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C-X-C chemokine receptor type 4 and cytokine expressions in cows of a dairy herd with high prevalence of calves persistently infected with bovine viral diarrhea virus

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Abstract

Animals persistently infected (PI) with bovine viral diarrhea virus (BVDV) play an important role in the spread of BVDV. Alteration of maternal C-X-C chemokine receptor type 4 (CXCR4) expression has been suspected as closely concerned with the production of PI calves. It is not clear what the influence of CXCR4 response to the prevalence of PI calves. We have previously reported a dairy herd with high prevalence of PI calves within a short period having a single origin of infection. CXCR4 and cytokine expressions in cows of this herd were investigated. There were no significant differences in CXCR4 and cytokine expressions between the dams of PI calves and the dams of non PI calves in the herd. In the comparison among the herds, CXCR4 expressions in the PI producing herds were significantly lower than the BVDV-free herd. Moreover, CXCR4 expressions in the high prevalence herd and the low prevalence herd were similar. These findings among herds corresponded with the previously reported experimental production of persistent infection with BVDV in cows. Based on the cytokine profile of these herds, IL-10 was significantly higher in the high prevalence herd and the BVDV-free herd. The combination of low expression of CXCR4 and high expression of IL-10 might be closely concerned with some bias for the production of PI calves.

Key Words: BVDV, CXCR4, cytokine, PI

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Introduction

Bovine viral diarrhea virus (BVDV) is an economically important viral pathogen in cattle that is widespread throughout the world. BVDV is a single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family, genus *Pestivirus*⁹⁾. BVDV can infect cattle of all ages, with consequences of infection in immunocompetent cattle ranging from subclinical or mild disease to a highly fatal form⁴⁾. Infection of naïve pregnant cows with noncytopathic (ncp) BVDV may result in transplacental infection of the fetus. Adult immunocompetent pregnant cows seroconvert and clear the virus. But the outcome of the infection of the fetus depends on the stage of gestation at which the infection occurred. Infection of the fetus with ncp BVDV after about 120 days of gestation may result in transient infection of the fetus. When the developing fetus is infected with ncp BVDV before about 150 days of gestation, the infection may result in the development of immunotolerance to the infecting BVDV strain and persistent infection^{4,8,12,22,23,32)}. There has been a predominance of studies showing that the prevalence of persistently infected (PI) animals ranged from 0.5% to 2.0% under the uncontrolled conditions^{12,13,14)}.

Mechanisms of establishment of persistent infection in fetuses infected before the development of a competent immune system are not clearly identified. The immunological response to the infection in the pregnant cow could affect the transplacental transmission and persistent infection³⁰⁾. C-X-C chemokine receptor type 4 (CXCR4) expression was previously reported as concerned with BVDV infection^{30,33)}. Based on a genome-wide microarray analysis of BVDV experimentally infected heifers, CXCR4 was significantly down-regulated in the dams of PI fetuses³⁰⁾. On the other hand, in an *in vitro* study³³⁾, BVDV induced up-regulation of CXCR4 in naïve peripheral blood mononuclear cells following culture with serum from pregnant cows infected with ncp BVDV. The down-regulation of

maternal blood cell CXCR4 that occurred *in vivo* may require specific immune responses that are difficult to replicate *in vitro*³³⁾. The importance of chemokines in viral persistence, pathogenesis and infection of immune cell subsets has been clearly demonstrated for some members of the *Flaviviridae* other than BVDV such as Dengue virus and Hepatitis C virus^{5,7,11,15,16,20)}. Also in natural infection with BVDV, CXCR4 is suspected as closely concerned with the production of PI animals.

The cytokines, also, as mediators of the immune response to the infection had been involved in the production of BVDV PI fetuses. It was reported that interference with type I interferon (IFN) and associated pathway for evasion of the innate immune response was related to the mechanism of BVDV persistent infection^{3,30)}. Moreover, stimulation of a biased T helper 2 (Th2) cytokines response in pregnant cows may be of advantage to the virus in establishing persistent infection with BVDV²⁵⁾. The involvement of cytokines in the transplacental infection was previously reported in many pathogens as toxoplasmosis, leishmaniasis and neosporosis^{1,14,17,19,21,31)}.

Alteration of maternal CXCR4 expression has been suspected as closely concerned with the mechanism of production of BVDV PI fetuses as reported in previous experimental studies^{30,33)}. In the present study, we investigated the involvement of maternal CXCR4 and cytokine expressions in the natural occurrence of BVDV PI calves. We have previously reported a dairy herd with high prevalence of PI calves within a short period having a single origin of infection. CXCR4 and cytokine expressions were investigated in the cows of this herd and the suspected causes of high prevalence of PI calves were discussed.

Materials and Methods

Animals and herds: A dairy herd with high prevalence of BVDV PI animals estimated 7.0%¹⁰⁾

was used in the present study. The herd included approximately 50 milking dams and 40 heifers and calves. Nine BVDV PI animals included a milking cow and 8 newborn calves were diagnosed in the herd during 15 months of surveillance having a single origin of infection. Other two dairy herds were used for comparison. One herd with low prevalence of PI calves included approximately 34 milking cows and 18 heifers and calves. One PI was detected then surveillance continued for 9 months and no more PI calves were detected. Prevalence of PI animals in this herd estimated 1.5%. Another herd was BVDV-free herd included approximately 27 milking cows and 27 heifers and calves. No PI animals were detected in this herd after repeated BVDV examinations. All cows in this herd had been vaccinated against BVDV.

Samples, total RNA extraction and cDNA synthesis: Blood with anticoagulant was collected from 26 cows in the high prevalence herd including 6 dams of PI calves, 34 cows in the low prevalence herd and 25 cows in the BVDV-free herd. These samples included different periods of pregnancy and fetuses were not diagnosed at the sampling time. RNA was extracted from leukocytes using QIAamp RNeasy Mini Kit (Qiagen Inc., Tokyo, Japan) according to the manufacturer's instructions. The extracted RNA quantity was determined spectrophotometrically and stored at -80°C until use. Synthesis of cDNA was carried out using 8 μl of the RNA, Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen Inc., Tokyo, Japan) and a random hexamer (Promega Inc., Tokyo, Japan). The synthesized cDNA was stored at -30°C .

Quantification of CXCR4 and cytokine genes expression: CXCR4 expression and cytokine expressions including interleukin-4 (IL-4), IL-6, IL-10, IL-12p40, IFN- α , IFN- γ and transforming growth factor- β (TGF- β) of the cows in these herds were estimated using real-time polymerase

chain reaction (real-time PCR). Real-time PCR was performed using 7300 real-time PCR systems (Applied Biosystems, Tokyo, Japan) using 5 ng of cDNA in a total of 25 μl reaction mixture including 5 pmol of each primer and 12.5 μl of Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Tokyo, Japan). The conditions for real-time PCR were 50°C for 2 min., 95°C for 3 min., 50 cycles (denaturation at 95°C for 30 sec., annealing at $55\text{--}65^{\circ}\text{C}$ for 30 sec. according to the primer and extension at 72°C for 60 sec.) and a dissociation step for the confirmation of specific amplification. The details of primers used in real-time PCR were described in Table 1. The results of quantification of each gene were analyzed with the comparative threshold cycle (ΔCt) method and presented as $2^{-\Delta\text{Ct}}$ (28). ΔCt is the difference between the Ct of a given target and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The amplification efficiencies of the target genes and GAPDH were approximately equal. Statistical analysis of data was performed using Student's *t*-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant when $P < 0.05$.

Results

CXCR4 and cytokine expressions in the high prevalence herd

CXCR4 and cytokine expressions in the dams of PI calves and non PI calves in the herd were estimated. No significant differences were observed between both dams (Fig. 1). In spite of these non significant differences, there was tendency of IL-6, IL-10 and IFN- γ expressions to be higher in the dams of PI calves than the dams of non PI calves. Also, there was tendency of IL-12, IFN- α and TGF- β expressions to be lower in the dams of PI calves than the dams of non PI calves. IL-4 expression was very low in both dams.

Table 1. Details of nucleotide sequences of primers used for real -time PCR

Gene	Primer sequence (5'-3')	Size of amplified product (BP)	Annealing temp. (°C)	Reference or accession number
CXCR4	Sense: TATCGTCCATGCTACCAACA Antisense: GAGTCGATGCTGATCCCAAT	377	55	NM_174301
IL-4	Sense: TGCCCCAAAGAACAACAAGT Antisense: TTTAGCCTTTCCAAGAGGTC	200	55	26)
IL-6	Sense: TCC AGA ACG AGT ATG AGG Antisense: CAT CCG AAT AGC TCT CAG	236	55	18)
IL-10	Sense: TGCTGGATGACTTTAAGG Antisense: AGGGCAGAAAGCGATGACA	186	55	18)
IL-12p40	Sense: AGGTCGTGGTAGAAGCTGTG Antisense: CCTTGTGGCATGTGACTTTG	275	65	26)
IFN- α	Sense: GAAGGCTCAAGCCATCTCTG Antisense: CCAGGTGTGTGTCAGTCCTT	365	60	E00135
IFN- γ	Sense: ATAACCAGGTCATTCAAGG Antisense: ATTCTGACTTCTCTTCCGCT	218	55	18)
TGF- β	Sense: AGAGAGGAAATAGAGGGCTT Antisense: ATGAATCCACTTCCAGCCCA	306	55	6)
GAPDH	Sense: GGCGTGAACCACGAGAAGTATAA Antisense: CCCTCCACGATGCCAAAGT	120	55	27)

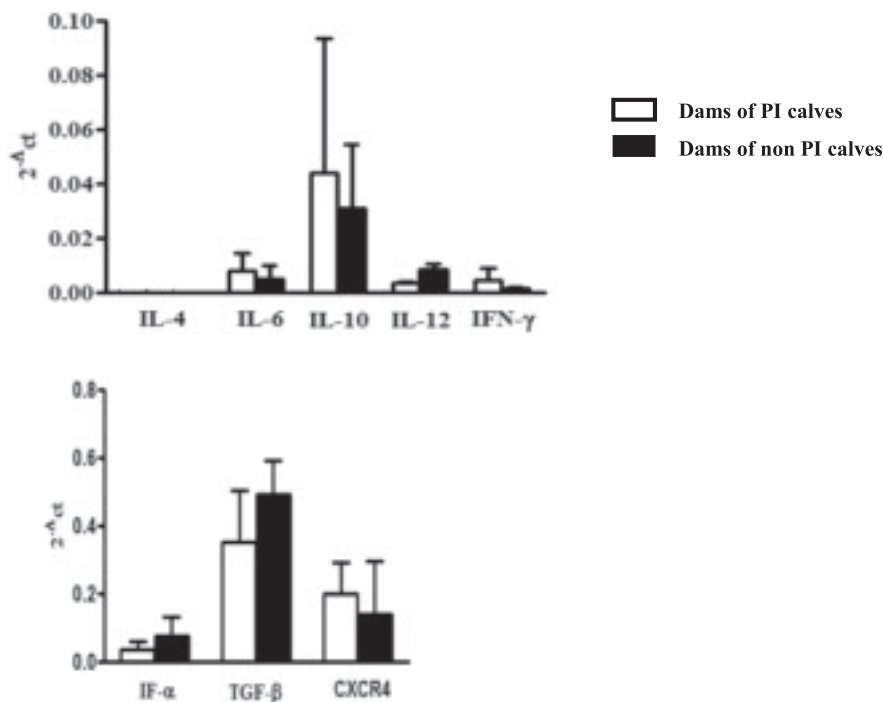


Fig. 1. CXCR4 and cytokine expression in the high prevalence herd. CXCR4 expression and cytokine expression including IL-4, IL-6, IL-10, IL-12, IFN- α , IFN- γ and TGF- β in 6 dams of PI calves (open column) and 20 dams of non PI calves (closed column). Expression values were calculated using comparative Ct method ($2^{-\Delta C_t}$). Bars indicated standard deviation. Data were analyzed using Student's *t*-test.

Comparison of CXCR4 and cytokine expressions among herds

Because CXCR4 expression in the dams of PI calves was not significantly different from the dams of non PI calves in the high prevalence herd, the expression of CXCR4 was compared to a low prevalence herd and BVDV-free herd. As shown in Fig. 2, CXCR4 expressions in the PI producing herds including the high prevalence and low prevalence herds were significantly lower than the BVDV-free herd. CXCR4 expressions in the high prevalence and the low prevalence herds were similar. There were no significant differences in cytokine expressions among herds except for IL-10. IL-10 was significantly higher in the high prevalence herd and the BVDV-free herd than the low prevalence herd. In the high prevalence herd, IL-6, IL-12, IFN- γ and IFN- α had tendency to be higher and

TGF- β had tendency to be lower. These results indicated some bias toward Th2 cytokines including IL-10 and IL-6 in the high prevalence herd and the BVDV-free herd.

Discussion

In the present study, we investigated the involvement of maternal CXCR4 and cytokine expressions in the natural occurrence of PI calves. CXCR4 expression in the dams of PI calves was not significantly different from the dams of non PI calves in the high prevalence herd. This was not corresponding to a previous study reported that CXCR4 was significantly down-regulated in blood of experimentally infected dams carrying PI fetuses³⁰. In the comparison among herds, CXCR4 expressions in

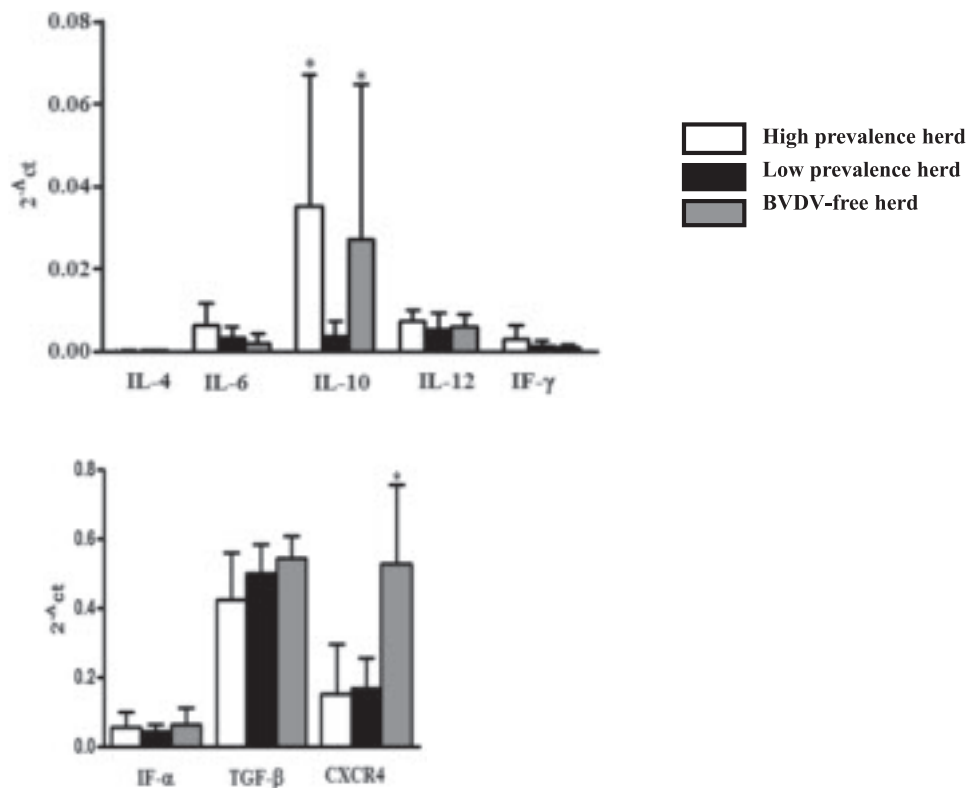


Fig. 2. Comparison of CXCR4 and cytokine expressions among herds: CXCR4 expression and cytokine expression including IL-4, IL-6, IL-10, IL-12, IFN- α , IFN- γ and TGF- β of 26, 34 and 25 cows in the high prevalence, low prevalence and the BVDV-free herds respectively. Expression values were calculated using comparative Ct method ($2^{-\Delta C_t}$). Asterisk (*) indicated the significant higher difference ($p < 0.05$) to other columns within the same gene. Bars indicated standard deviation. Data were analyzed using one-way analysis of variance (ANOVA).

PI producing herds were significantly lower than the BVDV-free herd. This result suggests that the cows in PI producing herd had down-regulated CXCR4 expression whether they were dams of PI or not. It was reported in an *in vitro* study³³⁾ that BVDV induced up-regulation of CXCR4 in naïve peripheral blood mononuclear cells following culture with serum from pregnant cows infected with ncp BVDV. However, the down-regulation of maternal blood cell CXCR4 that occurred *in vivo* may require specific immune responses that are difficult to replicate *in vitro*³³⁾.

Cytokines might also play a role in increasing susceptibility of transplacental transmission of BVDV and production of PI animals^{25,30)}. It was reported that Th2-biased cytokines may be of advantage to the virus in establishing persistent infections *in utero*²⁵⁾. This report might not be coincided with our results as there was a bias toward Th2 cytokines observed in both dams of PI calves and non PI calves in the high prevalence herd. Moreover, in the comparison among herds, a bias toward Th2 cytokines was indicated in the high prevalence herd and the BVDV-free herd. These findings indicated that not only Th2 biased cytokines were involved in the persistent infection with BVDV but also other factors might be included. Previous studies indicated a relationship between the high expression of IL-10 and the transplacental infection in other diseases such as Leishmania infection in mice and neospora caninum in cattle^{1,19)}. In the present study, no significant differences in IL-10 expression between the dams of PI calves and non PI calves in the high prevalence herd. On the other hand, significant higher expression of IL-10 was observed in the cows of the high prevalence herd and the BVDV-free herd compared to the low prevalence herd. These findings indicated a combination of low expression of CXCR4 and high expression of IL-10 in the high prevalence herd. IL-10 and CXCR4 expressions in the high prevalence herd showed no significant differences between dams

of PI calves and non PI calves. These findings indicated that both dams had an equal risk for PI production inspite of production of non PI calves. Other factors might also be responsible for birth of non PI calves. In the low prevalence herd, CXCR4 expression was similar to the high prevalence herd. However, IL-10 expression was significantly lower than the high prevalence herd and the BVDV-free herd. This situation might influence the prevalence of PI calves. More detailed profiling of cytokines would be needed.

The relationship between type I IFN and the mechanism of persistent infection with BVDV was previously reported^{3,30)}. Other reports stated that the type I IFN was associated with expression of CXCR4^{29,33)}. In contrast to these reports, our results indicated no significant differences of IFN- α between the dams of PI calves and non PI calves in the high prevalence herd. Furthermore, IFN- α expression showed no significant differences among herds. Further studies are necessary to clarify the involvement of IFN- α in the persistent infection and its relationship with CXCR4.

It was reported that maternal CXCR4 was up-regulated in sheep during early pregnancy due to the implantation and placentation²⁾. Additionally, it was previously indicated that CXCR4 expression was up-regulated in cows carrying normal fetuses in the period between 75 and 160 days of pregnancy³⁰⁾. This variation in the degree of CXCR4 expression during pregnancy was estimated in the present study because cows within the same herd were in different periods of pregnancy. In the present study, the changes in CXCR4 expression estimated during pregnancy in the same cow had no specific tendency (data not shown). Moreover, the change of cytokine expressions estimated during pregnancy in the same cow had no specific tendency (data not shown). It was not clear that this tendency might depend on the influence of BVDV infection or not. Further investigations should be necessary for it.

In conclusion, in the present study, we tried

to investigate the involvement of maternal CXCR4 and cytokine expressions in the natural occurrence of persistent infection with BVDV. A dairy herd with high prevalence of PI calves was used for this investigation. The combination of low expression of CXCR4 and high expression of IL-10 might be closely concerned with some bias for production of PI animals and increase the prevalence of PI calves.

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