Clinical impact of tumor-infiltrating CD45RO$^+$ memory T cells on human gastric cancer

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Abstract. Memory T cells survive for months and even years and are critical for host defense in humans. They have been recently suggested to play a significant role in tumor immunity. In this study, we aimed to investigate the clinical impact of tumor-infiltrating memory T cells on human gastric cancer. We evaluated CD45RO$^+$ T cells infiltrating into primary gastric cancer tissues by immunohistochemistry in 101 patients with gastric cancer. Patients were classified into 2 groups (CD45RO^{+Hi} and CD45RO^{+Lo}) based on the number of positively stained T cells. There was no significant correlation observed between CD45RO status and post-operative prognosis in early gastric cancer. By contrast, in advanced cancer, the post-operative overall and disease-free survival of patients with CD45RO^{+Hi} were significantly improved compared to those of patients with CD45RO^{+Lo}. In addition, CD45RO status in the primary tumors significantly correlated with the development of post-operative recurrence, particularly peritoneal recurrence. Furthermore, the local expression of interferon-γ (IFN-γ) in the CD45RO^{+Hi} tumors was significantly higher than that in the CD45RO^{+Lo} tumors, suggesting that CD45RO$^+$ T cells induced local immune activation. Multivariate analysis indicated that the CD45RO$^+$ status was an independent prognostic factor in advanced gastric cancer. In conclusion, tumor-infiltrating CD45RO$^+$ memory T cells are functional and have significant prognostic value in human gastric cancer. Our data suggest that adaptive immune response is clinically critical in gastric cancer.

Introduction

Recent progress in the treatment of gastric cancer has had a significant impact on the survival and quality of life of patients. This includes the introduction of novel chemotherapeutic reagents and various therapeutic modalities in gastric cancer (1-4). Although complete cure can be expected in the majority of patients with early-stage disease, the prognosis of advanced gastric cancer remains poor. Therefore, the development of novel therapeutic approaches for advanced gastric cancer is required in order to improve patient prognosis.

Tumor immunology has been extensively investigated over the past decade, leading to the discovery of a number of approaches that may lead to novel anticancer treatments. Among them, tumor-infiltrating lymphocytes (TILs) have gained much attention in a variety of human cancers (5,6). T cells recognize cancer antigen and induce immune responses for the elimination of cancer cells. These lymphocytes that infiltrate tumors are defined as TILs (7,8). TILs include a variety of subpopulations of T cells (9-11). Originally, CD4$^+$ or CD8$^+$ TILs have been evaluated and suggested to play critical roles in tumor immunity (12-23). CD45RO$^+$ memory T cells have been further demonstrated to play specific and significant roles in a number of human cancers. CD45 is the leukocyte common antigen and functions as a tyrosine phosphatase in leukocyte signaling (24). The expression of different CD45 isoforms is cell-type specific and depends on the stage of differentiation and the state of activation of cells (25-28). CD45RO is one of the most suitable single markers for human memory T cells, that can finely represent the activation status of T cells (29).

Memory T cells are known to be generated during cell-mediated immune responses, and survive for months and even years after the antigen is eliminated (30-32). These memory T cells are responsible for more rapid and amplified responses to second and subsequent exposures to antigens. It is well known that they play critical roles in host defense against infection. Furthermore, recent studies have shown that they have significant prognostic value in several malignant tumors, including colorectal, gastric and esophageal cancer (33-38). However, the mechanisms by which the memory phenotype of T cells has actually influenced the clinical outcome ofz cancer patients are largely unknown. A previous study has shown that the densities of CD45RO$^+$ T cells, as well as those of CD3$^+$ or CD8$^+$ T cells are independent prognostic factors in gastric cancer (34). However, the precise clinical significance of those T cells was not elucicated.

In this study, we evaluated CD45RO$^+$ memory T cells infiltrating into gastric cancer tissues and further investigated

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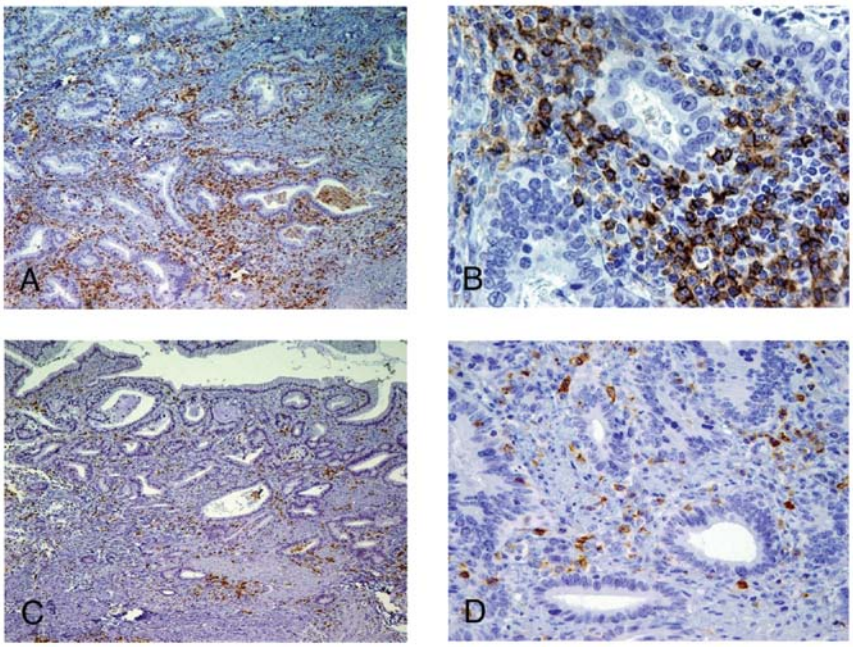

Figure 1. Immunohistochemical staining of human gastric cancer tissue for CD45RO$^+$ T cells. By immunohistochemistry, we evaluated CD45RO$^+$ T cells infiltrating into gastric cancer tissues. Representative case of (A) CD45RO^{+Hi}, original magnification, x100; (B) CD45RO^{+Hi}, x400; (C) CD45RO^{+Lo}, x100; and (D) CD45RO^{+Lo}, x400.

the pathological and clinical impact of these T cells on human gastric cancer in order to gain a deeper understanding of the mechanisms involved.

Materials and methods

Patients. We examined 101 patients with gastric cancer who underwent surgery at the Department of Surgery, Nara Medical University, Nara, Japan between 2000 and 2006. The median age of the patients was 65 years, with a range of 36-84 years. Twenty-seven patients classifed as T1 early-stage gastric cancer, while 74 patients were classifed as advanced-stage gastric cancer (T2 stage or greater). In addition, 57 patients had lymph node metastasis and 13 had distant metastasis. All the patients had received radical gastrectomy with lymph node dissection. None of the patients had received any pre-operative anticancer treatment, and the majority of patients with advanced cancer pathologically defined as stage III or IV had received adjuvant chemotherapy. Clinicopathological findings, such as gender, depth of tumor invasion, histological type, lymph node metastasis, lymphatic invasion, venous invasion and stage were reviewed according to the Japanese Classification of Gastric Carcinoma, 2nd English edition and the Tumor Node Metastasis (TNM) Classification of Malignant tumors, 6th edition.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were cut into 4-μm-thick sections, deparaffinized and rehydrated in a graded series of ethanols. After the sections were pre-treated with citrate buffer (pH 6.0) for 20 min at 110˚C in an autoclave, endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 10 min. The sections were then incubated for 30 min at 37˚C with anti-human CD45RO antibody (UHL1, monoclonal mouse; DakoCytomation, Kyoto, Japan). The labeled antigens were detected by a Dako envision system (DakoCytomation) and visualized by 3,3'-diaminobendizine tetrahydrochloride as the chromogen. Hematoxylin was used as for counter staining.

Evaluation of immunostaining. By immunohistochemistry, we evaluated CD45RO$^+$ T cells infiltrating into gastric cancer tissues and counted positively stained cells in each tissue under x400 magnification (5 fields analyzed per tissue; Fig. 1). We defined the average number as the cut-off value and classified CD45RO$^+$ status into 2 groups: a high density of tumor-infiltrating CD45RO$^+$ T cells into tumors (CD45RO^{+Hi}) and a low density of tumor-infiltrating CD45RO$^+$ T cells into tumors (CD45RO^{+Lo}).

Extraction of total RNA and real-time reverse-transcription PCR. Total RNA was isolated from the advanced-stage gastric cancer tissues using the guanidine isothiocyanate method (RNeasy Protect Mini kit; Qiagen, Tokyo, Japan) and was transcribed into cDNA using the cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed using the ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA). All primer/probe sets were purchased from PE Applied Biosystems. PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 1 μl of cDNA in a 20-μl final reaction volume. The PCR thermal cycle conditions were as follows: an initial step at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The expression level of the housekeeping gene, β2-microglobulin, was measured as an internal reference with a standard curve to determine the integrity of the template RNA for all specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β2-microglobulin).

Table I. Association between clinicopathological features and CD45RO$^+$ status for the 74 patients with advanced gastric cancer.

Features	Total	CD45RO^{+Hi}	CD45RO^{+Lo}	P-value
Gender				0.60
Male	54	26	28	
Female	20	11	9	
Age (years)				0.59
Median (range)	67 (31-84)	68 (31-79)	65 (46-84)	
Histological type				0.48
Differentiated	33	18	15	
Undifferentiated	41	19	22	
Primary tumor				0.03
T2	53	31	22	
T3	17	6	11	
T4	4	0	4	
Regional lymph nodes				0.18
N0	24	13	11	
N1	29	17	12	
N2	14	6	8	
N3	7	1	6	
Distant metastasis				0.36
M0	61	32	29	
M1	13	5	8	
Lymphatic invasion				0.06
Positive	55	24	31	
Negative	19	13	6	
Venous invasion				0.77
Positive	15	7	8	
Negative	59	30	29	
UICC stage				0.17
I	16	10	6	
II	25	14	11	
III	15	8	7	
IV	18	5	13	

CD45RO^{+Hi}, high density of tumor-infiltrating CD45RO$^+$ T cells into tumors; CD45RO^{+Lo}, low density of tumor-infiltrating CD45RO$^+$ T cells into tumors; UICC, Union for International Cancer Control.

Statistical analysis. The cancer-specific survival time was calculated from the date of surgery to the date of death from gastric cancer. The experimental data were analyzed by the χ^2 test, the Mann-Whitney U test and the Student's t-test. Survival curves were estimated using the Kaplan-Meier method, and the differences between the curves were evaluated by the log-rank test. Multivariate analysis was carried out using the Cox regression model to evaluate 4 factors [tumor status (T factor), nodal status (N factor), metastatic status (M factor) and CD45RO$^+$ status.] Differences at $P<0.05$ were considered to indicate statistical significance.

Results

CD45RO$^+$ memory T cell expression in gastric cancer. The expression patterns of CD45RO^{+Hi} and CD45RO^{+Lo} in gastric cancer are shown in Fig. 1. We evaluated CD45RO$^+$ T cells infiltrating into gastric cancer tissues and counted the positively stained cells in each tissue. The average number of positive cells was 133. This number was then defined as the cut-off value. As a result, we determined that 48 patients were CD45RO^{+Hi} and 53 were CD45RO^{+Lo}.

Correlation between CD45RO$^+$ status and clinicopathological characteristics. We analyzed the correlations between CD45RO$^+$ status and various clinicopathological factors. There were no significant correlations observed between CD45RO$^+$ status and clinicopathological factors in the 101 patients with all stages of gastric cancer. However, there was a significant correlation observed between CD45RO$^+$ status and the T factor in 74 patients with advanced gastric cancer (Table I, P=0.03). In more advanced tumors, i.e., T3 and T4 stage, significantly

Table II. Multivariate survival analysis for prognostic significance of pathologic features and CD45RO$^+$ status in advanced gastric cancer.

Prognostic features	HR	95% CI	P-value
CD45RO$^+$ status			0.04
CD45RO^{+Hi}	1.0	Referent	
CD45RO^{+Lo}	2.17	1.04 to 4.52	
Primary tumor			0.002
T2a	1.0	Referent	
T2b	2.76	0.89 to 8.49	
T3	2.48	0.76 to 8.05	
T4	17.36	3.93 to 76.76	
Regional lymph nodes			NS
Negative	1.0	Referent	
Positive	1.93	0.45 to 5.36	
Distant metastasis			0.001
Absence	1.0	Referent	
Presence	3.56	1.64 to 7.75	

HR, hazard ratio, CI, confidence interval; CD45RO^{+Hi}, high density of tumor-infiltrating CD45RO$^+$ T cells into tumors; CD45RO^{+Lo}, low density of tumor-infiltrating CD45RO$^+$ T cells into tumors; NS, not significant.

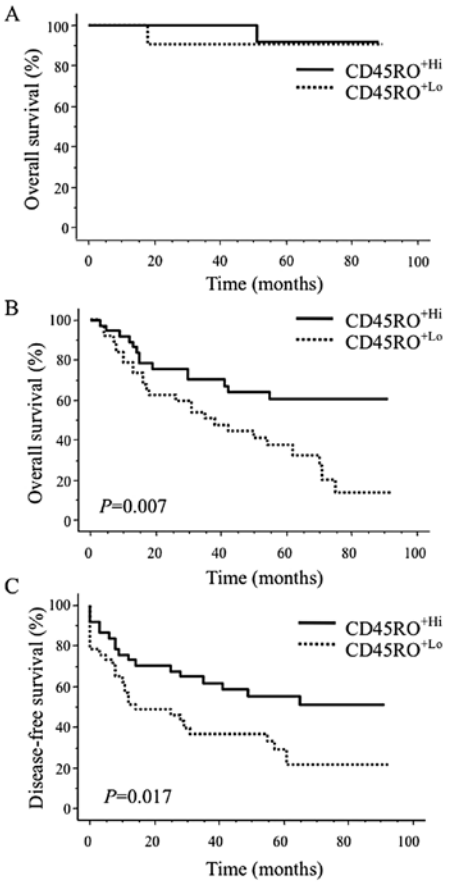

Figure 2. Impact of tumor-infiltrating CD45RO$^+$ T cells on post-operative prognosis. (A) There was no difference observed in post-operative overall survival in early-stage gastric cancer. (B and C) There was a significant difference observed in post-operative overall survival (P=0.0072, CD45RO^{+Hi} vs. CD45RO^{+Lo}) and disease-free survival (P=0.0174) in the 74 patients with advanced gastric cancer.

fewer CD45RO$^+$ T cells were observed. The other factors did not significantly correlate with CD45RO$^+$ status.

Impact of CD45RO$^+$ status on post-operative prognosis. There was no significant difference between CD45RO$^+$ status in the 27 patients with early-stage gastric cancer as regards post-operative overall survival (Fig. 2A). On the other hand, there was a significant difference in post-operative overall survival (CD45RO^{+Hi} vs. CD45RO^{+Lo}; P=0.007l; Fig. 2B) and disease-free survival (CD45RO^{+Hi} vs. CD45RO^{+Lo}; P=0.02; Fig. 2C) in the 74 patients with advanced gastric cancer. Furthermore, multivariate survival analysis with Cox's proportional hazards model indicated that CD45RO$^+$ status, as well as the T factor and M factor, were independent prognostic factors in advanced gastric cancer (Table II; P=0.04).

Influence of tumor-infiltrating CD45RO$^+$ T cells on post-operative recurrence. We further analyzed the influence of CD45RO$^+$ T cells infiltrating primary cancer tissue on post-operative recurrence. The number of CD45RO$^+$ T cells in primary tumors was significantly lower in the cases that developed recurrence compared to the cases with no recurrence (P=0.02) (Fig. 3). By the analysis of each recurrence pattern, the difference was significant, particularly as regards peritoneal recurrence (P=0.01) (Fig. 3). However, no significant differences were observed in lymph node and hepatic recurrence.

Correlation between CD45RO$^+$ T cells and local immune status in primary gastric tumors. Finally, we examined the local immune status in advanced gastric cancer tissues by the quantification of interferon-γ (IFN-γ), granzyme B, perforin and forkhead box P3 (Foxp3) related to the adaptive immune response using real-time PCR analysis. As a result, there were no statistically significant differences observed between CD45RO$^+$ T cell status and the mRNA expression of granzyme B, perforin and Foxp3. On the other hand, the mRNA expression of IFN-γ in CD45RO^{+Hi} tumors was significantly higher than that in CD45RO^{+Lo} tumors (P=0.02) (Fig. 4).

Discussion

We and others have reported that the presence of CD45RO$^+$ memory T cells in tumors is an independent prognostic factor in a number of human malignancies, including colorectal, gastric and esophageal cancer (33,34,37). CD45RO is the most suitable single marker for the memory T cell population in humans (29). These cells include both CD4$^+$ and CD8$^+$ lymphocytes that have been exposed to antigen (32,39-41). Lee *et al* (34) previously reported that the densities of CD45RO$^+$, as well as those of CD3$^+$ and CD8$^+$ TILs are independent prognostic factors in gastric cancer. Our study corroborated their findings. On the other hand, as regards the correlation between the clinicopathological characteristics and the status of CD45RO$^+$ TILs, our results were considerably different from those presented in the

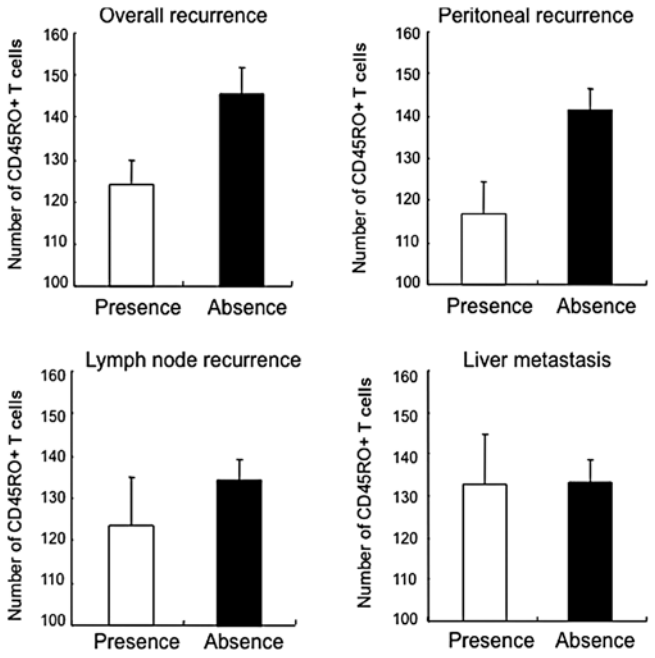

Figure 3. Correlation between CD45RO$^+$ status in primary tumors and post-toperative recurrence. The number of CD45RO$^+$ T cells in the primary tumors of patients who did not develop recurrence was significantly greater than that in the tumors of patients who developed recurrence (P=0.02). Furthermore, the difference was significant, particularly as regards peritoneal recurrence (P=0.01). By contrast, no significant differences were observed in lymph node and hepatic recurrence.

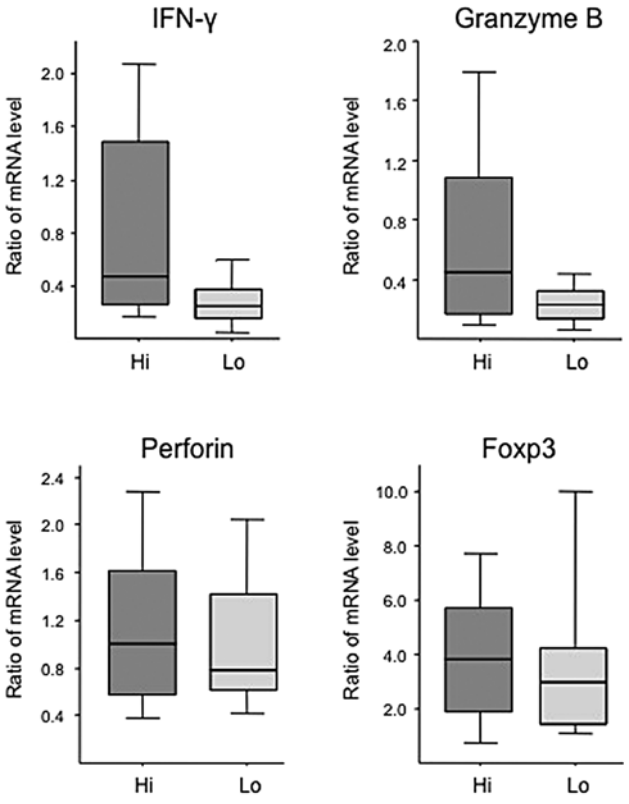

Figure 4. Local immune status in advanced gastric cancer tissues quantified using real-time PCR. Although there were no significant correlations observed between CD45RO$^+$ status and the local expression of granzyme B and perforin, IFN-γ expression in CD45RO^{+Hi} tumors was significantly higher than that in CD45RO^{+Lo} tumors (P=0.02). Furthermore, there was no difference observed in Foxp3 expression between CD45RO^{+Hi} and CD45RO^{+Lo} tumors.

study by Lee *et al* (34). In our study, CD45RO status significantly correlated with the T factor in advanced gastric cancer. By contrast, Lee *et al* reported that CD45RO was a significant predictor of lymph node metastasis. Although the reasons for the differences between our studies remain unknown, a larger-scale study is required for a more definitive conclusion to be reached.

In order to gain a deeper understanding of the functions of CD45RO$^+$ memory T cells in gastric cancer, we further analyzed the correlations between CD45RO$^+$ T cells infiltrating primary gastric cancer tissues and post-operative recurrence and local immune activation. Of note, there was a significant correlation observed between post-operative recurrence and the status of CD45RO$^+$ memory T cells in the primary tumor tissues. Our results suggest that memory T cells in primary tumors may play a role in controling the development of recurrence. In particular, peritoneal recurrence was significantly associated with the number of memory T cells. Taken together, these dasta suggest that memory T cells residing in the primary tumor may inhibit tumor growth and invasion at the primary tumor site, thereby resulting in the prevention of peritoneal dissemination.

Our additional analysis of the local immune status indicated that IFN-γ expression in the CD45RO^{+Hi} tumors was significantly higher than that in the CD45RO^{+Lo} tumors, while there was no difference in Foxp3 expression between the 2 groups. These findings indicate a few important facts relating to memory T cells in human gastric cancer. First, CD45RO$^+$ memory T cells are functional and play critical roles in the prevention of tumor progression and recurrence. These T cells with the property of effector cells may produce IFN-γ, leading to local immune activation. Second, since there was no significant difference in Foxp3 expression between the 2 groups, these memory T cells may not, either directly or indirectly, be related to regulatory or suppressive function. However, further integral studies on the acquired immune mechanisms in gastric cancer are warranted.

Immunotherapy has been long expected to become a powerful anticancer treatment that is tumor-specific and less toxic (42). This type of therapy includes cancer vaccines, adoptive cell therapy and monoclonal antibody-based treatment. Based on our data, as well as data from previous reports, it is evident that any type of anticancer treatment should not prevent tumor-specific memory T cells in cancer patients (33,34,36). Furthermore, if the intentional induction or maintenance of memory T cells became possible, such a novel strategy could lead to a breakthrough in cancer treatment. However, to date, only a few studies have evaluated dynamic of memory T cells before and after any specific anticancer therapy. From a series of our previous studies on T cell-negative and regulatory pathways in several types of cancer, a promising strategy may be targeting T cell-negative pathways, including cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) (43-45). In fact, a recent large-scale randomized clinical trial demonstrated the significant impact of immunotherapy using anti-human CTLA-4 monoclonal antibody on patient overall survival in metastatic melanoma (46). Other treatments

including cytokine therapy and cancer vaccine should be also evaluated from the point of view of memory T cells.

In conclusion, the results from the present study demonstrate that tumor-infiltrating $CD45RO^+$ memory T cells have significant prognostic value in human gastric cancer, and suggest that adaptive immune response is functionally critical in human gastric cancer. Our data may prove useful for the development of novel therapeutic approaches for gastric cancer.

Acknowledgements

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