

Short communication

CELLULAR IMMUNE RESPONSE TO INTERFERON- τ IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF JAPANESE BLACK CATTLE WITH BOVINE LEUKEMIA VIRUS INFECTION

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IFN- τ is a type I interferon, and it is known to be non-virus inducible in ruminants. IFN- τ reduced syncytium formation by PBMC obtained from BLV infected cattle *in vitro*. In order to clarify the effects of IFN- τ on cellular immune function in Japanese Black (JB) cattle with bovine leukemia virus (BLV) infection, immune related factors of peripheral blood mononuclear cells (PBMC) were analyzed using IFN- τ as a stimulator. Thirty-two JB cattle were used in this investigation, and these cattle were divided into three groups: cattle with enzootic bovine leucosis (EBL) (EBL Group, N=7), clinically healthy cattle with BLV infection (Carrier Group, N=13), and clinically healthy cattle without BLV infection (non-Carrier Group, N=12). A number of mRNA expressions of interleukin-12 and interferon (IFN)- γ as immune cell activating cytokines, perforin and granulysin as cytotoxic factors, and myxovirus resistance protein (MX)-1 and MX-2 as anti-virus factors of PBMC were analyzed after culturing cells with phytohemagglutinin (PHA) or IFN- τ . The basal mRNA levels of perforin and granulysin in the Carrier Group were significantly higher than those in the non-Carrier Group. Also, significantly higher basal mRNA levels of MX-1 and MX-2 in the EBL Group were detected compared with the non-Carrier Group. The mRNA expressions of perforin and granulysin in PBMC stimulated with PHA were higher in the Carrier Group than those in the non-Carrier Group. There were significantly higher mRNA levels of MX-1 and MX-2 in PBMC stimulated with IFN- τ in the EBL Group compared with those in the non-Carrier Group. These results suggest an enhanced sensitivity of anti-virus reaction in PBMC by IFN- τ treatment in JB cattle with EBL.

Key words: bovine leukemia virus, cellular immune factor, interferon- τ , Japanese Black

INTRODUCTION

Enzootic bovine leucosis (EBL), a disease caused by the Bovine leukemia virus (BLV), is the most common neoplastic disease of cattle. Persistent lymphocytosis (PL),

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identified as a polyclonal expression of B lymphocytes, occurs in BLV-infected cattle and develops into lymphoma. These lymphomas in EBL are observed at multiple sites including peripheral as well as visceral lymph nodes, which affect clinical symptoms. Enlarged peripheral lymph nodes can be recognized visually and palpably [1].

Progression of the disease in BLV cattle may be affected by the degree of cellular immune response. Amills et al. [2] reported that the expressions of IFN- γ , IL-2, and IL-4 mRNAs were significantly lower in the peripheral blood mononuclear cells (PBMC) from BLV+PL+ cows compared to BLV- Holstein cows. They demonstrated that the progression of BLV infection to PL was associated with a lower expression of classical Th1 and Th2 cytokines by CD4⁺ T cells. Since IFN- γ mRNA expression of PBMC was inversely correlated with the number of lymphocytes, the decrease in this cytokine expression caused by increased virus load in BLV infected cattle might have resulted in immunosuppression increasing the risk of EBL [3].

IFN- τ , a member of the type I IFN family, is considered as a non-virus inducible cytokine in ruminants. But this IFN is known as an effective antiretroviral cytokine, and thus an interest in using IFN- τ for therapy of retroviral infections in humans has been described [4,5]. A previous study demonstrated that recombinant bovine IFN- τ reduced syncytium formation by PBMC obtained from BLV infected cattle *in vitro* [6]. Type I IFNs induce the production of cytolytic and antiviral factors in cytotoxic lymphocytes (CTL), as well as natural killer (NK) cells. Granulysin and perforin molecules are contributing factors for lysis of tumor or cells with intracellular pathogens CTL and NK cells. Myxovirus resistance protein (MX) is a potent inhibitor of human retrovirus infection, that serves as a key effector of IFN-mediated resistance to this virus infection [7].

Japanese Black (JB) is the most common breed of beef cattle in Japan. Although the incidence of BLV infection has been known in JB cattle [8], the immune response in JB cattle with BLV has not been reported. A previous report showed that the mean BLV titers in cattle decreased after administration of recombinant bovine interferon (IFN)- τ in Japanese Black (JB) [6]. However, the cellular immune function in JB cattle has not been clarified after administration of IFN- τ . In order to evaluate the immune function in BLV-infected cattle, analysis of PBMC was conducted in several previous studies [2,3,6]. In order to clarify the immune modulatory effect of IFN- τ in JB cattle with BLV infection, we compared the mRNA expression of cellular immune parameters after treatment with IFN- τ between PBMC obtained from EBL and carriers of BLV.

MATERIALS AND METHODS

Animals

Thirty-two JB female cattle were used for this investigation. The animals were divided into three groups: seven cattle with EBL (EBL Group, age: 7.16 ± 1.14 (mean \pm standard error (SE))), 13 clinically healthy cattle with BLV infection (Carrier Group,

age: 7.37 ± 0.64), and 11 clinically healthy cattle without BLV infection (non-Carrier Group, age: 6.82 ± 0.42). BLV infection in the Carrier Group was diagnosed by agar gel immunodiffusion (AGID) test. Typical clinical signs of EBL such as depression, anorexia, inability to stand, enlarged lymph nodes and anemia were observed in the EBL Group, but there were no abnormal clinical signs in the Carrier Group and non-Carrier Group. The blood samples were collected into tubes containing heparin for analysis of cytokine production. White blood cell (WBC) counts were determined by a blood cell counter (Celltac α MEK-6358, NIHON KOHDEN, Tokyo, Japan).

Real-time PCR

The PBMCs suspended in RPMI 1640 medium (Invitrogen, Tokyo, Japan) supplemented with 10% fetal calf serum (Cansera International, Rexdale, Canada) were placed in 24-well plates (2×10^6 cells/ml/well), and treated with $5 \mu\text{g/ml}$ of phytohemagglutinin (PHA; AppliChem GmbH, Germany) or 5×10^4 IU/ml of IFN- τ (supplied from Nippon Zenyaku Kogyo Co.) for 12 h at 37°C . After incubation, the supernatants were removed, and the cells were re-suspended using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The PBMC were then subjected to RNA extraction and real-time PCR using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), as described previously [9].

Total RNA was transcribed into cDNA by using the GoScript reverse Transcription System (Promega, Mannheim, Germany) combined with oligoDT₁₅ primers, recombinant RNasin ribonuclease inhibitor, and GoScript™ reverse transcriptase according to the manufacturer's instructions. The primers used for the evaluation of gene expression of β -actin, IL-12p40, IFN- γ , perforin, granulysin, and MX-1 and MX-2 were designed as described in the literature (Table 1). Real-time PCR was set up using $2 \mu\text{l}$ first-strand cDNA template, $7.4 \mu\text{l}$ deionized H₂O, $0.3 \mu\text{M}$ of upstream and downstream primers, and $10 \mu\text{l}$ Power SYBR Green I master mix with ROX as a reference dye (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The thermal cycling conditions were 3 min at 95°C followed by 15 s at 95°C (40 cycles) and 1 min at 60°C . Melting curve analysis was constructed to verify the presence of a gene-specific peak and the absence of the primer dimer.

Table 1. Primers used for real-time PCR

Gene	Accession number	Product length	Primer designation	Sequence (5'-3')
IL-12p40	NM_174356.1	117	Forward	GGACATCATCAAACCAGACCC
			Reverse	AGGGAGAAGTAGGAATGCGG
IFN- γ	NM_174086	108	Forward	TCAAATTCGGTGGATGATCT
			Reverse	CTTCTCTCCGCTTTCTGAGG
granulysin	NM_001075143.1	136	Forward	GAGATCAGCCCAGATGAGAATAC
			Reverse	TTTCCAGCTACGATGTCCCTCA
perforin	NM_001143735.1	105	Forward	ACTCCGAGGATGCCAACITTC
			Reverse	TGTGCACCAGGTGAAAACCTGTA
MX-1	NM_173940.2	116	Forward	AGGATCACGCACATTTTCAGG
			Reverse	CAACAGGGGCAGAGTTTTACAA
MX-2	NM_173941.2	80	Forward	CCTACAAGTGCACAGGTGACAAC
			Reverse	CCTACAAGTGCACAGGTGACAAC

The final quantification of immune related molecule mRNA was carried out using the comparative CT (threshold cycle) method. This method was used after a validation experiment, which demonstrated that the efficiencies of the target and reference (β -actin) genes were approximately equal as previously described [9].

Statistical analysis

Results were expressed as mean \pm SE. Two-way analysis of variance (ANOVA) was used to compare mean mRNA levels among three culture conditions. When ANOVA was significant, Tukey's test was used as a post-hoc test. For all analyses, $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Lymphocyte counts were 9.72 ± 1.68 (Mean \pm SE $\times 10^9$ cells/L) in the EBL Group, 3.63 ± 0.52 in the Carrier Group, and 3.71 ± 0.21 in the non-Carrier Group. Counts were significantly higher in the EBL Group compared with the other two groups. In the previous report of adult JB cattle, the lymphocyte count was in the range of 0.37 ± 0.17 to $0.43 \pm 0.21 \times 10^9$ cells/L [8], which resembled our data in the Carrier and non-Carrier Group. Cattle in the EBL Group were diagnosed as having lymphocytosis. In the present study, levels of IL-12 mRNA expression treated with phytohemagglutinin (PHA) or IFN- τ were significantly higher in the Carrier Group compared with the non-Carrier Group (Figure 1). IL-12 is a cytokine, which enhances the cytotoxic activity of NK cells and CD8⁺ cytotoxic T lymphocytes, and stimulates the production of IFN- γ from T cells and NK cells. Expression of IL-12 p40 mRNA increased in cattle in the alymphocytotic stage, but decreased in PL animals [10]. A previous study reported that IFN- γ was elevated in the BLV-positive aleukemic cattle but not in the BLV-positive persistently lymphocytotic cattle [11]. The significant loss of cellular cytokine expression such as IL-12 p40 and IFN- γ was characteristic for cattle that developed PL status shortly after BLV infection [12]. In the present study, a higher IL-12 p40 mRNA was found, but lymphocytosis was not detected in the Carrier Group. Since a significant increase in IL-12 p40 mRNA was not detected in the EBL Group following lymphocytosis, IL-12 may be one of the important effective cytokines which inhibit BLV replication in the Carrier Group cattle.

Levels of perforin and granulysin mRNA were significantly lower in the Carrier Group than those in the non-Carrier Group, but a statistically significant difference was not detected in the EBL Group (Figure 1). Granulysin is an antimicrobial molecule and perforin is a cytolytic molecule released by NK cell or CTL. Both are released by NK cells and CTL when they are attached to virus-infected cells or tumor cells. The cytotoxic granule pathway is likely to be the principal mechanism by which CD8⁺ T cells eliminate virus-infected cells [13]. Functional impairment of retrovirus-specific CD8⁺ T cells has been associated with an expansion of CD25⁺FoxP3⁺ T cells in PL

cattle [3]. A previous study demonstrated that CD25⁺FoxP3⁺ T cells isolated from both lymph nodes and peripheral blood significantly suppressed human immunodeficiency virus (HIV)-specific CTL function [14]. A significantly lower mRNA level of cytolytic molecules was not detected in the EBL Group, although a significantly lower level of these mRNAs in PBMC with or without treatment of PHA was detected in the Carrier

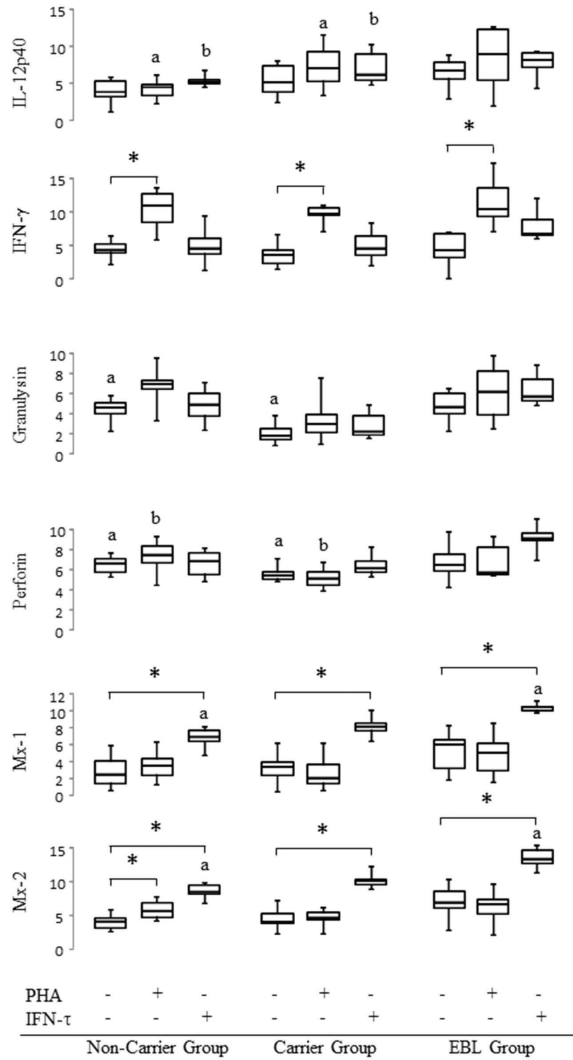


Figure 1. Immune markers mRNA expression of PBMC in BLV cattle. Asterisks indicate the time point where significant differences between each group was observed ($P < 0.05$). Same letters indicate significant differences between immune factors mRNA and control values ($P < 0.05$)

Group. Since defective functions of CTL or NK cells were linked to the development and progression of leukemia [15], there is a high risk of EBL in cattle with depression of cytolytic factors of CTL or NK cells. But a significant difference in these cytolytic factors of PBMC with IFN- τ stimulation was not detected among the three observed groups. Further investigation is needed to clarify the regulation of perforin and granulysin expression in cattle with EBL.

Table 2. Cytokine mRNA expression of PBMC in BLV cattle

Particle	Culture	Onset group	Positive group	Healthy group
		(N=7)	(N=13)	(N=12)
IL-12	Control	4.98±0.52	5.35±0.63	3.75±0.47
	PHA	5.76±0.85	7.46±0.87a	4.21±0.33a
	IFN- τ	6.78±0.68	7.25±0.68a	5.29±0.18a
IFN- γ	Control	4.43±0.0.97	3.80±0.45	4.31±0.42
	PHA	8.94±0.65	9.92±0.30	10.11±0.77
	IFN- τ	6.26±0.88	5.26±0.60	4.84±0.63
Granulysin	Control	3.66±0.42	2.11±0.28a	4.40±0.30a
	PHA	4.44±0.70	3.26±0.51a	6.85±0.43a
	IFN- τ	4.95±0.46	2.74±0.32	4.85±0.42
Perforin	Control	5.02±0.47	5.43±0.19a	6.46±0.25a
	PHA	4.50±0.63	5.02±0.25a	7.20±0.43a
	IFN- τ	5.99±0.90	6.17±0.23	6.64±0.36
Mx-1	Control	3.75±0.67	2.94±0.46	2.58±0.53
	PHA	4.08±0.71	2.46±0.49	3.48±0.43
	IFN- τ	7.98±0.13a	7.94±0.29	6.85±0.30a
Mx-2	Control	4.94±0.67	3.55±0.39	3.65±0.44
	PHA	4.16±0.57	3.75±0.30a	5.83±0.39a
	IFN- τ	10.13±0.37a	9.24±0.27	8.60±0.27a

Data are expressed as the mean \pm SE (target gene mRNA/ β -actin gene mRNA)

Values denote relative expression of each parameter mRNA

*Same letters indicate a significant difference between groups (P<0.05).

In the present study, mRNA expression of MX-1 and MX-2 increased significantly due to IFN- τ treatment in all three groups (Figure 1), which proved that MX mRNA was induced by IFN- τ in JB cattle. MX proteins confer selective resistance to the virus [16], and are a particularly useful indicator of Type 1 IFN activity, as it is not induced by other IFN or any other cytokine [17,18]. MX mRNA levels in lymphoid tissues were parallel with the magnitude of the increase in Type 1 IFN mRNA levels. Expression of mRNA of MX-1 and MX-2 by IFN- τ treatment was the highest in the EBL Group. The level of PBMC-associated MX protein suggested the association of the disease stage of the patient with retrovirus infection [19]. In the lymphoid tissues of monkeys with a similar immunodeficiency virus infection, higher viral RNA levels were associated with increased MX mRNA levels [20]. Since high titers of the virus were present in the PL stage of BLV infection [21], increased MX mRNA levels by

IFN- τ treatment might be consistently detected in PBMC from animals with EBL, even if mRNA levels of cellular immune cytokines or cytolytic factors are not high.

In this study, IFN- γ mRNA expression of PBMC was enhanced significantly by treatment with PHA, and MX mRNA expressions were enhanced significantly by treatment with IFN- τ in all three groups. PHA is used as a mitogen for the stimulation of PBMCs *in vitro*, and induced mainly the release of IFN- γ among IFNs from lymphocytes. Production of type-1 IFNs occurs mainly in response to dsRNA viral infection [22,23]. Treatment with type-1 IFNs resulted in an induction of MX protein expression of PBMC [24]. These results suggest that the reaction of cytokines or mRNA expression of cellular immune factors in PBMCs was different between PHA and IFN- τ stimulation.

In conclusion, we have demonstrated in this study that the mRNA expression of cytolytic mediators such as granulysin and perforin decreased in the Carrier JB cattle, but antiviral mediators such as MX-1 and MX-2 of PBMC increased by treatment with IFN- τ in the EBL cattle. Since specific antiviral activities of MX have been reported during experimental infections with RNA viruses [25], it is possible that type1 IFN such as IFN- τ plays an effective role in the host defense against BLV in cattle. These results may be associated with an enhanced sensitivity of anti-virus reaction of PBMC to IFN- τ , rather than cytolytic reaction in JB cattle with EBL. It is known that the kinetics of cytokine production measured by preceding mRNA expression was well correlated with the following corresponding protein synthesis [26]. Therefore, we chose mRNA expression to estimate targeting protein synthesis in the immune cells to evaluate the kinetics of immune response in cattle with BLV. This is the first report which demonstrates cellular immune factors in JB cattle with BLV infection. Further studies are needed to clarify the role of IFN- τ in cattle with EBL.

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IMUNSKI ODGOVOR MONONUKLEARNIH ČELIJA PERIFERNE KRVI NA INTERFERON- τ KOD INFEKCIJE VIRUSOM LEUKEMIJE JAPANSKOG CRNOG GOVEČETA

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IFN- τ je tip I interferon koji je poznat po tome da ga kod goveda ne indukuju virusi. IFN- τ je redukovao nastanak sincicijuma pomoću PBMC dobijenih od BLV inficiranih goveda *in vitro*. U cilju razjašnjavanja efekta IFN- τ na ćelijske imune funkcije Japanskog crnog govečeta (JB) inficiranih virusom govede leukemije (BLV), imuni faktori mononuklearnih ćelija periferne krvi (PBMC) su ispitivani koristeći IFN- τ kao stimulator. U ispitivanju su učestvovala 32 JB govečeta podeljena u 3 grupe: goveda sa enzootskom bovinom leukozom (EBL) (EBL grupa, n=7), klinički zdrava goveda sa BLV infekcijom (grupa kliconoša, n=13) i klinički zdrava goveda bez BLV infekcije (ne-kliconoše, n=12). mRNK ekspresije interleukina-12 i interferona (IFN)- γ kao aktivirajućih citokina imunskih ćelija, perforina i granulozina kao citotoksičnih faktora, proteina rezistencije na miksovirus (MX)-1 i (MX)-2 kao antivirusnih faktora PBMCa bili su ispitivani nakon kultivisanja ćelija sa fitohemaglutininom (PHA) ili IFN- τ . Osnovni nivoi mRNK perforina i granulozina u grupi kliconoša bili su signifikantno viši u odnosu na grupu ne-kliconoša. Takođe, zabeleženi su signifikantno viši osnovni nivoi mRNK MX-1 i MX-2 u okviru EBL grupe u poređenju sa grupom ne-kliconoša. Ekspresija mRNK perforina i granulozina kod PBMC stimulisanih sa PHA bile su više u grupi kliconoša u poređenju sa ne-kliconošama. Nivoi mRNK MX-1 i MX-2 bili su

znatno viši u PBMC stimulisanih sa IFN- τ u EBL grupi u poređenju sa grupom neklicionoša. Navedeni rezultati ukazuju na povećanu osetljivost antivirusnog odgovora PBMC nakon IFN- τ tretmana JB goveda inficiranih sa EBL.