Clinical Implications of CD4+CD25+Foxp3+Regulatory T Cell Frequencies After CHP-MAGE-A4 Cancer Vaccination

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Abstract. Background/Aim: The aim of this study was to explore whether the treatment effect or immune response to a cancer vaccine can be predicted by the percentage of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in peripheral blood mononuclear cells (PBMCs) after vaccination. Patients and Methods: Sixteen patients (9 men, 7 women; median age 61.5 years) enrolled in the CHP-MAGE-A4 cancer vaccine clinical trial who had a fixed dose (300 μg of CHP-MAGE-A4 cancer vaccine and 0.5 Klinische Einheit (KE) of OK432 and received at least four vaccinations were investigated. Safety, immune response, and clinical effects were assessed before and after the cancer vaccination. Results: Treg ratios that remained low both before and after vaccination were associated with a good prognosis, and a low Treg/CD4 lymphocyte ratio 7-weeks after the initial vaccination was correlated with a better prognosis. Conclusion: The Treg ratio following vaccination appears to have some utility for predicting patient prognosis.

Immunotherapy is a novel therapeutic approach for refractory malignant tumors and is expected to become a standard method of adjuvant therapy after radical surgical resection. Modern immunotherapy began with non-specific immunotherapy that elicits inflammation, such as Coley’s vaccine, and has evolved since the discovery of tumor-specific antigen by Thierry Boon. The emergence of new immunotherapy with treatment effects through the induction of immune responses against specific antigens is anticipated. These include: active immunotherapy, such as cancer vaccine therapy that uses peptides or proteins that elicit specific immune responses; and passive immunotherapy, such as genetically-modified T cell receptor therapy that targets p53 or melanoma antigen gene (MAGE)-A4, as well as chimeric antigen receptor (CAR) T cell therapy that targets GD2 in neuroblastoma (1-7).

However, while the efficacy of immunotherapy has been demonstrated, there has been criticism that the effects observed at the basic experimental level are not fully reproduced in clinical studies. Several reasons have been postulated for this. First, the issue with cancer vaccine therapy itself is that, unlike preventive vaccination for an infection, the patient already possesses the target antigen before cancer vaccination and has been exposed to the antigen long-term. For this reason, T cells that are normally responsive to antigens may be in an unresponsive state (8, 9). Second, regarding the patient, the presence of an immunosuppressive mechanism is a factor. Regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), cancer-associated fibroblasts (CAFs), and inhibitory cytokines such as IL-6, IL-10, and TGF-β have been shown to impair anti-tumor immune responses (10). To overcome such processes, attempts have been made to develop various immune adjuvants and drug delivery systems or use neo-antigens, and improvements in treatment effects have been reported with each modality (11-15).

We previously conducted a cancer vaccine clinical trial and have searched for a biomarker that predicts treatment effect in order to introduce immunotherapy to selected patients who show favorable treatment effects and immune responses. Specifically, we examined the antigen-specific antibody response and reported that elevation in the Th2-mediated antibody titer may be associated with disease progression, and that the antigen spreading pattern may reflect the treatment-induced reduction in tumor size (16, 17).
The focus of the present study was on Tregs, a type of immunosuppressive cell, and whether it is possible to predict the treatment effect or immune response by measuring the percentage of CD4+ CD25+ Foxp3+ Tregs in peripheral blood mononuclear cells (PBMCs) after cancer vaccination.

Patients and Methods

Study design. This study investigated 16 patients who were enrolled in the CHP-MAGE-A4 cancer vaccine clinical trial, a phase I and II clinical trial evaluating the safety, immune response, and clinical effects of CHP-MAGE-A4 cancer vaccine therapy. The clinical trial is registered on the University Hospital Medical Information Network-Clinical Trials Registry (UMIN-CTR). This clinical study (UMIN ID: 000001999) was approved by the Human Ethics Committees of Hokkaido University (March 2009), and written, informed consent was obtained from all patients before treatment. All patients underwent CHP-MAGE-A4 cancer vaccine therapy at Hokkaido University Hospital between 2009 and 2012, and the treatment protocol is presented on UMIN-CTR (https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000002369). The inclusion and exclusion criteria of enrolled patients were described in detail in previous reports (16, 17).

Vaccine protocol and sample collection. A total of 24 patients were registered in phases I and II of this clinical trial. Of these patients, 16 patients who had a fixed dose (300 μg of CHP-MAGE-A4 cancer vaccine and 0.5 Klinische Einheit (KE) of OK432) and were able to receive at least four vaccinations were selected. The basic CHP-MAGE-A4 cancer vaccination schedule entailed a total of six vaccinations given at two-week intervals. Safety, immune response, and clinical effects were assessed four weeks after the final vaccination. Additional vaccinations were subsequently given based on the patient’s wishes.

Immune responses during vaccination were evaluated using peripheral blood samples collected from patients at baseline and at each vaccination time point. PBMCs were collected using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), washed twice with RPMI1640 media containing glutamic acid, penicillin, and streptomycin, and cryopreserved in CELL BANKER (serum containing cryopreservation medium, Nihon Seiyaku Kogyo, Co., Ltd) at −130°C until further use. The Treg assay in the present study used PBMC samples collected before vaccination and 7, 11, and 15 weeks after the initial vaccination (Figure 1).

The CHP-MAGE-A4 cancer vaccine is a conjugate of 300 μg of MAGE-A4 recombinant protein and 3.6 mg of cholesteryl hydropullulan (CHP) with 0.5 Klinische Einheit (KE) of OK-432 (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) added as an immunologic adjuvant. These were kindly provided by the Department of Immuno-Gene Therapy of Mie University. The method of vaccine preparation was described in detail in a previous report (17).

Evaluation of safety and clinical response. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE v3.0). Computed tomography (CT) was performed before and after vaccination to assess the clinical effects. Every measurable region was evaluated by the modified Response Evaluation Criteria In Solid Tumors (mRECIST) (18).

Antibody staining of PBMCs. After thawing the frozen PBMC samples for 1 min at 37°C, the samples were immediately transferred to 10 ml of Stain Buffer (2% fetal bovine serum (FBS) + phosphate-buffered saline (PBS)) and centrifuged at 1500 RPM for 5 min at 20°C to remove DMSO. After resuspending the cell pellet in 10 ml of Stain Buffer, the cells were stained with 0.4% Trypan Blue solution and then quantified using light microscopy, centrifuged (1200 RPM for 10 min at 20°C), and resuspended in Stain Buffer to a concentration of 5x10⁶ cells/ml. Antibody staining was performed following the manufacturer’s instructions (BD Biosciences Pharmingen, San Diego, CA, USA), and the following experiments were conducted at room temperature. To each test tube (12×75 mm, 5 ml Round Bottom Polystyrene Test Tubes) containing 50,000 cells, cell surface markers CD4-FITC, CD25-PE-Cy5 (BD), and CD3-PE (eBioscience, San Diego, CA, USA) and each isotype antibody were added and incubated for 20 min in the dark. Samples were washed with Stain Buffer, centrifuged (1,500 RPM for 10 min at 20°C), and decanted. Next, for cell fixation, 1 ml of Human Foxp3 Buffer A (BD) was added to the cell suspension, incubated for 10 min in the dark, washed, centrifuged (3,000 RPM for 5 min at 20°C), resuspended in 0.5 ml Human Foxp3 Buffer B (BD) diluted with Human Foxp3 Buffer A for membrane permeabilization, and incubated for 30 min in the dark. Following incubation, 2 ml of Stain Buffer was added, and the samples were centrifuged at 3,000 RPM for 5 min at 20°C. The supernatant was removed, and the cells were washed with Stain Buffer. Next, human Foxp3-PE (Clone: 259D/C7, BD) or PE mouse IgG1 κ isotype control was added, and the cell suspension was incubated for 30 min in the dark. After washing, the cells were resuspended in 0.6 ml of Stain Buffer and subsequently analyzed using flow cytometry.

Flow cytometric analysis of PBMCs. All samples were analyzed using a FACSCaliburTM (Becton Dickinson, San Jose, CA, USA) and CellQuest software (Becton Dickinson). CD4+ CD25+ Foxp3+ T lymphocytes were examined to analyze the prevalence of Tregs. Negative controls were plotted, and the forward scatter (FSC) and side scatter (SSC) voltages were adjusted such that the lymphocyte population could be viewed. A CD3-PE single stain control was used to define the lymphocyte population. The CD4+ lymphocyte population was determined using a CD4-FITC single stain control, and the quadrant boundaries were defined by plotting the double stain controls using CD4-FITC, Foxp3-PE, and CD25-PE-Cy5. Color voltage and compensation settings were set based on the single stain control, and the same settings and conditions were used for all samples. A total of 10,000 events of CD4+ lymphocytes were acquired, the CD4+ cells were gated, the CD25+ and Foxp3+ cells were quantified, and the Treg ratio was expressed as a percentage of all CD4+ cells. Data analysis was performed using the FACSCaliburTM flow cytometer and CellQuest software.

MAGE-A4 humoral immune responses. MAGE-A4 antigen-specific immune responses were determined by ELISA in patient serum samples. Detailed materials and methods for this assay have been described previously (16, 17). Patients with 1) a MAGE-A4-specific IgG antibody titer at or below baseline before vaccination that increased after starting the vaccination, or 2) a high antibody titer at baseline that increased at least two-fold after vaccination were considered to be those with an MAGE-A4 antigen-specific humoral immune response.
Statistical analysis. The data obtained are expressed as medians and standard deviation, and p<0.05 was considered significant. The survival of all patients was analyzed from the first day of vaccination to the day the patient died, and survival analysis by group was conducted using the log-rank test. Treg ratios were compared between patients and healthy donors at each time point with box-and-whisker plots and analyzed statistically with the Mann-Whitney U-test. All analyses were performed with StatView statistical software (version 5.0; SAS Institute Inc., Cary, NC, USA).

Results

Patients’ characteristics. Twenty-four patients were enrolled in the CHP MAGE-A4 cancer vaccine clinical trial. Sixteen of these patients who received CHP-MAGE-A4 (300 μg) and OK-432 (0.5 KE) at least four times were analyzed in the present study. The characteristics of the 16 patients are summarized in Table I.

The 16 patients consisted of nine with colorectal cancer, three with breast cancer, and four with other types of cancer (bile duct cancer, gallbladder cancer, pancreatic cancer, and mesothelioma). There were 9 men and 7 women, and their median age was 61.5 (range=34-79 years) years. All 16 patients received the vaccine at least four times, and 12 patients completed six vaccinations. The median number of vaccinations was six (range=4-12 vaccinations). Vaccination could be continued for as long as the patient desired until the condition exacerbated. Grade 3 or greater CTCAE adverse events were not observed. The most frequent side effect was Grade 1 redness of the skin that developed in all patients at the injection site; however, this resolved on its own without special treatment.

The clinical effect evaluated according to mRECIST criteria after four vaccinations was stable disease in 7 patients and PD in 9 patients. There were no cases of complete response or partial response (Table I). The median survival of all patients was 4.8 (range=1.9-17.0 months) months. The survival curve for all patients is shown in Figure 2.

Transitions over time of CD4⁺CD25⁺Foxp3⁺ Tregs. PBMC samples collected from 16 patients were analyzed using flow cytometry. Figure 3 shows a typical example of gating. The left figure shows the lymphocytes, and the center figure shows the CD4⁺ gate area in the lymphocytes. As shown in the right figure, the CD25⁺ and Foxp3⁺ area was defined as the CD4⁺CD25⁺Foxp3⁺ Treg area. The percentage of Tregs in CD4⁺ lymphocytes as measured and designated the Treg ratio.

The median Treg ratio in all 16 patients before vaccination was 10.28%, which was significantly greater than the ratio in the nine healthy donors (6.6%). At other measured time points (7, 11, and 15 weeks after starting the vaccination), the median Treg ratios were 10.21%, 8.43%, and 7.74%, respectively, and they were significantly greater than the ratios in the healthy donors (Figure 4A).
The association between the Treg ratio and patient survival. To investigate whether the Treg ratio could become a prognostic predictor in treated patients, a survival analysis was conducted at each time point (pre-vaccination, and 7, 11, and 15 weeks after vaccination) by dividing all 16 patients into a high or low Treg group based on the Treg ratio. The cut-off value for dividing the high and low groups was set at 10.28%, which was the median Treg ratio before the initial vaccination. A significant difference in prognostic was not observed between the two groups based on the Treg ratio before vaccination (Figure 5A; log-rank test, \( p=0.318 \)). At the 7-week time point, the 16 patients were divided equally into each group, and survival was significantly longer in the low group (Figure 5B; log-rank test, \( p=0.020 \)). Median survival was 8.5 months in the low group and 3.2 months in the high group.

There were no differences in prognostic between the two groups at the 11-week time point (Figure 5C; 12 patients total, with 7 patients in low group and 5 patients in the high group), or at the 15-week time point (Figure 5D; 9 patients total, with 8 patients in the low group and 1 patient in the high group).

For further examination, the Treg ratios before vaccination and 7 weeks after vaccination were compared in each patient (Table II). The results showed that one patient (Case 5) was in the high group before vaccination but converted to the low group at the 7-week time point, and, conversely, another patient (Case 8) converted from the low to the high group from pre-vaccination to 7 weeks. Aside from these 2 patients, 7 patients (Cases 1, 3, 6, 10, 11, 13, and 14) were in the low group both before vaccination and 7 weeks after vaccination, and the remaining 7 patients (Cases 2, 4, 7, 9, 12, 15, and 16) were in the high group at both time points.

Case 5, who converted from the high group to the low group, survived 470 days, while Case 8, who converted from the low group to the high group, survived 101 days; the median survival of all patients was 144.5 days. Patients who were in the low group from pre-vaccination to the 7-week time point included Cases 1, 3, 6, 10, 11, 13, and 14; and they survived for 96, 148, 469, 509, 141, 255, and 257 days, respectively. Patients who remained in the high group included Cases 2, 4, 7, 9, 12, 15, and 16, and they survived for 88, 88, 56, 69, 317, 105, and 167 days, respectively. The patients that remained in the high group had a significantly shorter survival (Mann-Whitney U-test, \( p=0.047 \)).

Association between the CD4+CD25+Foxp3+ Treg ratio and MAGE-A4-specific humoral immune responses. We previously examined the MAGE-A4-specific humoral immune response in patients before and after they received the CHP-MAGE-A4 cancer vaccine, and we reported whether an immune response was induced by the sixth vaccination (16, 17). Among the 16 patients in the present study, 10 showed induction of a MAGE-A4-specific immune response (IgG). These 10 patients included 8 patients who had no increase in antibody titer before vaccination but displayed an increase in antibody titer with vaccination (Cases 1, 3, 7, 10, 11, 12, 14, and 16) and 2 patients who were positive for the antibody titer from baseline and showed a two-fold or greater increase with vaccination (Cases 6 and 9) (Table II). To examine the correlation between the ability of the cancer vaccine to induce specific immunity and the Treg ratio, the patients were divided into two groups based on the presence or absence of MAGE-A4-specific IgG elevation, and the Treg ratios were compared between the groups. No significant differences were observed between the two groups at any time point before vaccination and 7, 11, and 15 weeks after vaccination (Figure 6; Mann-Whitney U-test, \( p=0.515, 0.386, 0.089, \) and 0.770).

Discussion

With the goal of developing a method for immunological monitoring of patients who have received the CHP-MAGE-A4 cancer vaccination, the present study was designed to analyze whether measuring the percentage of CD4+CD25+Foxp3+...
Tregs within the CD4+ lymphocyte population of peripheral blood samples is useful in predicting the treatment effect and prognosis. Methods for immunological monitoring of cancer vaccine therapy that have been previously shown to be useful include IgG ELISA using serum samples (antigen-specific humoral immunity evaluation), intracellular cytokine analysis and the ELISPOT assay using PBMCs (cell-mediated immunity evaluation) (19-21).

We have also previously reported antigen-specific responses in patient serum samples using ELISA (16, 17), and we found that antigen-specific responses were observed in more than half of the patients. Nonetheless, anticipated clinical effects could not be attained, and the acquisition of an antigen-specific humoral immune response was not correlated with clinical effects.

To identify potential biomarkers, we also conducted a comprehensive investigation of antigen spreading. Of the 12

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**Figure 3. Differentiation of regulatory T cells by flow cytometry.** Peripheral blood mononuclear cells were stained with anti-CD4-FITC, anti-CD25-PE-Cy5, and anti-Foxp3-PE for three-color flow cytometric analysis. The lymphocyte population was gated (left panel), and further gated for the anti-CD4-antibody-positive population (center panel). The CD4+ lymphocyte population extracted following this process is plotted (right panel), and the cell population positive for anti-CD25 antibody and anti-Foxp3 antibody was defined as CD4+CD25+Foxp3+ regulatory T cells (Tregs). Ten thousand CD4+ lymphocyte events were acquired, and Tregs were quantified and expressed as a percentage of all CD4+ lymphocytes (Treg ratio).

**Table II. Detailed profiles of patients.**

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Pre: Pre-vaccination; 7w: seven weeks after the first vaccination; H: high Treg ratio group; L: low Treg ratio group.
patients who received the MAGE-A4 cancer vaccine, eight showed responses to anti-MAGE-A4 antibody. Furthermore, antibody responses to NY-ESO-1 and MAGEA3 were observed in 6 patients and 5 patients, respectively. Antigen spreading was frequently observed, and immune responses other than the response against the administered antigen were also induced after vaccination. However, neither the antigen-specific immune response nor antigen spreading was useful as a surrogate marker for determining the prognosis (16). We therefore decided to conduct an exploratory study of the Treg ratio in PBMCs, which a limited number of reports have demonstrated that they are associated with the prognosis of cancer vaccine treatment.

Figure 4. Treg ratios before and after administration of vaccine and comparison with healthy donors. A: HD indicates healthy donor, Pre-Tx indicates before vaccination, and 7w, 11w, and 15w indicate 7 weeks, 11 weeks, and 15 weeks after starting the vaccination, respectively. The Treg ratio is significantly greater in patients than in healthy donors at baseline and at all time points after starting the vaccination (Mann-Whitney U-test, p=0.001, 0.001, 0.019, 0.047). B: The Treg ratio is significantly lower at the 15-week time point compared to baseline (Mann-Whitney U-test, p=0.027). C: Changes in the Treg ratios of the nine representative patients whose data could be collected up to the 15-week time point are shown. The Treg ratio decreased in some patients, but increased in others. There are also those whose Treg ratio declined first, but increased later. These findings indicate the absence of a common and specific trend.
Tregs, originally identified in a pathogenic mechanistic study of autoimmune diseases, are an essential cell group in the maintenance of immunological self-tolerance (22). Their roles in cancer immunity have been previously elucidated in Treg-deletion mouse models, and they are widely known to suppress tumor-responsive T cells, as well as antitumor immunity (23-25). In humans, it has also been shown that CD4+CD25+Foxp3+ Tregs are increased in the peripheral blood and in tumor-infiltrating lymphocytes of many types of cancer (26-29), and that a greater ratio of Foxp3+ Tregs to CD4+ or CD8+ T cells is associated with a poor prognosis and tumor progression in patients with advanced cancer (30-32).

In the present study, the Treg ratio before vaccination was significantly greater in the 16 patients who received the cancer vaccination than in healthy donors. Because the patients in this clinical trial had advanced cancer and were resistant to standard therapy, the result that the Treg ratio was greater in these patients than in healthy donors even before vaccination does not contradict previous reports that demonstrated a correlation between elevated Treg ratios and cancer progression (33-35).

A significantly greater Treg ratio compared to healthy donors was also observed at 7, 11, and 15 weeks after the initial vaccination. Nonetheless, the median Treg ratio tended to decrease with a repeated vaccination at 7, 11, and 15 weeks.

Figure 5. Relationships between the Treg ratios before and after vaccination and survival. Patients were divided into the low Treg group and the high Treg group at each time point to analyze survival. The median Treg ratio of 10.28% before vaccination (baseline) was used as the cut-off value. A Pre-Tx indicates before treatment, while B 7w, C 11w, and D 15w indicate 7 weeks, 11 weeks, and 15 weeks after starting the vaccination. Kaplan-Meier survival curves at each time point are shown with patients divided into two groups based on the Treg ratio at each time point. Survival is significantly longer in the low Treg group than in the high Treg group at 7 weeks after starting the vaccination (Log-rank test, p=0.020).
weeks, with a significant decrease at the 15-week time point compared to pre-vaccination (Figure 4B). At 11 weeks, the sample size decreased from 16 to 12, as 4 patients dropped out from data collection due to disease progression or death (Cases 4, 7, 8, and 9, with survival of 88, 56, 101, and 69 days, respectively). At 15 weeks, the sample size decreased further to 9 patients as 3 patients dropped out for similar reasons (Cases 1, 2, and 15, with survival of 96, 88, and 105 days, respectively). In patients who continued the vaccination and were available for data collection without dropping out, the Treg ratio may have been maintained at a relatively low level (Figure 4C). Moreover, in the comparison of the high- and low-Treg ratio groups, divided at the cut-off value of 10.28% (median at baseline), patients who remained in the low group from pre-vaccination to 7 weeks had a significantly longer survival than those who remained in the high group. These data suggest that the maintenance of the Treg ratio at a low level during vaccination is associated with a better prognosis. Similar findings have been demonstrated in past clinical trials. Hansen et al. measured the Treg ratio in CD4 lymphocytes using PBMC samples from 33 patients in the telomerase peptide vaccine trial to examine the association between this ratio and survival. This study demonstrated an extension in overall survival with the vaccine in immune responders with low Treg ratios, and it suggested a mechanism in which this phenomenon is associated with a cytokine profile favorable for antitumor immunity, namely high INFγ/IL4 and INFγ/IL-10 ratios (30). In addition, since it had been indicated that the effects of vaccination could potentially be predicted by the pre-vaccination Treg ratio, Yoshitake et al. conducted a comprehensive examination of PBMCs from patients in the

Figure 6. Relationship between the Treg ratio and MAGE-A4-specific humoral immune responses. Patients were divided into those with or without an elevation in MAGE-A4-specific IgG to compare Treg ratios. Significant differences in the Treg ratios are not observed between the two groups at baseline or at any of the time points after starting the vaccination. P indicates patients with an increase in MAGE-A4-specific IgG, and N indicates patients without this increase.
phase II clinical trial for a peptide vaccine. They found that patients with a high Treg ratio before treatment had a poor prognosis, and reasoned that the induction of antigen-specific cytotoxic T lymphocytes (CTLs) may be difficult to induce in patients with a high Treg ratio (21). Murahashi et al. conducted a phase I study combining a cancer vaccine and cyclophosphamide in 18 patients and demonstrated that the patients with a lower number of Tregs at baseline and thereafter had a better prognosis (36).

There are two known types of Tregs: the naive type, where the cells circulate in the lymphatic tissues such as the lymph nodes; and the inducible type, where the cells become activated after receiving antigen stimuli and become mobilized to tissues (22). Although the mechanisms of how Tregs suppress the functions of effector T cells and cytotoxic T cells are not completely understood, several pathways via inhibitory cytokines such as IL10 and TGF-β, and via co-stimulatory factor CTLA4 have been postulated. CTLA4 is an inhibitory factor expressed by activated T cells, but it is constitutively expressed on Tregs (37). It has become clear that anti-tumor immunity increases with in vivo treatment with anti-CTLA4 antibody, which has been used clinically in recent years as an immune checkpoint inhibitor (38, 39). To increase the efficacy of cancer vaccine therapy, it is necessary not only to induce an antigen-specific immune response, but also to take an approach that focuses on the Treg ratio, suggesting that combined treatment with these immune checkpoint inhibitors and cancer vaccine therapy may be effective. It was postulated that the findings of the present study could facilitate the development of such novel treatment strategies.

As this study was limited by its small sample size and the unbalanced patient characteristics, it will be necessary to validate these as well as the effector factors in future studies. In summary, the present investigation showed that maintenance of the Treg ratio at a low level both before and after cancer vaccination is associated with a good prognosis, and that a low Treg/CD4 lymphocyte ratio at the 7-week time point after vaccination compared to baseline (median of 10.28%) in cancer patients is correlated with a better prognosis.

References


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