, it is thought that structural features such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for anti-tumour action through immune activation. The (1→3) and (1→6) β-glucans isolated from Basidiomycetes mushrooms have high viscosity and high molecular weight (over 2000 kDa) and are insoluble in water. In general, β-glucan readily forms gels that contain higher-order structures of single spirals or triplet spirals because of its unique primary structure. Purification is therefore extremely difficult, and consequently crude β-glucan fractions rather than purified β-glucan were used in many studies.

We have successfully isolated and produced on an industrial scale a water-soluble low-molecular-weight (LMW) β-(1,3-1,6) D-glucan from *Aureobasidium pullulans*, strain GM-NH-1A1 (black yeast, a mutant of the strain K-1).[13] Recently, we reported that a water-soluble LMW β-(1,3-1,6) D-glucan purified from *A. pullulans* strain 1A1 exerted anti-tumour and anti-metastatic effects by stimulating the immune system in the small intestine.[14] Furthermore, this β-glucan prevented an ova-albumin-induced allergic

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reaction by inhibiting reductions in interleukin (IL)-12 and interferon-γ in the spleen of ova-albumin-sensitised mice,[15] and it inhibited decreases in natural killer cell activity, IL-6 and IL-12 and an increase in blood corticosterone levels caused by restraint stress.[16]

5-Fluorouracil (5-FU), first synthesised by Duschinsky and colleagues in 1957,[17] is used extensively in the treatment of various types of cancer.[18–21] However, 5-FU is associated with gastrointestinal toxicity and myelotoxicity through its phosphorylation in the digestive tract[22] and bone marrow tissue.[23] We have previously reported that adverse reactions (myelotoxicity, gastrointestinal toxicity and immune toxicity) to chemotherapeutic drugs such as 5-FU, cisplatin and doxorubicin were prevented by a basic polysaccharide chitin/chitosan without any loss of anti-tumour activity.[24–26] Fujii and colleagues reported that the anti-itumour effect of 1-(2-tetrahydrofuryl)-5-fluorouracil (tegafur) on sarcoma 180 and AH130 tumours was enhanced by oral administration of uracil, and that the concentration of 5-FU in the tumour after the co-administration of tegafur and uracil was much higher than after administration of tegafur alone.[27,28] Consequently, the combination of tegafur and uracil, termed UFT, was developed as a new chemotherapeutic drug, and has been clinically used worldwide.[29,30] However, 5-FU derivatives cause severe gastrointestinal toxicity, with diarrhoea and mucositis, and haematological toxicity (leucopenia), which appear to be dose-limiting factors. The aim of this study was to determine whether a purified water-soluble LMW β-glucan enhances the anti-tumour activity of UFT and protects against adverse reactions in mice bearing colon 26 tumours.

Materials and Methods

Materials

LMW β-glucan was purified from *A. pullulans* 1A1 using previously described methods.[13–15]

Animals

Male Balb/c mice (5 weeks old) were obtained from SLC Japan (Shizuoka, Japan) and were housed for 1 week before the experiments in a room with controlled temperature and humidity. During this period and the experiments they had free access to laboratory chow (Oriental Co., Osaka, Japan) and water.

Mice were treated according to the ethical guidelines of the Animal Center, Ehime University Graduate School of Medicine. The Animal Studies Committee of Ehime University approved the experimental protocol.

Cells

The colon 26 cell line was obtained from the Institute of Development, Aging and Cancer, Tohoku University, and maintained in RPMI 1640 medium (Nissui Pharmacy Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (Gibco BRL, Auckland, New Zealand), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) (Sigma, St Louis, MO, USA). These cell were inoculated into mice to grow into solid tumours.

Measurement of anti-tumour activity and adverse effects of UFT

Approximately 1×10^5 cells were injected subcutaneously into the backs of mice. Treatment with UFT with or without LMW β-glucan was started after 7 days, when tumours were approximately 50 mm^3. UFT (50 mg/kg) was administered orally once daily (8:00 am) for 14 consecutive days starting on day 7 after implantation of tumour cells. LMW β-glucan (25, 50 and 100 mg/kg) was administered orally twice daily (8:30 am and 8:30 pm) for 14 consecutive days, starting on day 7 after implantation of tumour cells. Normal mice and untreated tumour-bearing mice (controls) were given distilled water alone on the same schedule. Tumour volume was determined every 2 or 3 days by direct measurement with callipers and calculated using the formula: volume = (length $\times$ width^2)/2. Food intake was measured every day after the morning drug administration. Body weight was measured every other day. The incidence of diarrhoea was determined daily after drug administration.

On day 22 after tumour inoculation (i.e. the day after the last treatment), blood was obtained by venous puncture under anaesthesia with diethylether, and the tumour, liver, small intestine, spleen and thymus were removed and weighed, for evaluation of anti-tumour activity and adverse effects. Blood samples were chilled in test tubes containing heparin, and the numbers of leucocytes, red blood cells and platelets, haemoglobin (Hb) content and the haematocrit were measured using a Coulter Counter (Japan Scientific Instruments Co. Ltd, Tokyo, Japan). Concentrations of glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) in the blood were measured using a Wako STA test kit (Wako Pure Chemical Co., Osaka, Japan).

Measurement of damage to the small intestinal mucosal membrane

The small intestine was washed with cold 0.9% NaCl to remove its contents. The mucosa (100 mg weight) was scraped off with a glass slide and homogenised with phosphate-buffered saline (PBS, pH 7.4) in a final volume of 1 ml. The protein content was measured by the method of Lowry and colleagues[31] using the DC protein assay reagent (Bio-Rad, Japan, Tokyo, Japan). To evaluate the extent of injury, the entire small intestine was fixed in 10% buffered formalin for at least 24 h, then progressively dehydrated in solutions containing an increasing percentage of ethanol (70, 80, 95 and 100%, v/v). The organic gas was cleared by ventilation with Histoclear (As-one, Tokyo, Japan). Samples were then embedded in paraffin under a vacuum, sectioned into 5 μm thick sections, de-paraffinised, and stained with haematoxylin and eosin.

Statistical analysis

All values are expressed as means ± SE. Data were analysed by one-way analysis of variance. Differences between means were analysed using Fisher's protected least-significant difference multiple comparison test. Differences were considered significant at $P < 0.05$.

Results

UFT (50 mg/kg once daily) significantly reduced tumour growth and final tumour weight and volume, when given alone or with the addition of LMW β-glucan (25, 50 and 100 mg/kg twice daily; Figure 1). There was no significant difference in tumour growth and tumour weight between the group receiving UFT alone and the groups receiving UFT plus LMW β-glucan.

Body weight was significantly reduced 9–14 days after administration of UFT (50 mg/kg) compared with control mice but there was no significant difference in body weight between the UFT-treated group and those that also received LMW β-glucan (data not shown). Food intake was also reduced 8–14 days after administration of UFT. LMW β-glucan (25, 50 and 100 mg/kg) tended to inhibit the reduction in food intake induced by UFT (Figure 2).

The reduction in small intestine weight caused by UFT was significantly inhibited by the highest dose of LMW β-glucan (100 mg/kg twice daily) (Table 1). The protein content of the small intestine mucosa was also significantly reduced by UFT compared with controls, but this was prevented by the administration of LMW β-glucan (25, 50 and 100 mg/kg; Figure 3A). The administration of UFT

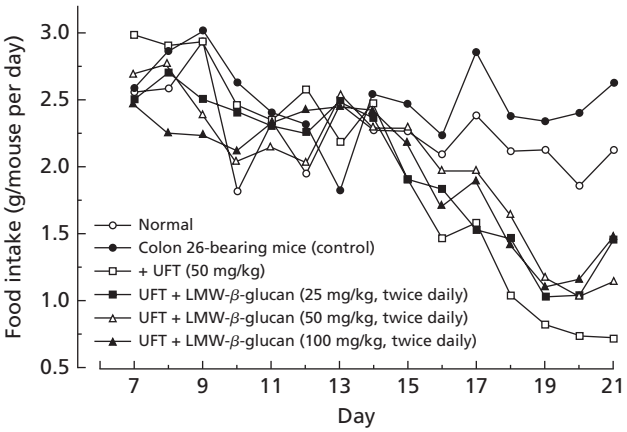

Figure 2 Food intake in colon-26-bearing mice treated with UFT. Mice were administered UFT (50 mg/kg) alone and in combination with water-soluble low-molecular-weight β-glucan, 25, 50 and 100 mg/kg twice daily for 14 days starting 7 days after tumour inoculation.

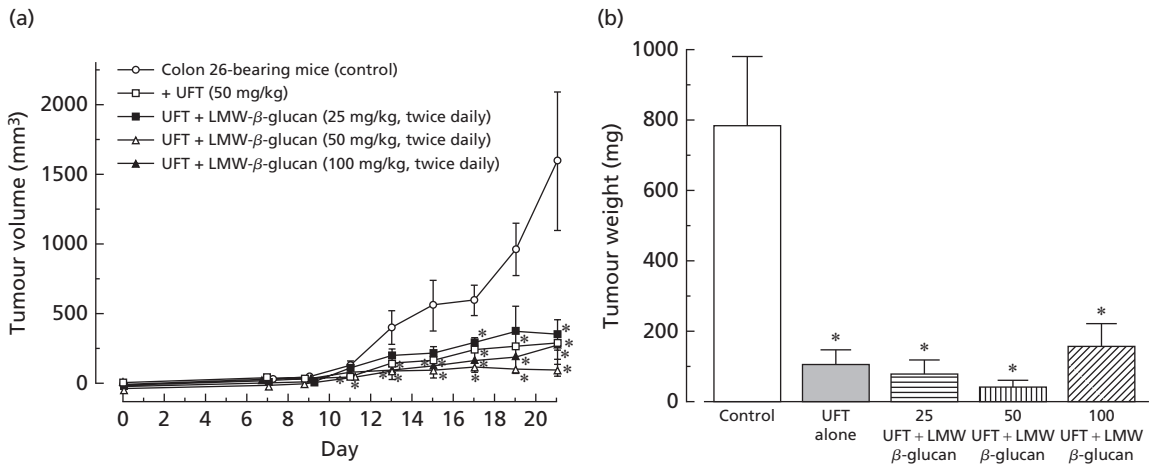

Figure 1 Tumour growth (a) and final tumour weight (b) in colon 26-bearing mice treated with UFT. Mice were administered UFT (50 mg/kg) alone and in combination with water-soluble low-molecular-weight β-glucan, 25, 50 and 100 mg/kg twice daily for 14 days starting 7 days after tumour inoculation. Values are means ± SE (n = 7 mice). *P < 0.05 vs vehicle-treated control mice.

Table 1 Effects of UFT alone and when co-administered with low-molecular-weight (LMW) β-glucan on the weights of various tissues in colon-26-bearing mice

	Liver	Spleen	Thymus	Small intestine
Normal	1059.7 ± 20.2	71.33 ± 2.28	33.50 ± 1.67	855.3 ± 25.5
Tumour-bearing mice				
Vehicle (water) controls	1165.9 ± 29.2	102.43 ± 9.52	33.29 ± 1.73	937.4 ± 26.0
UFT (50 mg/kg)	784.3 ± 45.0*	59.63 ± 4.54*	1.00 ± 1.00*	782.1 ± 22.3*
UFT + LMW β-glucan (25 mg/kg)	889.0 ± 77.0*	79.43 ± 18.1	2.14 ± 1.42*	888.9 ± 63.7
UFT + LMW β-glucan (50 mg/kg)	877.7 ± 60.3*	88.14 ± 20.23	3.71 ± 1.55*	854.9 ± 50.5
UFT + LMW β-glucan (100 mg/kg)	854.1 ± 65.7*	69.00 ± 12.04*	2.00 ± 1.36*	927.1 ± 36.2†

Values are tissue weight in mg, means ± SE (n = 7 mice). *P < 0.05 vs control, †P < 0.05 vs UFT-treated mice.

caused diarrhoea after day 9 but the onset of diarrhoea was delayed by the oral administration of LMW β-glucan (50 and 100 mg/kg twice daily; Figure 3B).

As shown in Figure 4, the surface epithelium of villi was remarkably damaged by UFT and the growth of intestinal villi markedly impaired compared with normal and control mice. The damage to the villi was prevented by oral administration of LMW β-glucan.

The weights of the liver, spleen and thymus were reduced by UFT compared with control values; this was not affected by co-administration of LMW β-glucan (Table 1). UFT reduced numbers of leucocytes and platelets in tumour-bearing

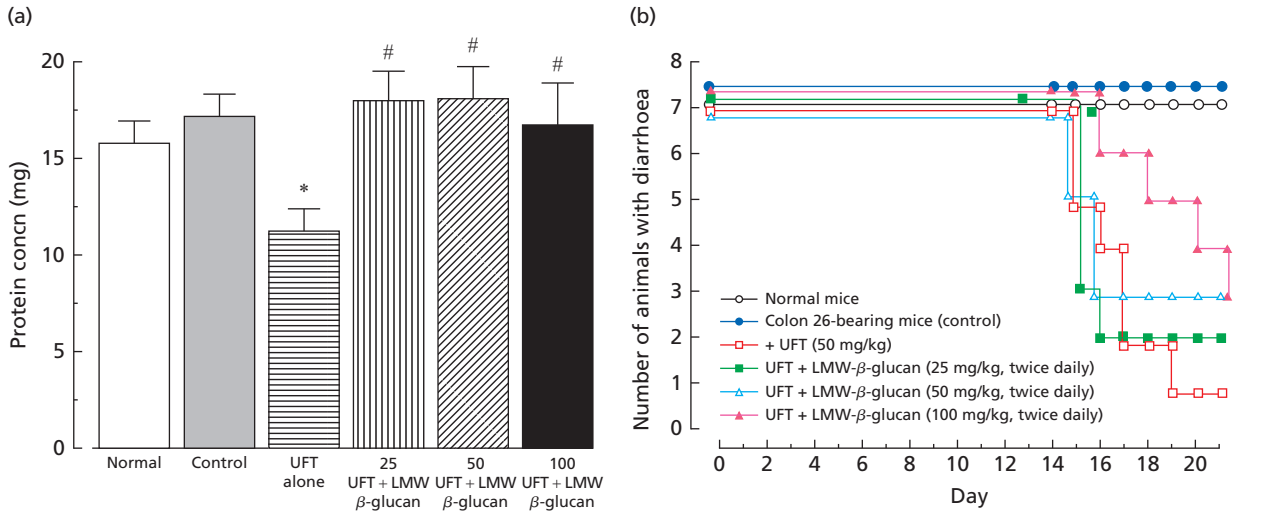

Figure 3 Small intestinal mucosal protein content (a) and incidence of diarrhoea (b) in colon-26 bearing mice treated with UFT. Values are means ± SE, $n = 7$ mice. LMW, low-molecular-weight. $^{*}P < 0.05$ vs vehicle-treated control mice; $^{\#}P < 0.05$ vs UFT-treated tumour-bearing mice. (b) Mice were administered UFT (50 mg/kg) alone and in combination with water-soluble low-molecular-weight β-glucan, 25, 50 and 100 mg/kg twice daily for 14 days starting 7 days after tumour inoculation.

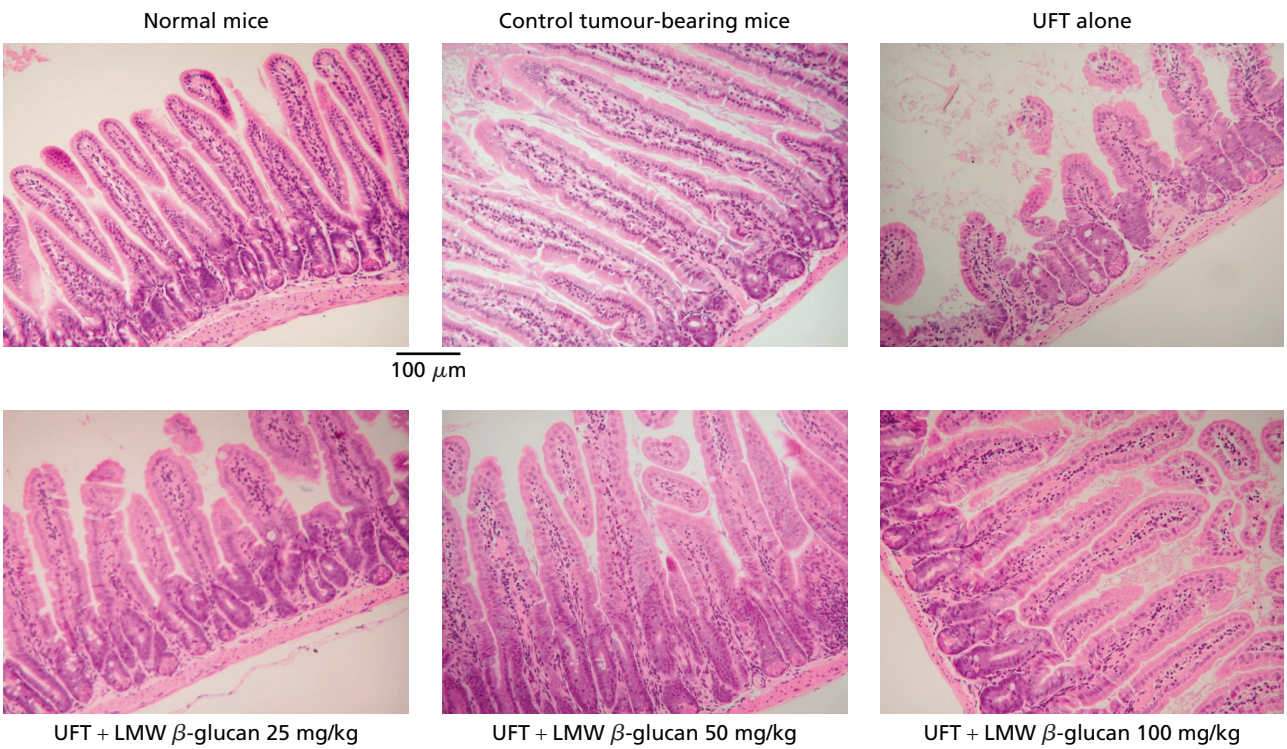

Figure 4 Light micrograph of small intestine stained with haematoxylin and eosin (×100 magnification) in colon-26-bearing mice. LMW, low-molecular-weight.

Table 2 Effects of UFT alone and when co-administered with low-molecular-weight (LMW) β-glucan on blood parameters of colon-26-bearing mice

	Red cell count ($\times 10^4$ per μl)	Leucocyte count ($\times 10^3$ per μl)	Platelet count ($\times 10^4$ per μl)	Haemoglobin concn (g/100 ml)	Haematocrit (%)
Normal	802.0 ± 10.0	5.17 ± 0.65	97.6 ± 2.51	13.7 ± 0.20	39.6 ± 0.48
Tumour-bearing mice					
Vehicle (water) controls	748.0 ± 14.1	4.74 ± 0.72	102.2 ± 3.35	13.2 ± 0.18	37.4 ± 0.57
UFT (50 mg/kg)	701.1 ± 20.9	0.66 ± 0.05*	53.6 ± 4.60*	12.1 ± 0.35	33.5 ± 0.92
UFT + LMW β-glucan (25 mg/kg)	685.4 ± 15.3	0.97 ± 0.21*	51.2 ± 11.9*	12.0 ± 0.18	33.2 ± 0.56
UFT + LMW β-glucan (50 mg/kg)	707.1 ± 8.1	1.07 ± 0.26*	53.8 ± 11.3*	12.4 ± 0.20	33.9 ± 0.36
UFT + LMW β-glucan (100 mg/kg)	717.0 ± 23.9	1.50 ± 0.40*	52.2 ± 8.78*	12.6 ± 0.36	34.7 ± 0.98

Values are means ± SE ($n = 7$ mice). *$P < 0.05$ vs controls.

mice compared with normal and control mice, but had no effect on the number of red cells, Hb concentration or haematocrit (Table 2) or GOT or GPT values (data not shown). At the highest dose (100 mg/kg twice daily) LMW β-glucan tended to partially inhibit the reduction in leucocyte number induced by UFT, but did not inhibit the reduction in platelet number (Table 2).

Discussion

The dose-limiting toxicities of most chemotherapeutic drugs are myelotoxicity and gastrointestinal toxicity. The development of colony-stimulating factors has reduced the severity and duration of hematopoietic toxicity.[32–34] However, mucositis still represents an important problem. In fact, severe mucositis (grade 3 and 4) that has not fully resolved at the time of re-treatment usually indicates that treatment should be withheld until the mucosa has healed, and that the drug dose must be decreased, with a subsequent reduction in therapeutic effect. Agents that protect the gastrointestinal tract against the toxicity of chemotherapeutic drugs are therefore likely to improve the tolerability of chemotherapy.

In this study, we found that oral administration of LMW β-glucan (50 and 100 mg/kg twice daily) to tumour-bearing mice reduced the incidence of diarrhoea caused by UFT by preventing damage to the small intestinal mucosa, but without interfering with its anti-tumour effects. LMW β-glucan partially inhibited the reduction in leucocyte number induced by UFT, but did not affect the reduction in platelet number. Thus, LMW β-glucan had greater preventive effects against UFT-induced gastrointestinal toxicity than UFT-induced myelotoxicity.

Further study is needed to clarify the mechanism behind the protective effect of LMW β-glucan on gastrointestinal toxicity. We have previously reported that chitin/chitosan prevented myelotoxicity and gastrointestinal toxicity without compromising anti-tumour activity.[24] The difference between chitin/chitosan and LMW β-glucan in terms of preventing 5-FU-induced adverse reactions may reflect differences between the basic polysaccharides of chitin/chitosan and the neutral polysaccharide of LMW β-glucan. Further work is needed to investigate the protective effects of neutral, acidic and basic polysaccharides against 5-FU-induced adverse reactions.

Conclusions

Water-soluble LMW β-1,3-D-glucan (branch β-1,6) purified from *A. pullulans* 1A1 might be useful for the protection of gastrointestinal toxicity caused by the oral anti-cancer drug UFT.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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