Induction and assay of interferon (IFN) from mitogen-stimulated bovine peripheral lymphocytes

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Abstract

The fundamental study on the interferon (IFN) production in bovine peripheral lymphocytes was carried out by plaque reduction method in MDBK cells using vesicular stomatitis virus (VSV). IFN was induced in bovine lymphocyte cultures by stimulation with 3 IFN inducers (Con A, PHA and OK-432). Con A was the most effective out of the three. The IFN titer had reachd a maximum level at five days incubation in 1×10^7 cells/ml of lymohocyte culture stimulated with 25μl/ml of Con A. Con A induced IFN was characterized by the pH and temperature stabilities. It was unstable at pH 2.0 and 56℃ compared with the relative stabilities of Newcastle Disease virus (NDV)-induced IFN. Moreover, Con A-induced IFN showed no heterologous activity, whereas NDV-induced IFN had a little. From these results, it was speculated that Con A-induced IFN was so called immune-IFN (γ-IFN). As the result of kinetics of IFN production and blastogenesis, there was a relation between IFN production and the blastogenic activity of lymphocytes.

Key words : interferon, cow, peripheral lymphocytes.

Introduction

Since interferon (IFN)was first demonstrated by Nagano et al.[1] and Isaacs and Lindermann [2], a number of studies on the property of IFN have been made.

It has been recognized that, according to the inducer and the cellular source of IFN, there are three types of IFNs (IFN-α, IFN-β, IFN-γ) that can be produced by mammalian cells. Production of IFN-α (leukocyte IFN) and IFN-β (fibroblast IFN) can be induced by a variety of agents but IFN-γ is generated only when lymphoid cells are stimulated by antigens or T cell mitogens [3, 4].

IFN was discovered by virtue of its direct antiviral activity and subsequent experiments have confirmed that IFN contributes to the antiviral defense mechanism by inducing an antiviral state in cells and tissues. In fact, this may be the major role of the IFN-α and IFN-β whose synthesis is usually induced by virus infecton.

However, INF-γ certainly can exert an antiviral activity on cells as a part of the function of the lymphokine produced by antigen-or mitogen-sensitized T lymphocyte. Including this antiviral

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activity, IFN－γ has three valuable properties : antiviral, immunomodulatory and antiproliferative effects [5]. On the other hand, IFN－γ has some differences in its physico-chemical characteristics compared to IFN－α and IFN－β ; it was shown to be relatively unstable at pH 2.0 and 56℃ [6].

Recently, a variety of animal species has been found to produce IFN. In human and mouse systems, mitogen-induced IFN (IFN－γ) as well as other types of IFN production have been well documented [7, 8, 9, 10].

In cattle, a few studies have been made on the production of mitogen induced IFN [11, 12], whereas a number of studies have been described about other types of bovine IFN.

The purpose in the present study was to establish the experimental conditions for the production and the assay of IFN titer induced by mitogens *in vitro*, and to compare the IFN production with the blastogenic activity in the bovine peripheral lymphocyte cultures stimulated with moitogens.

Materials and Methods

Blood samples :

Blood were obtained by caudal venepuncture from 14 healthy Holstein cows of 2 to 10 years old, raised on the farm of Obihiro University. They were all in on milking period during the experimental period. The heparinized blood was diluted 1 : 1 with phosphate buffered saline (PBS, pH 7.2) and layered over Ficoll Conray solution (specific gravity 1.077 g／㎤ at 25℃, Ficoll : Pharmacia, Sweden, Conray : Daiichi Pharm, Japan) in the 10㎖ glass tube. After the centrifugation of the tube at 400 g for 30 minutes, the mononuclear cell layer was aspirated. The cell were washed 3 times with PBS and twice with the culture medium (RPMI 1640 contained 10% fetal calf serum). The cells were resupended in the medium and examined for lymphocyte recovery rate, purity and viability. In this experiment, the mean recovery rete was about 75%, the lymphocyte population was more than 95% pure and there were more than 98% viable cells by trypan blue dye exclusion test.

IFN inducers :

Three mitogens were used for IFN inducers : Phytohaemagglutinin P (PHA P, Sigma, USA), Concanavalin A (Con A, Sigma, USA) and a Streptococcal preparation (OK－432, Chugai Pharm, Japan). Each mitogen was reconstituted with sterile PBS and divided into small aliquots and stored at －80℃ until used.

lymphocyte culture :

One milliliter of lymphocyte suspension containing varying number of cells and 100㎕ of IFN inducers were poured into each well of 12 wells plastic plates (Limbro, USA). This plate was incubated at 37℃ in 5% humidified carbon dioxide (CO_2) in the air for varying periods. After the incubation for the fixed time, culture fluids were harvested and centrifuged at 400 g for 10 minutes. The supernatant fluid were stored at －80℃ until the assay for IFN activity.

Preparation of bovine fibroblast IFN :

Newcastle disease virus (NDV) -induced IFN was prepared according to the method of Kono [13] : bovine testicles were collected aseptically from neonatal calves at Tokachi slaughter house. After trypsinization of the testicles, the cells were prepared. The monolayer cultures were allowed to grow in 50㎖ of culture bottles with 5% Eagle's minimum essential medium (EMEM) for IFN production. After the incubation for 72 hours at 37℃, the cultures were washed with Hank's balanced salt solution (HBSS) and inoculated with 5 pfu／cell of NDV (grown in 10days old embryonated eggs). After one hour, the inoculum was washed 3 times with HBSS and replaced by EMEM containing 5% fetal calf serum (5% EMEM). The culture fluid was centrifuged at 3,000 rpm for 20 minutes, and the supernatant fluid was collected. Salting out in 80% saturated solution with $(NH_4)_2SO_4$ centrifuged at 12,000 rpm for 30 minutes, the sediment was resuspended in 20㎖ of PBS. The fluid was dialyzed against

0. 05M glycine-HCl buffer (pH 2. 0) containing 0. 1M NaCl at 4 ℃ for 24 hours, and then redialyzed against PBS at 4 ℃ for 24 hours. The fluid was ultracenterifuged at 100, 000 g for 2 hours at 4 ℃, and the supernatant was stored at −80℃ until used.

Assay for IFN

IFN assay was performed by the plaque reduction method in MDBAK cells (Flow Laboratories Inc., USA) using vesicular stomatitis virus (VAV) as the challenge virus [14]. MDBA cells were cultured in 12−wells multiplates with Ham's F−12 medium (Flow Laboratories Inc., USA) contained 10% fetal calf serum (10% Ham's F−12). Serial three fold dilutions of IFN samples were mede in 1 % Ham's F−12 and 1 ㎖ of each dilution was added to triplicate cultures of MDBK cells.

After incubation for 24 hours, the cultures were washed with HBSS and inoculated with approximately 50 plaques of VSV. One hour later, the cultures were overlaid with 1 ㎖ of 1 % Ham's F−12 containing 1 % methylcellulose. After reincubation for 48 hours, the wells were rinsed and fixed with 5 % formol PBS and stained with 0. 1% crystal violet solution. IFN titers were calculated as a reciprocal of the dilution causing 50% reduction in the number of plaques.

Characterization of INF :

Characterization of INF produced in lymphocyte cultures by stimulation with Con A was performed by the following treatments.

Trypsin ; 10㎖ of IFN sample was incubated in the presence of 0. 5㎎/㎖ trypsin (Sigma, USA) for one hour at 37℃, and then 0. 25㎎/㎖ of trypsin inhibitor from soybean (Sigma, USA) was added to inhibit the residual trypsin actvity.

Centrifugation ; An aliquot of IFN sample was ultracentrifuged at 100, 000 g at 100, 000 g for 2 hours at 4 ℃, and the supernatant fluid was collected.

Acidification ; Five ㎖ of IFN sample was dialyzed against 0. 05M glycine-HCl buffer (pH 2. 0) containing o. 1M NaCl at 4 ℃ for 24 hours, and then redialyzed against PBS at 4 ℃ for 24hours.

Heating ; Five ㎖ of IFN sample was heated at 56℃ in a water bath for one hour.

Heterogeneous activity ; the heterogeneous activity of IFN samples were examined by titration of antiviral activity in murine fibroblast cell cultures (L−929).

Glucose consumption test :

The glucose concentration of the culture fluid was determined by using a commercial kit (GL−V, Kyokuto, Japan). The blastogenic activity of lymphocytes was estimated by glucose consumption test as described by ISHIKAWA and SHIRAHATA [15]. The blastogenic activity of lymphocytes was expressed by the glucose consumption index (GCI) as follows :

$$GCI = \frac{※(G)\text{medium} - (G)\text{mitogen stimulated culture}}{(G)\text{medium} - (G)\text{control culture}} \times 100$$

※(G) = glucose concentration

Results

1. Effect of concentration of IFN inducer on IFN production in boving lymphocyte cultures :

In the first experiment, each INF inducer was diluted with RPMI1640 medium in various concentrations and added to lymphocyte cultures of 5 x 10^6 cells/㎖, then the cultures were incubated for 4 days. As shown in Fig.1 the significant amount of IFN was produced in each culture. The dose response curves of IFN production by stimulation with PHA and Con A were similar, and dose-dependent each other. The maximum titrer of IFN were recognized in lymphocyte cultures stimulated with more than 20.0 ㎍/㎖ of PHA and with 25 ㎍/㎖ of Con A. In contrast, the response of IFN induced by OK−432 was not dose-dependent. The optimum dose was determined as 20 ㎍/㎖ in PHA, 25 ㎍/㎖ in Con A and 10 ㎍/㎖ in OK-432, respectively.

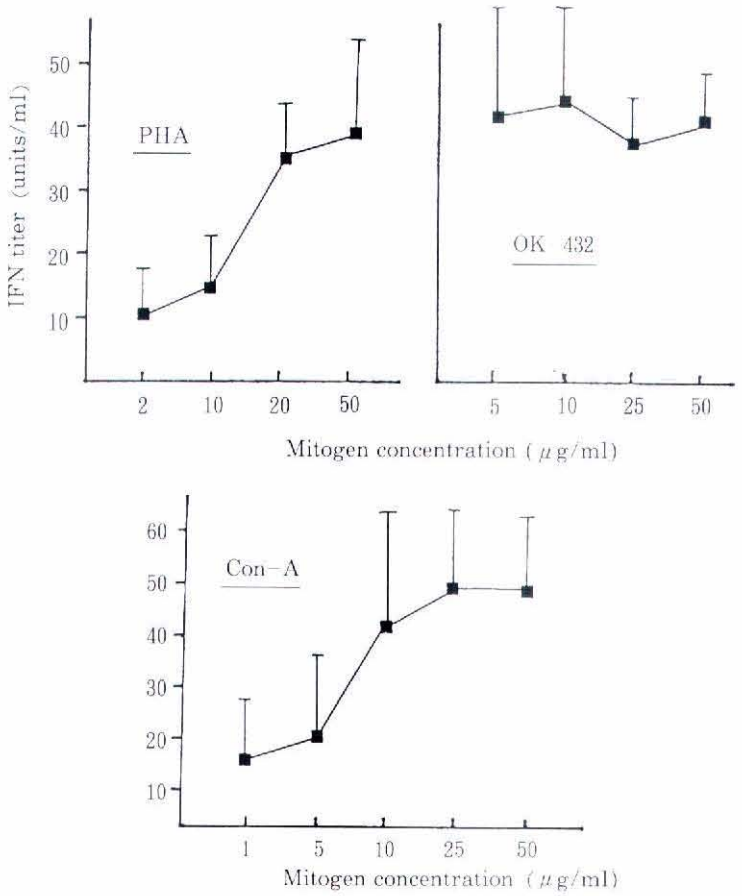

Fig.1 Effect of the concentration of IFN inducers (PHA, Con A and OK-432) on INF production in bovine lymphocyte cultures (N=5).

In the following experiment, the production of IFN stimulated with each optimum dose of the three inducers was compared in the identical bovine lymphocyte cultures. As shown in Table 1, the titer of IFN was the highest in the cultures stimulated with Con A, followed by PHA and OK 432. No IFN production was observed in the control cultures. Therefore, Con A was the most effective inducer out of the three, and used as the IFN inducer throughout the following experiments.

Table 1 Comparison of IFN titers in bovine lymphocyte cultres stimulated with PHA, Con A and OK-432

PHA (20 μg/mℓ)	Con A (25 μg/mℓ)	OK-432 (10 μg/mℓ)	Control
55.8±33.0*	93.0±34.9	31.4±17.8	0**

* units/mℓ (mean of 5 experiments)
** with culture medium

2. Effect of lymphocytes concentraion on IFN production :

The lymphocytes were diluted in the culture medium to cell concentrationd varying from 5×10^6 to 1×10^7 cells/㎖. IFN production in lymphocyte cultures were stimulated with 25 ㎍/㎖ of Con A for 4 days. As shown in Fig. 2, the titer of IFN elevated with the increasing of the cell concentration, and the highest titer was observed at 1×10^7 cells/㎖. Increasing the cell concentration of more then 1×10^7 cells/㎖ may result in much higher production of IFN. In the present study, however, the lymphocyte concentration of 1×10^7 cells/㎖ was used for considering routine ues.

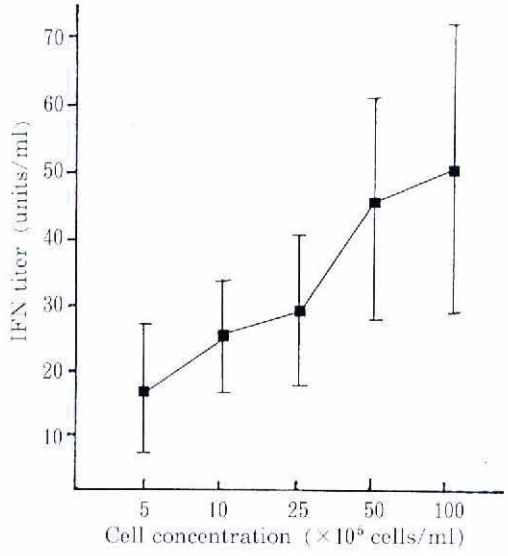

Fig.2 Effect of cell concentration on IFN production in bovine lymphocte cultures stimulated with Con A. (N=5)

3. Kinetics of IFN production and blastogenesis in the bovine lymphocyte culture stimulated with Con A, PHA and OK-432.

The influence of incubation period on IFN production were examined daily from 1 to 6 days in the lymphocyte cultures of 1×10^7 cells/㎖ stimulated with 25 ㎍/㎖ of Con A, 20㎕ of PHA and 10㎕ of OK-432, respectively.

As shown in Fig. 3, in kinetice of the IFN production, the titers of IFN by Con A, PHA and OK-432 were first detected after one day after the incubation and rose rapidly after that. They reached the maximum levels at 3 days in OK-432, 4 days in PHA and 5 days in Con A stimulated culture and subsequently declined. From these results, the IFN samples were collected at 5 days after stimulation with Con A in the following experiments. In the kinetics of blastogenesis (Fig. 4), the glucose consumption had increased rapidly in 3 days of incubation, and mildly after that. The titers of IFN increased almost in parallel with the glucose consumption of lymphocytes stimulated with Con A.

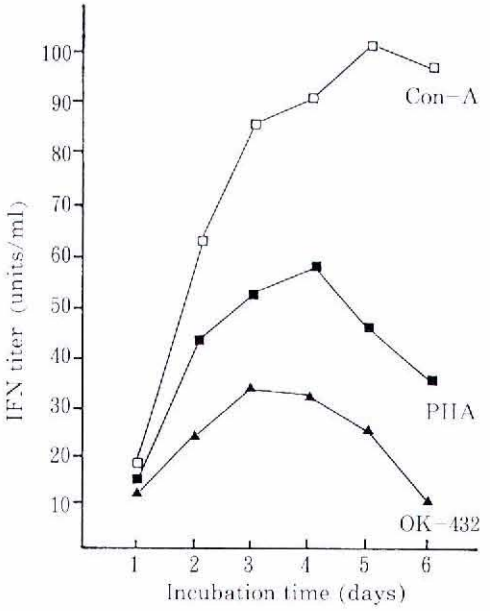

Fig.3 Effect of cell incubation period on IFN production in bovine lymphocyte cultures stimulated with Con A, PHA and OK-432. (N=5)

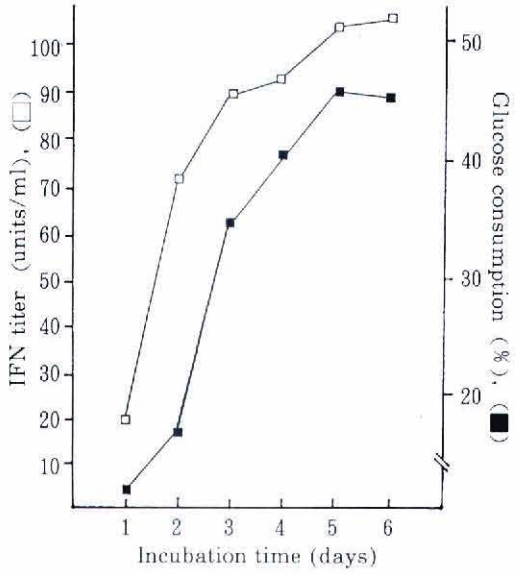

Fig.4 The co-relationship with INF production and lymphocyte blastogenesis in bovine lymphocyte cultures stimulated with Con A. (N=5)

4. Properties of Con A-induced and NDV-induced INFs :

The properties of two types of IFNs are shown in Table 2. Both IFNs were nondialyzable, inactivated by trypsin treatment, and not sedimented at 100,000 g. On the other hand, Con A-induced IFN was unstable at pH 2.0, whereas NDV-induced IFN was relatively stable. The titer of the former IFN retaind only below 4 % of original titer as compared with 54 % of the titer of the latter IFN. These IFNs were also shown to differ in their temperature stabilities. Con A-induced IFN was unstable at 56℃ for 60 minutes, though the titer of NDV-induced IFN maintained 35 % of original titer. In heterologous activity, Con A-induced IFN was no effective in protecting murine cells from VSV, but NDV-induced IFN was a litte.

Table 2 Properties of Con A induced IFN and Newcastle disease virus (NDV) induced IFN

Treatment	Relative titers of IFN (%)	
	Con A-induced	NDV-induced
None (original titer)	100	100
Dialyzation (PBS, 4℃, 24hr)	98*	100*
Trypsin (0.5mg / mℓ, 37℃)	<4	<4
Ultra-centifugation(100,000 g, 2 hr)	100	92
Acidification(pH 2.0, 24hr)	<4	54
Heating(56℃ 1 hr)	<4	35
Heterogenous activity (on murine cell, L-929)	<4	10
Virus specificity(Menngo-virus)	96	98

* relative IFN titer against original titer

Discussion

As the results of this study, the optimum conditions to obtain the higher IFN titers in the bovine peripheral lymphocyte cultures were determined as follows ;

(a) lymphocyte concentration was 1×10^7 cells / mℓ

(b) 25 μg / mℓ of Con A, 20 μg / mℓ of PHA and 10 μg / mℓ of OK-432 was added to each culture, respectively.

(c) incubation period of lymphocyte culture was 5 days (Con A), 4 days (PHA) and 3 days (OK-432), respectively.

Luna et al. [12] reported that the IFN production in response to Con A and PHA stimulation continued to rise for at least three days. In the present study, however, the kinetics of IFN production showed that the titer of IFN was still rising at 72 hours of incubation in the Con A and PHA simulated lymphocyte cultures and reached a peak level at 5 days (Fig. 3). So, it may suggest that the incubation period of 5 days is necessary for the significant amounts of IFN to

be obtained. In the present study, though the significant amounts of IFN were observed in the mitogen-stimulated lymphocyte cultures, the titers of IFN were consistentiy lower compared to other types of INF which have been reportrd in cattle [12, 13, 16]. The difference of these activities might be explained by the paper described in murine spleen cell culture [17] : the presence of an inhibitor for IFN-γ activity was responsible for low activity of the IFN-γ.

Recently, Hughes et al.[18] reported that bovine mitogen-induced IFN levels were only 27.3 units/$m\ell$. Whereas, in the present data, more amounts of IFN (89.0 units/$m\ell$) was observed in the lymphocyte cultures which was incubated under the optimum conditions. However, further studies are required to standardize the culture conditions for IFN assay.

Animal IFNs have been characterized by their pH and temperature stabilities [6, 19, 20]. Generally, in contrast to the virus-induced IFN, the mitogen-induced IFN is unstable at pH 2.0, and 56℃ for 60 minutes. In the present study, Con A-induced IFN was also unstable at pH 2.0 and at 56℃ indicating that so called immune-IFN (IFN-γ) was dominant, whereas NDV-induced IFN was comparatively stable under these conditions. This result agreed with the results of previous reports [11, 21, 22, 23], and suggested that these two types of IFN were shown to differ in their pH and temperature stabilities. As there has been no available specific antibodies to bovine IFNs, it was unable to identify and separate the IFN type in this study.

Both bovine NDV-and Con A-induced IFNs were examined for the heterologous activity to protect murine fibroblast cells to infection with the viruses. The results suggested that Con A induced IFN had no heterologous activities in murine cell, whereas NDV-induced IFN had a little (Table 2). Although a number of reports have described heterologous activities among mammalian IFNs [12, 16, 24, 25], the results have been variable. Babiuk and Rouse [16] reported that bovine immune-IFN, in distinction from bovine fibroblast-IFN, showed considerable heterologous activities in many species of cells. On the other hand, Lunaet al. [12] reported that bovine fibroblast-IFN was tha most effective in protecting murine cells (L-929) to infection with viruses among three types of IFNs. It is a well known fact that IFN-β and IFN-γ have shown a little heterologous activities, whereas IFN-α has shown marked ones [24, 25].

Green et al. [8] reported that marked quantitative difference existed between the degree of blastogenesis and the amounts of INF-γ in human lymphocyte culrures. In contrast with their reports, the data presented in this paper indicated that the most effective concentration of mitogen stimulating blastogenesis described in a previous paper [15] consisted with that of the mitogen for IFN induction (Fig. 1). Maximum production of Con A-induced IFN occurred during 5 to 6 days in the cultures (Fig. 3), and in these periods, the blastogenic activity also reached maximum level in the same cultures. Moreover, in the instance where very little or no blastogenesis had been induced, no IFN production was usually found (data not show). Friedman and Cooper [7] showed that IFN production stimulated with mitogens was related to the events involved in the mitotic cycle of lymphocytes. Therefore, the present data suggested that there was a relation between the mitogen-induced IFN production and the blastogenic activity of lymphocytes.

It has been well recognized that IFN-γ induction played a part of immune response, especially in the stimulation by mitogens or some antigens in human [8] and mouse [26]. The present study supports that such mechanism may be applied to bovine lymphocyte cultures, and indicates that the assay for mitogen-induced IFN in bovine lymphocyte cultures may provide one of the useful methods to evalutae bovine immune status *in vitro*.

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ウシ末梢血リンパ球のマイトジェン刺激によるインターフェロン産性能について

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インターフェロン(IFN)は，その抗ウイルス活性，抗腫瘍活性ならびに生体の免疫応答能の修飾など多様な作用を持つ生理活性物質として知られている。インターフェロンは特異抗原の他にも非特異的マイトジェン刺激によっても生産される。マイトジェン刺激によるインターフェロンの誘発はヒトおよびマウスにおいて数多く検討されているが，ウシではその報告は比較的少ない。そこで今回，ウシ末梢血リンパ球のマイジェン刺激によるインターフェロン産生能についての基礎的な検討を行った。

実験には本学付属農場に飼育されているホルスタイン乳牛のうち，乾乳期の2～10歳の乳牛14頭を用いた。インターフェロンの測定はMDBK細胞とVSVを用いたプラーク半減法により行った。すなわち，ヘパリン加静脈血より比重遠心法により分離したリンパ球にPHA，ConAおよびOK－432の3種類のマイジェンを加え培養し，その培養上清中に産生されたインターフェロンの力価を測定した。対照培養に対してプラーク数が半減した試料の希釈倍数をインターフェロン力価（unit／mℓ）とした。使用した3種のマイトージェンの内，ConA刺激では細胞数1 x 10^7／mℓの培養系に対して25μℓ／mℓのConA添加で120時間培養後に最大のインターフェロン力価を示した。これらの条件下で産生されるインターフェロンは，NDVにより産生されるインターフェロンの性状と比較した場合，酸（pH2）および温度（56℃）に対して不安定であることからインターフェロンγ（IFN－γ）であろうと推定された。また，これらのリンパ球培養と同一条件下で培養した培養系におけるリンパ球幼若化反応をグルコース消費試験で測定した結果，インターフェロン産生能はリンパ球幼若化能と良く相関することが認められた。

以上の成績より，ウシ末梢血のマイジェン刺激培養によるインターフェロン産生能の測定はウシの免疫機能評価の一手段として有効であると考えられた。

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