Interferon- γ Production of Mitogen Stimulated Peripheral Lymphocytes in Perinatal Cows

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ABSTRACT. The production of interferon- γ (IFN- γ) in bovine peripheral blood mononuclear cells (PBMC) during the perinatal period was studied. IFN- γ activity was neutralized with monoclonal antibodies (MoAb) to recombinant bovine IFN- γ . IFN- γ production was apparently suppressed by treatment of the PBMC with MoAb to bovine pan-T cell. IFN- γ was more susceptible to treatment with MoAb to CD4⁺ than that with MoAb to bovine CD8⁺, suggesting that the CD4⁺ T-cell subset was the major producer of the lymphokine formed in the culture. IFN- γ production by concanavalin A (Con A) stimulation was found to be significantly depressed throughout the perinatal period (from 5 weeks before to 7 weeks after parturition) when compared to that of the non-pregnant control group, but phytohemagglutinin (PHA)-induced IFN- γ production was markedly depressed from 3 weeks before to 2 weeks after calving. The addition of recombinant human interleukin 2 (100 IU/ml) to the PBMC cultures from perinatal cows resulted in the augmentation of IFN- γ production by stimulation with Con A or PHA.—**KEY WORDS:** IFN- γ , IL-2, immunosuppression, perinatal cattle.

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In general, the immune response of pregnant women is known to be depressed in the cellular and humoral systems so as to prevent the immunological rejection of the fetus by the mother. Kehrli *et al.* [12] also reported that in cattle both the phagocytic function of neutrophils and the blastogenic response of lymphocytes to T-cell mitogens were depressed in perinatal cows. Kashiwazaki *et al.* [11] demonstrated significant suppression of the blastogenesis of bovine lymphocytes against phytohemagglutinin (PHA) during the perinatal period, especially in multiparous cows. Ishikawa and Shirahata [8] reported that the lymphocyte blastogenic activity, percentage of E-rosette forming cells, and serum IgG concentration were depressed in perinatal cows. These immunosuppressive conditions of pregnant cows may cause them to be susceptible to infectious agents: mastitis or puerperal infection often occurs during the perinatal period.

Recently, a few reports have concerned the biological and physicochemical characteristics of bovine interferon (IFN) using preparations of the bovine recombinant IFNs (IFN- α 1, IFN- α 2, IFN- β and IFN- γ) [2, 4, 5]. It may be useful to investigate the induction and production of cell populations of IFN- γ in order to elucidate the immune status of perinatal cows.

In this study, we examined the cell population involved in the production of bovine IFN- γ , the fluctuation in the IFN- γ titer in perinatal cows and the effects of recombinant human interleukin 2 (rHuIL-2) on IFN- γ production.

MATERIALS AND METHODS

Blood samples: Thirty Holstein Friesian cows, ranging from 3 to 5 years old, kept at the Obihiro University Farm, were used. Blood samples were collected weekly in heparinized vacuum tubes (Venoject, Terumo, Japan) from the jugular or caudal veins of 7 pregnant cows in the

gestational and post-parturient period.

Preparation of bovine peripheral blood mononuclear cells (PBMC): The PBMC were prepared as described previously [9].

Induction of IFN: We used concanavalin A (Con A, Sigma Chemical Co., U.S.A.) and PHA (Sigma Chemical Co., U.S.A.) as IFN inducers. A cell suspension adjusted to a concentration of 5×10^6 /ml was cultured in 24 well-plastic plates (Falcon, New Jersey, U.S.A.) and included different concentrations of inducers for 1 to 7 days. At the end of the incubation, the cell suspension was collected from each well and centrifuged at $600 \times g$ for 30 min. The resulting supernatant was stored at -80°C until used as IFN samples.

Assay of IFN titer: The IFN assay was performed by the plaque reduction method [14] in MDBK (Flow Laboratories Inc., U.S.A.) with the vesicular stomatitis virus (VSV, Indiana strain) as the challenging virus. Details of the assay were described previously [8]. As a positive control, recombinant bovine IFN- α 1 (rBoIFN- α 1, containing a titer of 10^7 per mg protein, courtesy of Ciba-Geigy, Japan) was used and the IFN titers of samples were converted to international units (IU/ml).

Preparation of anti-rBoIFN- α 1 rabbit serum and the monoclonal antibody (MoAb) to rBoIFN- γ : The anti-rBoIFN- α 1 serum was prepared by the method of Wood *et al.* [20]: one ml of rBoIFN- α 1 (50 $\mu\text{g}/\text{ml}$) was mixed with 1 ml of Freund's complete adjuvant and injected into a rabbit subcutaneously or intramuscularly. A booster injection with Freund's incomplete adjuvant was repeated 30 and 60 days after the primary injection. Fourteen days after the final inoculation, the rabbits were bled and the serum was collected. The serum was inactivated by heating it at 56°C and then stored at -80°C until used. This antiserum showed activity neutralizing 10 IU of rBoIFN- α 1 even at a 50-fold dilution. The MoAb to rBoIFN- γ was kindly provided by Dr. Wood (CSIRO

Division of Animal Health, Australia) [20].

Stability of IFN at pH 2.0 and 56°C for 30 min: This was examined as described previously [9].

Neutralization test of IFN: The neutralization test was performed by the method of Fujii *et al.* [5]. The IFN sample was added to the same volume of a monoclonal anti-rBoIFN- γ solution diluted 50-fold or an anti-rBoIFN- $\alpha 1$ solution diluted 20-fold and allowed to stand for 1 hr at 37°C. The mixture was serially diluted with a 1% minimum essential medium (MEM) and tested for IFN activity by the assay procedure described above.

Treatment of T-cells and T-cell subsets with MoAbs: Three bovine T-cell specific MoAb, anti-pan T-cells (Lot # BAQ82A, VRMD Co., U.S.A.), anti-CD4⁺ cells (Lot # GD50A1, VRMD Co., U.S.A.) an anti-CD8⁺ cells (Lot # BAQ111A, VRMD Co., U.S.A.) were used in this study. Each MoAb was diluted with RPMI-1640 and absorbed by bovine red blood cells to exclude non-specific cell cytotoxicity. PBMC (2×10^6 /ml) was suspended in MoAb and centrifuged for 10 min at $240 \times g$. 0.2 ml of rabbit complement (Cedarlane Laboratories Co., Ltd., Canada) diluted 1:6 with MEM was added to the sediment and then the entire suspension was incubated for 1 hr at 37°C. After the incubation, the cells were washed twice and then resuspended in 0.4 ml of RPMI-1640. The cell suspension (5×10^6 /ml) was then incubated with 50 μ g/ml of an IFN inducer for 96–120 hr in 5% CO₂ in air on 24 well-microplates.

The effect of rHuIL-2 on IFN production in perinatal cows in vitro: Seven pregnant cows were bled at weekly intervals from 5 weeks before to 7 weeks after parturition. The PBMC of each cow were cultured with 50 μ g/ml of Con A for 120 hr, or with 50 μ g/ml of PHA for 96 hr. In order to examine the effect of IL-2 on the production of IFN, 100 IU/ml of rHuIL-2 (Lot # RIL-2, 1 mg/10,000 IU, Zeym, U.S.A.) was added to the cultures of the PBMC 1 week before, at the time of, and 1 week after parturition.

Statistical analysis: A Student's *t*-test was used to examine the significance of differences. Differences giving $P < 0.05$ were regarded as significant.

RESULTS

The characteristics of IFN induced by Con A and PHA: As shown in Table 1, the activity of bovine IFN induced by Con A or PHA was neutralized by anti-rBoIFN- γ (MoAb) but not by anti-BoIFN- $\alpha 1$ (MoAb). On the other hand, the activity of rBoIFN- $\alpha 1$ was not affected by treatment with anti-BoIFN- γ but was neutralized by anti-rBoIFN- $\alpha 1$. The activity of IFN induced by Con A or PHA was unstable at pH 2.0 and 56°C for 30 min (Table 1).

The effect of bovine T-cell specific MoAb on IFN production: In order to determine the cell populations involved in IFN production by Con A or PHA, the PBMC of non-pregnant cows were treated with 3 bovine T-cell specific MoAb and complement, respectively. The production of IFN was almost completely depressed (below 5 IU/ml) by anti-pan T-cell serum in both Con A and PHA stimulated cultures. Furthermore, the IFN titer was lowered to 13% of the control titer in the Con A ($P < 0.01$) cultures and to 29% of the control titer in the PHA cultures when the PBMC of non-pregnant cows were treated with anti-CD4⁺ cells. In comparison, the IFN titer was depressed to 20% of the control titer in the Con A cultures and to 37% of the control titer in the PHA ($P < 0.01$) cultures when the PBMC of the cows were treated with anti-CD8⁺ cells (Table 2).

IFN production in bovine PBMC during the perinatal period: The fluctuation in IFN production in perinatal cows is shown in Fig. 1. The IFN titer began to decline from 4 weeks before parturition and the minimum IFN titer was observed at 1 week before parturition in both Con A and PHA stimulated cultures. The IFN titer gradually increased after parturition and reached the control level at 7 weeks after parturition. The IFN titer was comparatively low during the perinatal period in the Con A stimulated PBMC cultures ($P < 0.01$ and $P < 0.05$), but in the PHA stimulated PBMC cultures the IFN titer was significantly depressed ($P < 0.05$) from 3 weeks before to 2 weeks after parturition.

Table 1. Characteristics of IFNs produced by bovine PBMC in response to Con A and PHA

IFNs	IFN titer (IU/ml) ^{a)}				
	After neutralization with		Control	After treatment with	
	anti rIFN- γ	anti rIFN- $\alpha 1$		pH 2.0	Heating (56°C, 30 min)
Con A-induced ^{b)}	<5	79.0 $\pm$ 26.3	77.5 $\pm$ 20.5	<5	<5
PHA-induced ^{b)}	<5	83.5 $\pm$ 24.6	84.5 $\pm$ 29.4	<5	<5
rIFN- $\alpha 1$ ^{c)}	82.5 $\pm$ 5.7	<5	89.5 $\pm$ 7.1	34.5 $\pm$ 15.2	87.5 $\pm$ 9.0

a) Titers are the mean $\pm$ S.D. for 5 cows.

b) Cells (5×10^6 cells/ml) were incubated with 50 μ g/ml of PHA for 4 days or of Con A for 5 days.

c) Recombinant bovine IFN- $\alpha 1$ was used as a reference for bovine IFN.

Table 2. Effects of T subset-specific MoAbs on IFN production in bovine PBMC

Treatment	IFN titer (IU/ml) induced by ^{a)}	
	Con A	PHA
Control (MEM)	57.0 $\pm$ 12.5	77.6 $\pm$ 29.5
Complement (C')	57.6 $\pm$ 13.7[-1 $\pm$ 24] ^{b)}	75.0 $\pm$ 23.1[0 $\pm$ 14]
anti pan T+C'	<5	<5 [>95]
anti CD4 ⁺ +C'	7.0 $\pm$ 2.5[87 $\pm$ 4]**	21.8 $\pm$ 5.1[71 $\pm$ 4]
anti CD8 ⁺ +C'	46.2 $\pm$ 13.4[20.6 $\pm$ 6]	48.4 $\pm$ 16.9[37 $\pm$ 8]**

- a) Cells (5×10^6 cells/ml) were incubated with 50 μ g/ml each of mitogens for 4 days as described in MATERIALS AND METHODS, and titers are the mean $\pm$ S.D. for 6 cows.
- b) Figures in brackets indicate the percent inhibition of IFN activity after treatment with monoclonal antibodies plus rabbit complement in comparison with control. **: P<0.01.

Fig. 1. Depression of IFN production in bovine PBMC during the periparturient period. Bovine PBMCs (5×10^6 cells/ml) during the parturient period were incubated with 50 μ g/ml of Con A (○) for 5 days or PHA (●) for 4 days, and the resultant supernatants were assayed for IFN activity. Data are the mean $\pm$ S.D. for 5 to 7 cows at each point. ★: P<0.05, ★★: P<0.01.

The effects of rHuIL-2 on the production of IFN in perinatal and non-pregnant cows: Various concentrations of rHuIL-2 (25 to 500 IU/ml) were added to the PBMC cultures of the non-pregnant cows. In both the PHA and Con A stimulated cultures, there was no significant difference between the control and any other PBMC cultures with respect to the production of IFN (Fig. 2A). On the other hand, with the perinatal cows IFN production in the PBMC cultures significantly increased at parturition (P<0.05) in Con A stimulated PBMC cultures and at 1 week after parturition in Con A (P<0.01) and PHA (P<0.05) stimulated PBMC cultures with the addition of 100 IU/ml of rHuIL-2 (Fig. 2B).

DISCUSSION

Most of the IFN activity induced by Con A and PHA was labile at pH 2.0 and 56°C for 30 min and when

Fig. 2. Effects of rHuIL-2 on IFN production from nonpregnant (A) and periparturient cows (B). PBMC (5×10^6 cells/ml) from nonpregnant and periparturient cows were incubated with 50 μ g/ml of Con A for 5 days or PHA for 4 days in the absence (□ □) or presence (▨ ▨) of rHuIL-2, and the resultant supernatants were assayed for IFN activity. Data are the mean $\pm$ S.D. for 5 to 7 cows at each point. ★: P<0.05, ★★: P<0.01.

neutralized with anti-rBoIFN- γ (MoAb), indicating that IFN- γ was the predominant form of IFN in bovine PBMC cultures (Table 1).

There have been few reports concerning the mechanism of IFN- γ production in perinatal cows. In this paper, the authors showed that IFN- γ production in perinatal cows was apparently depressed, especially around parturition. Con A-stimulated PBMC produced a lowered titer of IFN- γ throughout the observation period, but PHA-stimulated PBMC were depressed only from 3 weeks before to 2 weeks after parturition (Fig. 1). This may be attributed to a difference in IFN- γ producing cells responding to each mitogen. As the major producing cells are CD4⁺ cells both in Con A and PHA stimulated PBMC (Table 2), CD8⁺ cells are more prevalent in the PHA stimulated PBMC [10].

IFN- γ plays an important role in the immune response, especially cellular immunity, and the production of IFN- γ induced by PHA or Con A corresponds to the degree of lymphocyte blastogenesis [9].

There are some reports that lymphocyte blastogenesis by T-cell mitogens was depressed in perinatal cows [8, 11, 12]. Ishikawa and Shirahata [8] suggested that both the percentage and the blastogenic activity of bovine T-lymphocytes were depressed by an increase in serum cortisol during the perinatal period. This suggested the participation of the T-lymphocyte population in the immune suppression mechanism in perinatal cows. The depression of IFN- γ production during the perinatal period may also be caused by a decrease in CD4⁺ cells which produce this cytokine [6].

The production of IFN- γ was reconstituted by the addition of rHuIL-2 to the cultures of the perinatal cows (Fig. 2B), but there was no significant increase in IFN- γ production in the non-pregnant cows [Fig. 2A]. It is known that the production of mitogen-induced IFN- γ is augmented by the addition of IL-2 in man and mice [16,

21] and that Con A- and PHA-induced human IFN- γ is significantly suppressed by the addition of the anti-IL-2 receptor antiserum [18], suggesting that the production of bovine mitogen-induced IFN- γ is IL-2 dependent.

An increase in immunosuppressive factors including steroids, pregnancy zone protein (PZP), early pregnancy factor (EPF), α -2 globulin and blocking antibodies etc. in serum has been known to occur in pregnant women [7, 19]. Serum containing these factors also inhibits the production of IL-2 [15, 17].

It is possible that the production of IFN- γ is also suppressed in perinatal cows because of the decreased production of IL-2, and an increase in immunosuppressive factors occurs, as mentioned above.

Further studies are necessary to clarify the mechanism of the decreased production of IFN- γ during pregnancy in cattle.

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