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—Original Article—

Evaluation of the immune status of peripheral blood monocytes from dairy cows during the periparturition period

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Abstract. Calving is a critical but stressful event required for milk production in dairy cows. In the present study, we investigated the immune status of peripheral blood mononuclear cells (PBMCs) isolated from periparturient cows to better understand and, thus, possibly prevent stress during the periparturient period. To evaluate the immune response of PBMCs, we assessed their proliferation with or without a mitogen (concanavalin A, ConA). Blood samples were collected 24 h before and after calving and 1 week after calving. The proliferation of non-treated cells remained unchanged throughout the examination period. The immune response of PBMCs isolated from the cows before calving was relatively low, even after ConA stimulation; however, the immune response of PBMCs collected at both time points after calving was significantly higher than those of non-stimulated controls. Next, we examined the expression patterns of T cell related and inflammatory cytokine genes in PBMCs. We found that the mRNA expression levels of both *CD4* and *CD8* showed decreasing trends after calving. The expression of the Th1 cell marker gene *IFNG* also decreased after calving. The mRNA expression level of the inflammatory cytokine gene *TNFA* increased after parturition. Overall, our results suggest that the PBMC immune response was weakened in cows before delivery and part of the expression of the immune cell-related genes in these cells is altered 24 h before and after calving.

Key words: Cow, Immune response, Parturition, Peripheral blood mononuclear cells

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The periparturient period is critical for dairy cows because they are sensitive to reduced immune competence, negative energy balance, systemic inflammatory response, and oxidative stress during this period [1]. Periparturient cows face various types of stresses. These diverse stresses can have harmful and complex effects on the immune system [1]; it is, therefore, very important that the physical condition of periparturient cows be evaluated and managed during this period. A comprehensive evaluation of the immune status of periparturient cows is required to effectively protect them from the effects of such stresses. Periparturient cows are characterized by inflammatory-like conditions [2]. Uncontrolled oxidative stress leads to immune dysfunction and inflammatory response in the animal, which result in increased incidence of infectious diseases [3, 4]. The results of previous studies suggest that the immune system in periparturient cows is suppressed before (for 1 to 2 weeks) and after (for 2 to 3 weeks) calving [5, 6]. In addition, the ability of lymphocytes to respond to mitogens and produce antibodies is impaired around the parturition period in cows [7, 8].

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The above-mentioned alterations to the immune status have been associated with several diseases, such as mammary infections, retained placenta, mastitis, and metritis [1, 5, 9]. The inflammatory conditions may contribute to low dry matter intake before and after calving, and the performance of cows, including their reproductive efficiency, is impaired [10, 11]. A comprehensive understanding of the immune status of cows will, therefore, help to prevent or ameliorate diseases and thus prevent production losses.

Several studies have suggested that the many stresses prevalent during the periparturient period increase the susceptibility of dairy cows to postpartum metabolic diseases [4, 12, 13]. In general, the transition period, i.e., 3 weeks before and after calving, is the most critical stage for dairy cows [14, 15]. Undoubtedly, parturition itself is a stress factor; however, changes in the immune status of peripheral blood mononuclear cells (PBMCs) in dairy cows just before and after calving have not been studied in detail. In the present study, we examined the immune status of cows during the periparturient period by assessing alterations in the blastogenic activity of PBMCs, i.e., the lymphocytes that play major roles in immune responses, 24 h before and after calving and 1 week after calving. In addition, to understand the immune activity of parturient cows, we examined the expression patterns of T cell- and inflammatory cytokine- related genes in PBMCs during the same period.

Material and Methods

Animals and blood sampling

All the study procedures were conducted in accordance with the Hokkaido University guidelines for the care and use of animals (approval No. 16-0019). Holstein cows (age: 3–7 years, n = 12) were used as study animals and maintained at the Hokkaido University farm. The selected cows were clinically assessed for diseases before and during the experimental period, and no diseases were detected. Blood samples were collected from the cows 24 h before and after calving and 1 week after calving through the external jugular vein using sterile vacuum tubes that contained heparin (Terumo, Tokyo, Japan).

Preparation of PBMCs

PBMC preparation was conducted on the same day as sampling. PBMCs were isolated from the whole-blood samples by density gradient centrifugation on Lymphocyte Separation Medium 1077 (TaKaRa Bio, Shiga, Japan), according the manufacturer's instructions. Whole-blood samples (10 ml) were diluted with an equal volume of phosphate-buffered saline (PBS), carefully layered onto the Lymphocyte Separation Medium 1077 and centrifuged at 450 × *g* for 40 min at room temperature (23 ± 2°C). Thereafter, PBMCs were collected, and erythrocytes were lysed in NH_4Cl-base lysis buffer. The PBMCs were then washed twice with PBS and suspended at a concentration of 1×10^5 cells/ml in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan). The separated cells were used for proliferation assays and RNA extraction.

Proliferation assays for PBMCs

The separated PBMCs were seeded into a 96-well plate (1×10^4 cells/well) in RPMI-1640 medium supplemented with 5% fetal bovine serum and antibiotic-antimycotic solution (Thermo Fisher Scientific, Yokohama, Japan) with or without the mitogen concanavalin A (ConA, 5 μg/ml) and cultured for 72 h under a humidified atmosphere of 5% CO_2 at 38.5°C. Cell proliferation assays were conducted using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. The PBMCs were cultured as described above. After culturing, the cells were treated with 20 μl of RPMI-1640 medium with CCK-8 reagent (1:10, v/v) and then incubated for 1.5 h. To estimate the number of proliferated cells, absorbance was measured for each well at a wavelength of 450 nm using an auto-microplate reader (ARVO X; PerkinElmer Japan, Kanagawa, Japan). The stimulated index (SI) was calculated as the ratio of the average absorbance value of wells containing mitogen (ConA)-stimulated cells to that of wells containing non-stimulated cells. All assays were performed in triplicate.

RNA extraction and analysis

Total RNA was extracted from the separated PBMCs on the same day as sampling using ISOGEN II (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. For quantitative real-time PCR (qPCR) analysis, the isolated total RNA (total, 500 ng) was first reverse-transcribed to cDNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's instructions; the resulting cDNA was stored at –30°C until further use. Then, the cDNA was diluted (1:5, v/v) in deionized distilled water, and 1 μl of the solution was used for each amplification reaction. The relative expression levels of the target genes were determined through qPCR using a LightCycler® 480 System II (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD™ SYBR® qPCR Mix (Toyobo); the final concentration of each primer (Table 1) was 0.5 μM. The thermal cycling conditions were as follows: 1 cycle at 95°C for 30 sec, followed by 50 cycles at 95°C for 10 sec, 60°C for 15 sec, and 72°C for 30 sec. The relative mRNA abundance was calculated on the basis of the geometric mean of the expression levels of bovine *ACTB*, *GAPDH*, and *H2AFZ*, which were used as the reference genes. Each run was completed with a melting curve analysis to confirm the specificity of the amplification and absence of primer dimer formation. All qPCR experiments were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines [16].

Statistical analyses

The results were expressed as mean ± standard error of the mean (SEM) values. The data were analyzed using repeated-measures analysis of variance (ANOVA) and Bonferroni test, using StatView statistical analysis software (Abacus Concepts, Berkeley, CA, USA). The differences with P-values < 0.0166 [0.05/3 (number of considerations) = 0.0166] were considered statistically significant, and those with P-values < 0.033 [0.1/3 (number of considerations) = 0.033] were considered to indicate a tendency.

Results

Proliferation and immune response of PBMCs

To evaluate the immune response (blastogenic response) of PBMCs, we performed a cell proliferation assay in the presence or absence of the mitogen ConA. The proliferation of non-stimulated control cells did not change throughout the examined period (Fig. 1A). The blastogenic response of PBMCs after ConA stimulation remained low before calving and significantly increased immediately after ($P < 0.0166$) and 1 week after calving when compared with that of the non-stimulated controls ($P < 0.0033$, Fig. 1B).

T cell marker gene expression in PBMCs

We conducted qPCR analyses to examine the expression of T cell marker genes in PBMCs during the same period (Fig. 2). The expression of *CD4* mRNA, which express in helper T cells, tended to be lower after parturition than just before parturition ($P = 0.0317$). Its expression in 1 week after parturition did not change when compared with the other day points. The expression level of *CD8* mRNA, which is expressed in cytotoxic T cells, was significantly decreased immediately after ($P < 0.0033$) and 1 week after ($P < 0.0166$) parturition when compared with that before parturition. The expression ratio of these mRNA (CD4/CD8) did not change throughout the examined period.

Expression of inflammatory cytokine genes in PBMCs

We examined the expression levels of Th1- (*Interferon gamma*, *IFNG*) and Th2- (*interleukin* (*IL) 4, IL10*) related genes in PBMCs during the same period (Fig. 3). The expression level of *IFNG* was

Table 1. Primers for real time PCR

Name (GenBank accession No.)	Sequence (5'-3')	Product length (bp)	References
For T cell marker genes			
CD4 (NM_001103225)	F: AGCAGAAAGTGAAACTCGTGG R: ACCAACTTCGGCTGATTTGAG	88	[34]
CD8 (NM_174015)	F: CGCAGACTAGGTCGGTCTCT R: GTTCCGGCGGTAGCAGAT	176	[35]
For Th1- and Th2- related genes			
IFNG (FJ263670)	F: TTGAATGGCAGCTCTGAGAAAC R: TCTCTTCCGCTTTCTGAGGTTAGA	150	
IL4 (EU276069)	F: TTGGAATTGAGCTTAGGCGTAT R: CCAAGAGGTCTTTCAGCGTACT	186	[36]
IL10 (NM_174088)	F: ATGCGAGCACCCTGTCTGAC R: TGCAGTTGGTCCTTCATTGAAAG	124	[37]
For inflammatory cytokine genes			
IL1B (EU276067)	F: AAACAGATGAAGAGCTGCATCCAA R: CAAAGCTCATGCAGAACACCACTT	394	[38]
IL2 (NM_180997)	F: ACATTTGACTTTTACGTGCCCAAG R: AATGAGAGGCACTTAGTGATC	307	[39]
IL6 (EU276071)	F: TAAGCGCATGGTCGACAAAA R: TTGAACCCAGATTGGAAGCAT	150	
TNFA (NM_173966)	F: TGACGGGCTTTACCTCATCT R: TGATGGCAGACAGGATGTTG	137	[40]
For internal control			
ACTB (AY141970)	F: TGGACTTCGAGCAGGAGATG R: GTAGAGGTCCTTGCGGATGT	222	
GAPDH (NM_001034034)	F: CACCCTCAAGATTGTCAGCA R: GGTCATAAGTCCCTCCACGA	103	
H2AFZ (NM_174809)	F: AGAGCCGGTTTGCAGTTCCCG R: TACTCCAGGATGGCTGCGCTGT	116	

F: Forward, R: Reverse.

significantly decreased immediately after parturition when compared with its expression before parturition ($P < 0.0166$). Its expression 1 week after parturition did not change significantly compared with the other time points. The expression levels of *IL4* and *IL10* tended to increase 1 week after parturition when compared with their expression levels before parturition ($P = 0.0238$).

Expression of inflammatory cytokine genes in PBMCs

We examined the expression patterns of inflammatory cytokine genes in PBMCs during the same period (Fig. 4). The expression levels of *IL1B*, *IL2*, and *IL6* did not change throughout the examined period. The expression level of tumor necrosis factor alpha (*TNFA*) did not change immediately after parturition, but it was significantly increased 1 week after parturition when compared with the level immediately after parturition ($P < 0.0033$).

Discussion

We performed a cell proliferation assay to evaluate the immune response of PBMCs. This assay has been widely used to assess

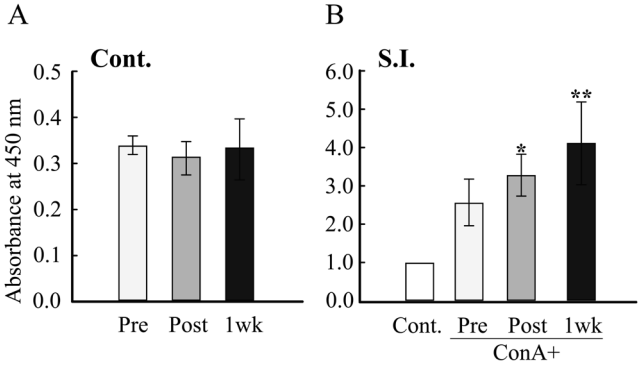


Fig. 1. Measurement of (A) proliferation of peripheral blood monocytes (PBMCs) isolated from cows 24 h before (Pre), 24 h after (Post), and 1 week (1 wk) after calving under non-stimulated conditions, and (B) immune responses in the presence (ConA+) or absence (Cont.) of the mitogen concanavalin A (ConA). SI: stimulation index (absorbance from stimulated wells/absorbance from non-stimulated wells; the value of non-stimulated cells in each group considered Cont. = 1.0). Asterisks indicate significant differences in absorbance levels: * $P < 0.0166$; ** $P < 0.0033$.

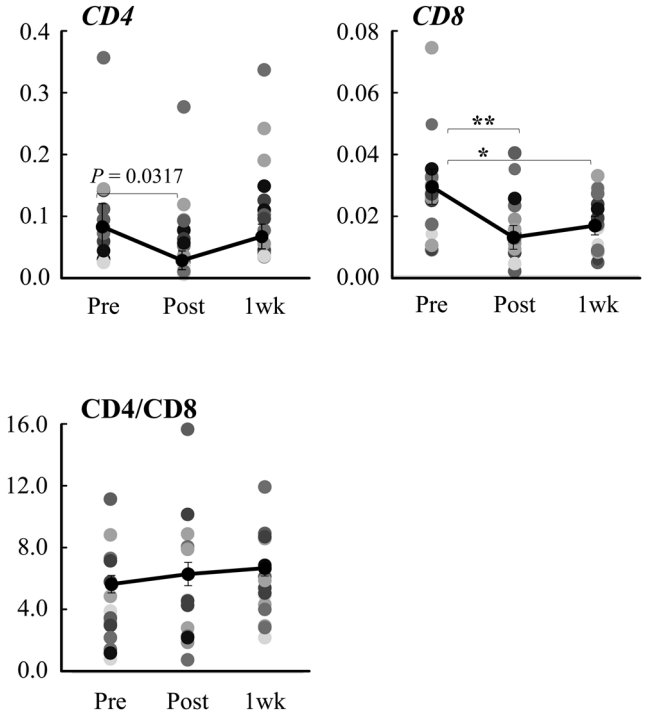


Fig. 2. Expression of T cell marker genes (*CD4* and *CD8*) in peripheral blood monocytes (PBMCs) isolated from cows 24 h before (Pre), 24 h after (Post), and 1 week after (1 wk) calving analyzed using quantitative real-time polymerase chain reaction. The values represent the expression levels relative to those of three internal control genes (i.e., geometric means of *ACTB*, *GAPDH*, and *H2AFZ*). The data are presented as mean ± standard error of the mean (SEM). Each dot represents an individual data point. The asterisk (*) indicates a significant difference ($P < 0.0166$) in the expression levels.

cellular immunity and it revealed the mitogen-induced blastogenic response of PBMCs [17]. Under non-stimulated conditions, cell proliferation did not change throughout the examined period. The blastogenic response of PBMCs isolated from cows immediately before calving was not significantly induced, even after mitogen stimulation. However, ConA stimulation significantly induced the blastogenic response of the PBMCs isolated from the cows after calving. This result may reflect the fact that immunocompetence was weakened in cows just before delivery, and it was gradually recovered after parturition. This finding is consistent with that of a previous study [7], in which cows were reported to be immunosuppressed before calving. However, some aspects of the present study differed from those of the previous study. Kehrli *et al*. [7] reported that the immunosuppressed state of cows lasted for 1 week after calving. A possible reason for this contradictory result is the difference in study animals used. Kehrli *et al*. [7] used heifers and steers, and we used multiparous cows. The first calving event may have been highly stressful for the heifers. With respect to animals, multiparous cows would reflect the general field data. However, in this study, we focused only on the periods just before and after calving. Further examination is necessary to extend the period by 1 or more weeks.

ConA is a T cell-specific mitogen [17–19]; thus, we examined whether the proportion of T cells (helper T cells and cytotoxic T cells) in the PBMCs changed. The expression level of *CD4* mRNA tended to be low, and *CD8* mRNA was significantly lower in the PBMCs 24 h after calving than before calving. No significant differences were observed in the ratio of these expression levels. Mehrzed *et al*. [20] reported that the imbalance of *CD4* and *CD8* (increased levels of CD4 and decreased levels of CD8) is involved in the immune response of lymphocytes. In this study, however, CD4 and CD8 showed the same expression patterns. Thus, the low blastogenic response of PBMCs during the periparturition period may have been caused by a decrease in the total T cell number rather than by change in the T cell populations.

The ratio of Th1 to Th2 cells is generally believed to play an important role in immune functions [21, 22]. Inflammatory cytokines also seem to play a central role in immune functions [23]. In this study, expression of the Th1-related gene, *IFNG*, decreased after calving, whereas that of the Th2-related genes, *IL4* and *IL10*, tended to increase after calving. As a result, the Th1/Th2 (IL4/IFNG) ratio tended to decrease, but it was not significant. We also examined the expression patterns of the genes that encode the inflammatory cytokines *IL1B*, *IL2*, *IL6*, and *TNFA*. The relationship between stress and these inflammatory cytokines has been thoroughly investigated in several mammals [24–30]. In this study, only the expression of *TNFA* mRNA significantly increased 1 week after calving. TNFA levels have also been shown to increase in response to stress in humans [26], mice [27], and rats [29]. Despite the differences in species and types of stresses used in these previous studies, our results were consistent with those reported, at least for *TNFA*. Therefore, the expression pattern of the inflammatory cytokine *TNFA* may be an indicator that could be effectively used to assess the stress conditions in cows during the periparturient period.

In the present study, we examined the immune status of PBMC samples collected from cows 24 h before and after calving and 1 week after calving. The immune response and gene expression of PBMCs suggest changes in their immune state just before calving. One of the causes for these changes may have been the changes in hormone concentrations, namely, progesterone, estradiol, and cortisol, which accompany parturition. Progesterone has been reported to inhibit many leukocyte functions [22, 31]. However, it is an important factor in the immune system and is unlikely to have caused these changes at calving because its concentration in the plasma decreases at parturition [7]. In contrast, estrogens have a suppressive effect on cell-mediated immunity and glucocorticoids have long been used as powerful immunosuppressive agents [32]. Miyaura and Iwata reported that progesterone and glucocorticoids inhibit Th1 development and enhance Th2 development in mice [22]. Therefore, increased levels of estrogen and fetal cortisol prior to parturition [33] may be involved in causing the change in the immune status observed at calving.

In conclusion, our results show that the immune response of cows was weakened before delivery, and part of the expression of immune cell-related genes could be altered before and after calving. Further studies are needed to better understand the changes in the immune status of periparturient cows and their causes; these findings would be helpful in detecting and preventing health risks of cows and reducing production losses.

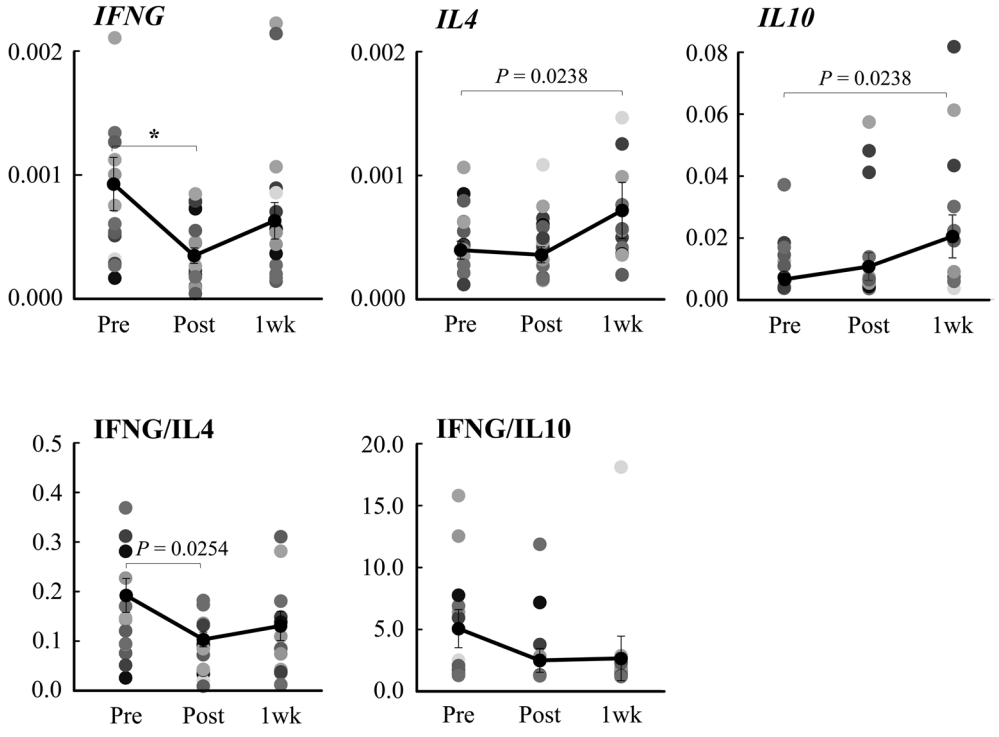
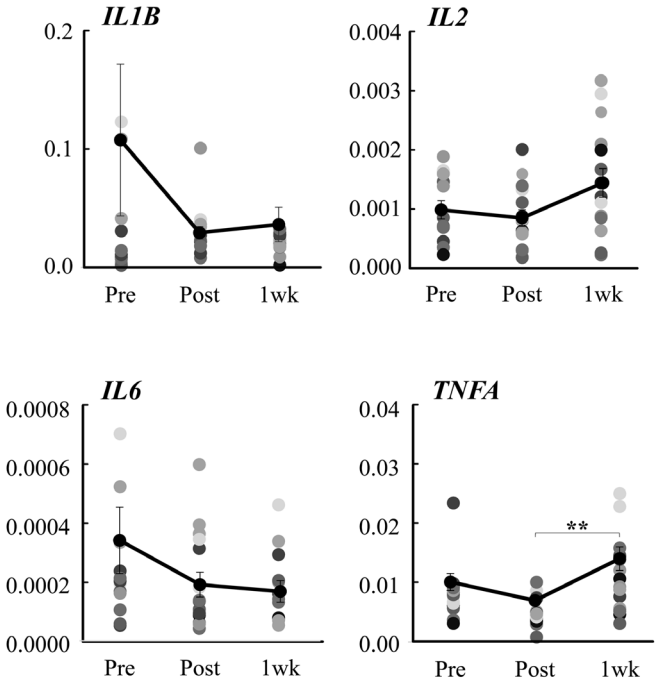


Fig. 3. Expression levels of Th1- (*IFNG*) and Th2- (*IL4* and *IL10*) related genes in peripheral blood monocytes (PBMCs) analyzed using quantitative real-time polymerase chain reaction. The values represent the mRNA expression levels relative to those of three internal control genes (i.e., geometric means of *ACTB*, *GAPDH*, and *H2AFZ*). The data are presented as mean ± SEM. Each dot represents an individual data point. The asterisk (*) indicates a significant difference ($P < 0.0166$) in the expression levels.



Declaration of conflict of interests: None.

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Fig. 4. Expression levels of inflammatory cytokine genes (*IL1B*, *IL2*, *IL6*, and *TNFA*) in peripheral blood monocytes (PBMCs) analyzed using quantitative real-time polymerase chain reaction. The values represent the mRNA expression levels relative to those of three internal control genes (i.e., geometric means of *ACTB*, *GAPDH*, and *H2AFZ*). The data are presented as mean ± SEM. Each dot represents an individual data point. The asterisk (*) indicates a significant difference ($P < 0.0166$) in the expression levels.

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