Preoperative immunonutrition regulates tumor infiltrative lymphocytes and increases tumor angiogenesis in gastric cancer patients

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Abstract

Introduction: An increased number of tumor infiltrative lymphocytes (TILs) is considered a favorable prognostic factor in various cancers because it is a marker of antitumoral activity of the immune system. In this prospective, non-randomized clinical trial, we evaluated the impact of preoperative immunonutrition on tumor infiltrative lymphocytes and neoangiogenesis in cancerous tissue in patients with locoregional and resectable gastric adenocarcinoma.

Material and methods: Patients with locoregional and resectable gastric adenocarcinoma were divided non-randomly into two study groups. The first (control) group included patients who had standard nutrition, and the second group included those who had immunonutrition for 7 days before surgery. The biopsy samples taken endoscopically in the preoperative period, as well as the gastrectomy samples, were subjected to immunohistochemical staining for quantitative analysis of CD4, CD8, CD16, CD56, CD31 and CD105 antibodies. Main outcome measures were CD4-to-CD8 ratio and CD105 levels.

Results: Fifty patients were included in the study between January 2013 and December 2014. Twenty-five patients were assigned to each of the first and second group. The CD4-to-CD8 ratio and CD105 levels determined in endoscopic biopsy samples were similar in both groups. The CD4-to-CD8 ratio in gastrectomy samples was significantly higher in the first group (p = 0.0001). The CD105 levels in gastrectomy samples were significantly lower in the first group (p = 0.01).

Conclusions: Seven-day preoperative immunonutrition use regulates TILs in gastric cancer patients, but prolonged use increases tumor angiogenesis.

Key words: immunonutrition, tumor infiltrative lymphocyte, angiogenesis, neovascularization, gastric cancer.

Introduction

In the inflammatory response key factors including cell division, neovascularization and angiogenesis regulate tumor progression in addition to controlling tumor growth. In the presence of excessive pro-inflamma-

tory cytokines, neovascularization develops and rapid tumor growth occurs when the balance of pro-inflammatory and anti-inflammatory cytokines is disturbed.

The common consideration in the use of immunonutrition in patients with cancer is to regulate the host's immune response and to control cancer by using the potential immune system present in the host against the tumor to inhibit malnutrition. It is difficult, however, to interpret the observed effects. In this study, we aimed to evaluate the effect of immunonutrition on TILs and neo-angiogenesis.

Material and methods

The study was designed as a single-center, open label, prospective non-randomized clinical trial. The study was initiated after the approval of the institutional review board. Patients who were diagnosed with gastric tumor between January 2013 and December 2014 were recruited for the study. After the diagnostic work-up and preoperative staging had been completed, patients who were found to have locoregional and resectable gastric adenocarcinoma according to the NCCN Clinical Practice Guidelines in Oncology for Gastric Cancer were included in the study.

All patients were informed about the study and were requested to sign an informed consent form.

To exclude severe malnutrition, all patients were initially evaluated with anthropometric methods including deviation from ideal body weight, total weight loss within the last 3 months and body mass index.

All patients were hospitalized seven days before surgery. Patients in the first or control group were given a regular diet without any nutritional support, whereas those in the second group had immuno-modulating substrates in addition to the regular diet. For this purpose, patients in the second group were given 237 ml of Impact RTD (Nestle, Istanbul, Turkey) solution, which was given three times a day. Impact RTD (Nestle, Istanbul, Turkey) solution contains arginine, omega-3 fatty acids and nucleotides.

The endoscopic biopsy materials obtained at the initial gastroscopy and the gastrectomy specimens were fixed with formalin, alcohol, xylol and liquid paraffin for 15 h. Afterwards the specimens were embedded in paraffin blocks, which were then sliced into 2–4 micron cross-sections with a microtome (Thermo Finesse ME+). All pathological preparations were subjected to conventional

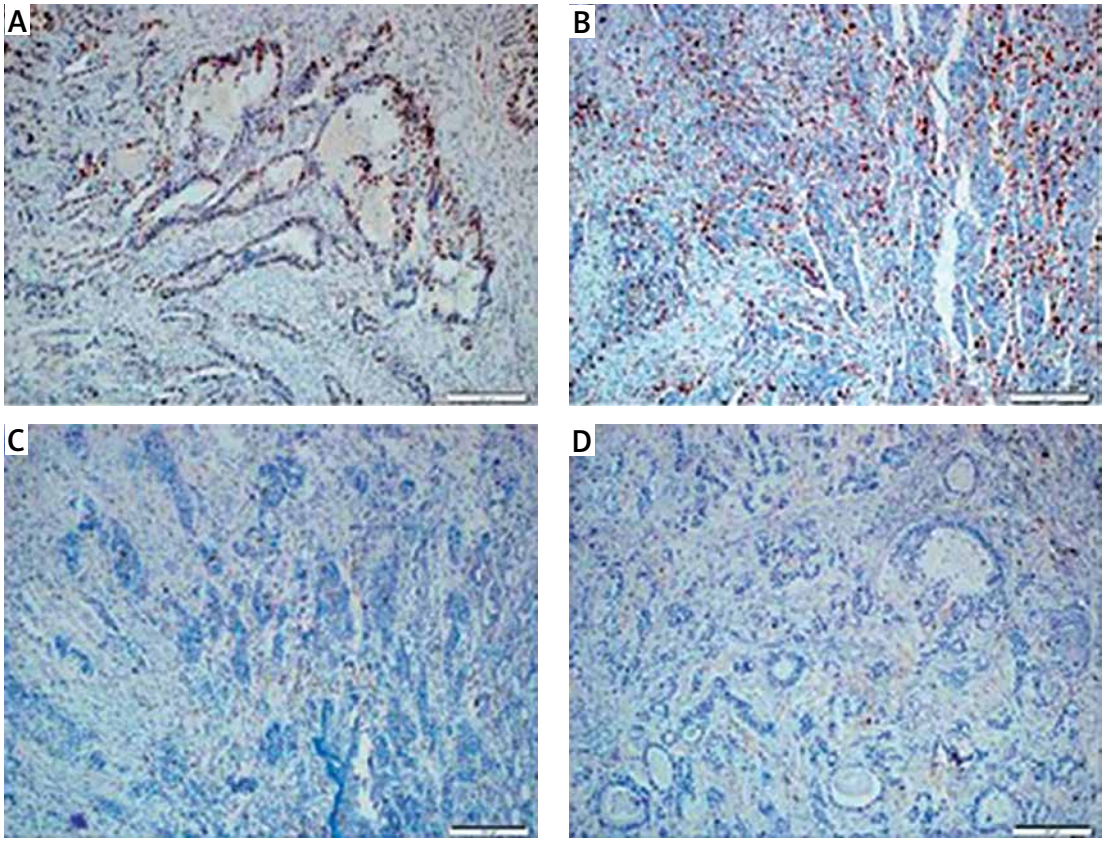

Figure 1. The intraepithelial tumor infiltrative lymphocytes within the tumor are in gastric cancer demonstrated through immunohistochemical staining (brown-stained cells): **A** – CD4 + stained cells, **B** – CD8 + stained cells, **C** – CD16 + stained cells, **D** – CD56 + stained cells

staining with hematoxylin-eosin in a fully automated staining device (Leica ST5020, Nussloch, Germany) as well as to immunohistochemical staining with CD4, CD8, CD16, CD56, CD31 and CD105 mouse monoclonal antibody (Novocastra, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) in an immunohistochemical staining device. Product and producer appear to be muddled) in four different dilutions (1/20, 1/20, 1/80, 1/1, 1/50 and 1/50, respectively).

After the procedures were completed, the hematoxylin-eosin-stained preparations were examined for histopathological diagnosis (Figures 1–3).

The immunohistochemically stained samples were examined under a light microscope (Olympus BX-51). The assessment focused on tumor ar-

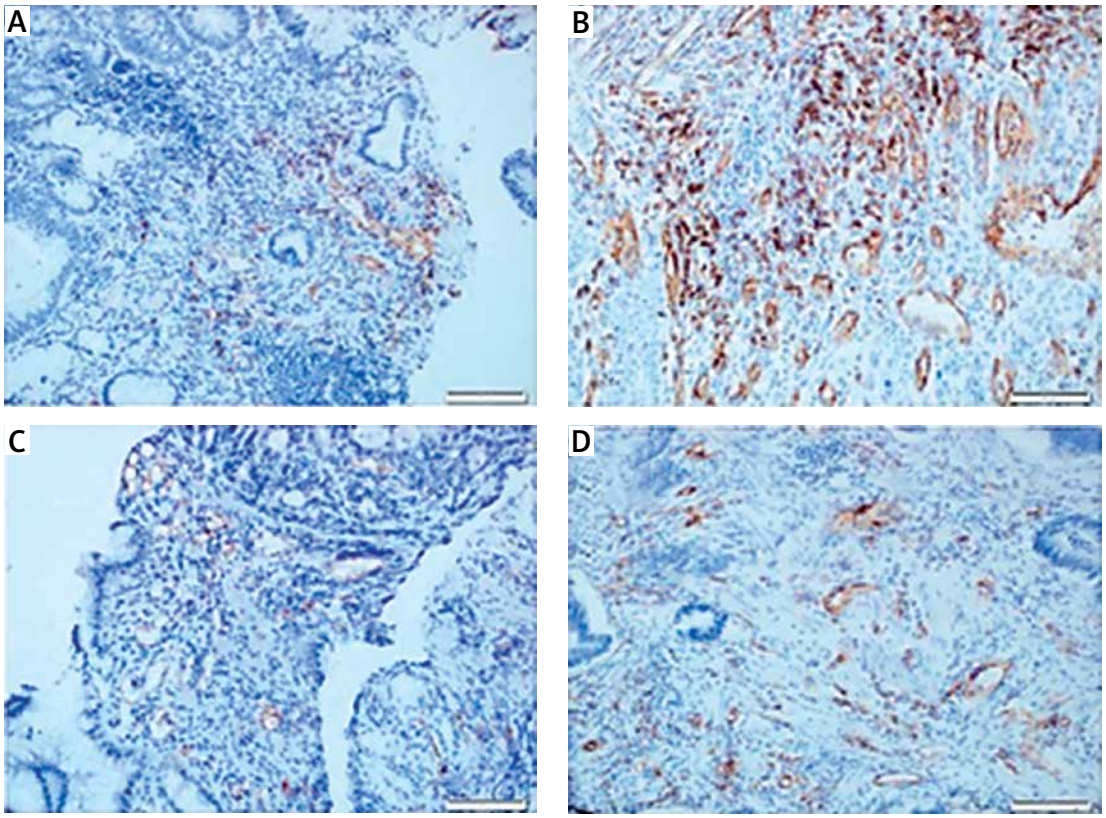

Figure 2. The endothelial cells within the gastric malignant tumor demonstrated immunohistochemical staining with antibodies against CD31 (brown-stained cells) (the most intensely stained samples are presented). Before standard nutrition (**A**), after standard nutrition (**B**), before immunonutrition (**C**), after immunonutrition (**D**)

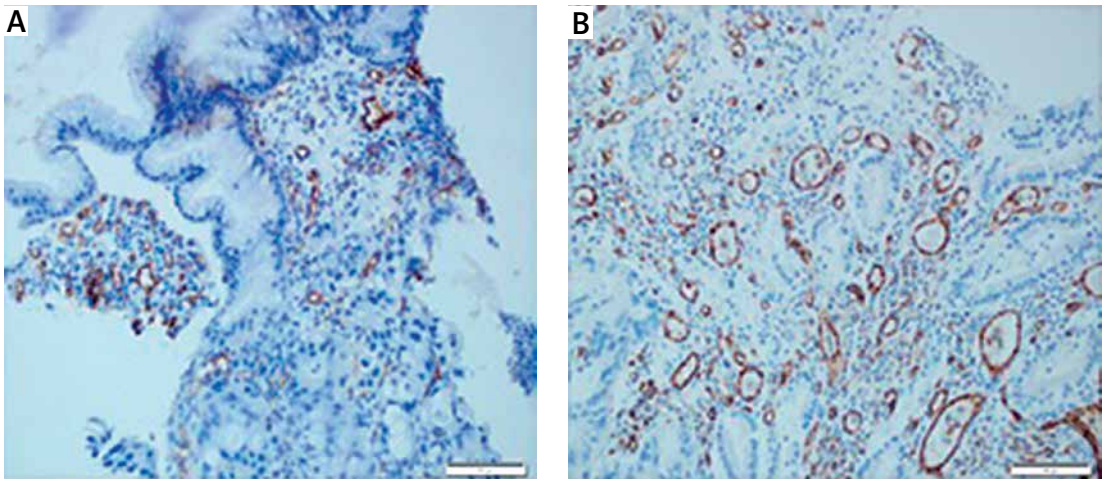

Figure 3. The endothelial cells within the gastric malignant tumor demonstrated immunohistochemical staining with antibodies against CD105 (brown-stained cells) (the most intensely stained samples are presented). After standard nutrition (**A**), after immunonutrition (**B**)

eas and ulcers and ulcer floor areas were excluded from the evaluation. For each immunohistochemically stained tissue, the tumor infiltrating lymphocytes and vessels were counted in 5 different areas under 400× magnification. Only the intraepithelial lymphocytes within the tumor area were accepted as tumor infiltrating lymphocytes. Since immunoreactivity was observed also in the tumor epithelium during the evaluation of CD4, only the cells in lymphocyte morphology were counted.

Statistical analysis

The statistical analyses were performed using the NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA) package. The data were evaluated using definitive statistical methods (mean, standard deviation, median, interquartile range) as well as the independent *t*-test to compare paired groups of variables showing a normal distribution, the Mann-Whitney *U* test to compare paired groups of variables not showing a normal distribution, and the χ^2 test to compare the qualitative data. The results were evaluated at the significance level of $p < 0.05$.

Results

No statistically significant differences were found between the standard nutrition and immunonutrition groups in terms of mean age, gender distribution, surgery type, differentiation distribution, T stage, N stage or TNM stages (Table I). No statistically significant differences were found between the standard nutrition and immunonutrition groups in terms of the numbers of removed lymph nodes and the numbers of metastatic lymph nodes (Table II). No statistically significant differences were found between the standard nutrition and immunonutrition groups in terms of endoscopic biopsy CD4, CD8, CD16, CD56, CD4/CD8, CD31 and CD105 values (Table III). No statistically significant differences were found between the standard nutrition and immunonutrition groups in terms of CD4, CD8, CD16, CD56 and CD31 values of the surgical pieces ($p > 0.05$).

The CD4/CD8 values of the surgical pieces were significantly higher in the standard nutrition group than in the immunonutrition group ($p = 0.0001$). The CD105 values of the surgical pieces were significantly lower in the standard nutrition group than in the immunonutrition group ($p = 0.01$) (Table IV).

Discussion

The guideline on enteral nutrition in surgery published by the European Society for Clinical Nutrition and Metabolism (ESPEN) in 2006 states that pre-operative use of oral nutrition support enhanced with immunomodulating agents reduc-

Table I. Demographics and stage*

Parameter		Immunonutrition		Standard nutrition		*P*-value
Age		57.92 ±10.35		59.12 ±13.32		0.846
Gender	Male	16	64.00%	12	48.00%	0.254
	Female	9	36.00%	13	52.00%	
Surgery	Distal	16	64.00%	17	68.00%	0.765
	Total	9	36.00%	8	32.00%	
Differentiation	G2	9	36.00%	8	32.00%	0.765
	G3	16	64.00%	17	68.00%	
T	T2	6	24.00%	11	44.00%	0.136
	T3	19	76.00%	14	56.00%	
N	N0	10	40.00%	10	40.00%	0.983
	N1	6	24.00%	5	20.00%	
	N2	6	24.00%	7	28.00%	
	N3a	3	12.00%	3	12.00%	
Stage	1B	3	12.00%	6	24.00%	0.503
	2A	9	36.00%	5	20.00%	
	2B	5	20.00%	8	32.00%	
	3A	5	20.00%	3	12.00%	
	3B	3	12.00%	3	12.00%	

**Independent t test – χ^2.*

Table II. Lymph nodes*

Parameter		Immunonutrition	Standard nutrition	*P*-value
Number of removed lymph nodes	Mean ± SD	24.84 ±5.68	24.96 ±5.62	0.907
	Median (IQR)	25 (19.5–29.5)	25 (19.5–29)	
Number of metastatic lymph nodes	Mean ± SD	3 ±3.71	2.32 ±3.41	0.446
	Median (IQR)	1 (0–6)	1 (0–4.5)	

**Mann-Whitney U test.*

Table III. Endoscopic biopsy results*

Variable		Immunonutrition	Standard nutrition	*P*-value
CD4	Mean ± SD	22.88 ±13.46	18.72 ±10.66	0.256
	Median (IQR)	25 (10–32.5)	17 (10.5–23.5)	
CD8	Mean ± SD	17.2 ±9.94	15.64 ±7.27	0.606
	Median (IQR)	15 (7–25)	15 (10–20.5)	
CD16	Mean ± SD	1 ±3.23	0.84 ±1.8	0.238
	Median (IQR)	0 (0–0)	0 (0–1)	
CD56	Mean ± SD	0.2 ±0.71	0.24 ±0.52	0.288
	Median (IQR)	0 (0–0)	0 (0–0)	
CD4/CD8	Mean ± SD	1.34 ±0.24	1.17 ±0.34	0.052
	Median (IQR)	1.25 (1.2–1.6)	1.1 (0.95–1.45)	
CD31	Mean ± SD	46.24 ±36.44	44.96 ±17.84	0.431
	Median (IQR)	40 (24.5–53)	45 (28–56)	
CD105	Mean ± SD	0.64 ±0.91	0.6 ±1.19	0.472
	Median (IQR)	0 (0–1)	0 (0–1)	

**Mann-Whitney U test.*

Table IV. Surgical piece results

Variable		Immunonutrition	Standard nutrition	*P*-value
CD4	Mean ± SD	35.8 ±21.59	37 ±19.2	0.778
	Median (IQR)	40 (12.5–50)	40 (20–52.5)	
CD8	Mean ± SD	62.8 ±36.17	45.4 ±26.14	0.064
	Median (IQR)	60 (40–72.5)	45 (20–65)	
CD16	Mean ± SD	8.4 ±20.8	2.2 ±4.8	0.181
	Median (IQR)	0 (0–5)	0 (0–0)	
CD56	Mean ± SD	0.28 ±1.06	0.28 ±1.02	0.682
	Median (IQR)	0 (0–0)	0 (0–0)	
CD4/CD8	Mean ± SD	0.56 ±0.23	0.84 ±0.22	0.0001
	Median (IQR)	0.6 (0.38–0.73)	0.8 (0.71–1)	
CD31	Mean ± SD	73.44 ±40.09	58.04 ±30.07	0.101
	Median (IQR)	66 (46.5–85)	47 (40.5–76)	
CD105	Mean ± SD	10.76 ±6.11	6.64 ±3.46	0.01
	Median (IQR)	9 (7–15.5)	7 (4–8.5)	

**Mann-Whitney U test.*

es post-operative morbidity and the hospitalization period after major abdominal cancer surgery. Therefore, the guideline recommends pre-operative use for 5–7 days regardless of nutritional risks in patients who will undergo major upper abdominal surgery [1]. On the other hand, nutritional support was only used for the energy need to prevent immune weakness and muscle destruction by supplying essential micronutrition and proteins, but today it is used for modulating immune functions [2–8]. Arginine, omega-3 fatty acids, glutamine, and nucleotides that have positive effects on immune functions have been studied extensively.

Presence of TILs in the tumor microenvironment is an indicator of the immune response of the host, and forms the basis of the cancer immunoediting [9–13]. Presence and increased numbers of lymphocytes are known to be directly associated with survival in many tumors [14]. But the prognostic role of tumor-infiltrating immune cells in patients with gastric cancer is largely unknown. Only a few reports have been issued on the association between tumor infiltrating immune cells and the clinical outcome in gastric cancer. Ishigami *et al.* [15] reported that patients showing a high level of natural killer cell infiltration in tumor tissues have a better prognosis, and Maehara *et al.* [16] showed that a high density of dendritic cell infiltration is associated with the absence of lymph node metastasis. On the other hand, the group of Fukuda [17] found no significant difference in survival between patients with marked or slight TIL infiltration. However, they detected TILs by immunostaining in gastric cancer patients, classified cases into groups with marked or slight TIL infiltration, and did not determine TIL numbers.

T-cell-mediated adaptive immunity is considered to play a major role in antitumor immunity. In mouse models, it has been demonstrated that adaptive immunity prevents the development of tumors and inhibits tumor progression [13].

Accordingly, recent data [18] showed that in gastric cancer high densities of immune cells related to adaptive immunity, especially cytotoxic T cells and memory T cells, are associated with favorable survival and indicate that adaptive immunity plays a role in the prevention of tumor progression.

The ratio of CD4/CD8 T cells has indeed been used as an indicator for evaluating an individual's immune function. Though some investigations have demonstrated an immunologic antitumor effect of CD4 and CD8 [19], the clinical significance of the CD4/CD8 ratio in tumor infiltrating lymphocytes and/or in peripheral blood as an indicator of progressive gastrointestinal tumor and/or worse prognosis of patients has been occasionally reported [19]. Diederichsen *et al.* [20] reported that a low CD4/CD8 ratio in tumor infiltrating lymphocytes is an independent prognostic indicator in patients with colorectal carcinoma. Decrease of the CD4/CD8 ratio is correlated with progressive behavior of the tumor indicated by such tumor-related factors as stage of the tumor, tumor invasion, lymph node metastasis, and size of the tumor in gastric cancer [21].

Another factor associated with survival and metastasis is tumor vascularity. The relationship between tumor vascularity and prognosis of a number of solid tumors is still being investigated. Proteins such as angiogenic cytokines, proteolytic enzymes, and migratory factors are considered to have an effective role in neovascularization. Endoglin (CD105) is one of these factors. It is a member of the transforming growth factor β (TGF-β) family, which is active in regulation of cellular activities such as proliferation, migration, production of extracellular matrix and hematopoiesis. Endoglin binds with TGF-β1 and TGF-β3 receptors with high affinity and induces angiogenesis by antagonizing the inhibitory effect of TGF-β1 on endothelial cell [22]. Recent studies have shown that there is a strong association between endoglin and angiogenesis and it is crucial in vascular diseases and tumor progression [23, 24]. Endothelial cells in tumor, inflammatory and regenerative tissues with active angiogenesis exhibit more intense CD105 staining than normal tissues. Additionally, its expression in vessels of preneoplastic lesions is less than in tumor vessels [23]. Pan-endothelial cell markers, such as CD31, CD34 and factor VIII, react with not only newly forming vessels but also normal vessels within tumor tissues. Anti-CD105 antibodies preferentially bind with activated endothelial cells in angiogenic tissues but do not stain or minimally stain endothelial cell in normal tissues. Thus, anti-CD105 antibody is superior to the other endothelial cell markers in evaluation of angiogenesis or angiogenic potential [22–26]. Minhajat *et al.* investigated organ-specific endoglin in human cancers' angiogenesis and reported that it is specifically expressed in lung, brain, liver, colon, breast and stomach cancers [27]. Wang *et al.* investigated the expression levels of sinusoidal endothelial cell antibodies (SE-1), CD31 and CD105 in rats with hepatocellular carcinoma and found that there is a strong association between all three markers and angiogenesis [28]. All these findings indicate that CD105 is a specific marker of angiogenesis and is superior to the pan-endothelial cell markers in evaluation of neovascularization. Saad *et al.* studied the prognostic value of endoglin and vascular endothelial growth factor in esophageal cancers and reported that CD105 is a specific and sensitive prognostic factor for esophageal can-

cers [29]. Tachezy *et al.* proposed that the ratio of CD105/CD31 is an index of angiogenesis and is a prognostic factor for intraductal papillary mucinous neoplasm [30]. However, microvascular intensity in cancers, either stained with pan-endothelial marker or CD105, is an indicator of poor prognosis [31–33]. Thus, CD105 and CD31 are used as indicators of neovascularity.

In conclusion, in our study, we found decreased CD4/CD8 ratios in the immunonutrition group compared to the group that received standard nutrition calculated based on the calorie requirement before surgery. This suggests that immunonutrition regulates the balance between Th1 and Th2, and may increase survival based on the other studies on this matter. However, as an interesting result, the CD105 amount was higher in the immunonutrition group than in the standard nutrition group. This led to the conclusion that, based on the studies with endoglin in which immunonutrition was used during the pre-operative period in a manner not compliant with the ESPEN recommendations, this may be associated with metastasis and shorter recurrence rates. These findings can be best interpreted when larger patient series and 5-year survival are investigated.

Conflict of interest

The authors declare no conflict of interest.

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