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0014-4754/84/121407-04$1.50 + 0.20/0
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Properties of bovine interferons[1]

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Summary. This study was done in an attempt to elucidate some of the properties of bovine IFNs. Maximum levels of both fibroblast and leukocyte IFNs occurred prior to 24 h whereas maximum levels of immune IFN were not reached until after 72 h. The latter species of IFN was unstable at either pH 2 or 56 °C whereas both the fibroblast and leukocyte IFNs were more stable under these conditions. Studies of cross-species protection between fibroblast and leukocyte IFNs indicate that the former was more protective for other species than the latter.
Key words. Interferon; bovine interferon.

A number of studies have been published on the production of bovine interferons (Bov IFN). These reports have described some of the properties of these IFNs, however, no definitive effort has been made to separate and classify them. Because studies have not been published on the molecular species we will use the 'classical' terms of leukocyte, fibroblast and immune to describe the properties of these IFNs. This paper is an attempt to elucidate some of the properties of Bov IFNS.

Materials and methods. Media used for growth and maintenance of fibroblast cells were Eagles MEM with 10 and 2% fetal calf serum (FCS), respectively[3]. RPMI with 10% FCS was used for leucocyte cultures. All media contained 0.25% gentamicin. Viurses used were Sendai, Newcastle disease virus (NDV), and vesicular stomatitis virus, Indiana strain (VSV). Sendai virus and NDV were grown in embryonated eggs. VSV was grown in cultures of bovine cells and titrated by plaque formation (PFU) in MDBK cells. Confluent cultures of bovine kidney cells (MDBK) were the primary source of fibroblast IFN and were used in the IFN assays. Other bovine cells employed included bovine embryonic kidney (BEK), embryonic bovine trachael cells (EBTr), and bovine turbinate (BT). All of which were purchased from the American Type Culture Collection, Rockville, MD. Nonlactating dairy Holstein cows were used as the source of bovine leukocytes. Leukocytes were separated from whole blood by means of a selective hypotonic lysis procedure developed in this laboratory[4]. Briefly, bovine blood was collected by jugular venipuncture and mixed with distilled water containing 0.3% galactose in a 1:1.5 ratio of blood to galactose solution respectively. After 3 min, 1.5 parts of cold buffered saline gelatin (BSG) at pH 7.2 was added. Leucocyte suspensions were centrifuged, washed in BSG and resuspended in RPMI-1640 to a final concentration of 5×10^6 cells per ml. When only lymphocytes were needed, aliquots of leukocytes, obtained by the 'lysis' procedure described above, were centrifuged on Ficoll-Paque (Pharmacia, Piscataway, NJ).

For the production of fibroblast IFN four bovine cell types were employed: BEK, MDBK, EBTr, and BT. Triplicate cultures of each cell type were established on plastic tissue culture flasks. The virus (NDV or Sendai) was added to cell monolayers as previously described[1]. After adsorption the virus was decanted, the monolayers washed and 10 ml of MEM were added. After 24 h incubation, the fluids were collected and the virus inactivated by irradiation for 15 min at 12 cm from an UV light (15 W) source. Poly I:C was used at a concentration of 0.01 mg/ml along with DEAE-dextran at 0.1 mg/ml. IFN production during the assay was prevented by one of the following: removing the inducers, UV inactivation or RNAase treatment.

For the production of leukocyte IFN the virus was added to the leukocyte suspensions and allowed to adsorb for 1 h. The cells were then washed, resuspended in RPMI-1640, and incubated for 24 h at 37 °C under 5% CO_2. The fluids were collected and frozen for subsequent assay.

Immune type IFN was obtained from lymphocytes induced with con A. The lymphocyte population was obtained as previously described. The mitogen was added at a concentration of 0.01–0.05 mg/ml. Tissue culture fluids, collected at various intervals post-induction, were frozen at −20 °C until assayed. The pH stability of the different IFNs was evaluated by dialyzing preparations against 100 volumes of pH 2 buffer. After dialysis for 24 h, the samples were redialyzed against pH 7.2 buffer for 24 h. IFN preparations were also subjected to 45 °C and 56 °C for different time periods, then assayed along with control preparations. The activity of IFN on both homologous and heterologous cells was determined using infection by VSV. This protection was compared with that obtained using MDBK cells. Prior to the assays, the stock VSV was titered on each cell type to determine the amount of virus required for the production of 30–50 PFU per well.

IFN was assayed using MDBK cells in a microplaque reduction assay using VSV. IFN preparations were serially diluted directly in the microtiter dishes. MDBK cells were then added, and the plates were incubated overnight at 37 °C under 5% CO_2. The cells were challenged with 30–50 PFU of VSV which

were allowed to adsorb for 1 h. The virus was decanted and the cells overlaid with 0.5% methylcellulose in maintenance medium and incubated for an additional 18–24 h. IFN titers were calculated as a reciprocal of the dilution causing 50% reduction in the number of plaques.

Results and discussion. Preliminary studies were concerned with the selection of the appropriate IFN inducers and the best producer cells. NDV was found to be the most effective IFN inducer in both MDBK cells and in leucocytes (fig.). Exposure of NDV to UV light reduced its effectiveness as an IFN inducer. In addition two immune IFN inducers, con A and PHA, were also tested in leukocytes, with con A being the most effective. Con-A induced IFN was used throughout the balance of these studies. It was noted that IFN concentrations induced by either the viruses or double stranded RNA generally reached maximum levels prior to the 24 h, whereas IFN concentrations induced by either con A or PHA continued to rise for at least three days (fig., b).

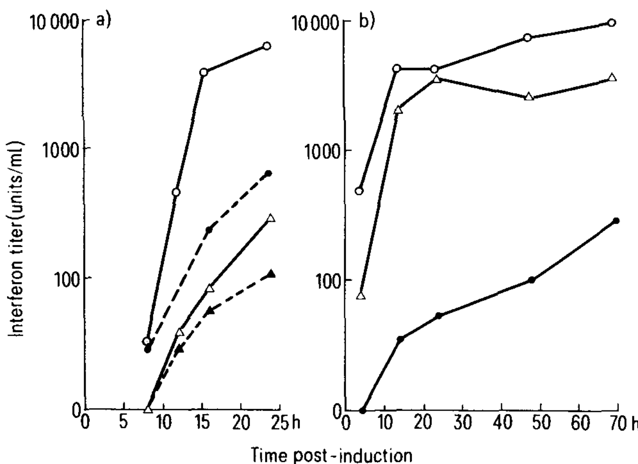
Throughout these studies, mixed leukocyte populations were used to produce the leukocyte IFN. Because of the low titers obtained as well as the possibility that there may be inhibitors released by granulocytes, the population was separated by centrifugation. Lymphocytes were approximately four times more effective producers of immune interferon when separated from other leukocytes (table 1). Stability of the various IFNs differed following exposure to 56°C. Immune IFN was the least stable with much of its activity lost by 15 min at this temperature. Leukocyte IFN was the most stable and fibroblast IFN was intermediate at this temperature (table 2). Immune IFN was the least stable at pH 2. There was little difference between the activity of leukocyte and fibroblast IFN following exposure to pH 2. Cross-species protection by bov IFNs has been reported previously. IFN produced by fibroblasts protected heterologous cells greater than leukocyte IFN (table 3). This was quite noticeable in virtually all of the species tested including other bovine cell types.

The IFN preparations used in these studies were 'crude' tissue culture fluids. It is possible that some of their 'activities' could be altered by factors other than IFN. All three types of IFN were nondialyzable or sedimentable at 105,000 × g and appeared to be proteins since activity remained intact after ribonuclease treatment but was lost after exposure to trypsin.

In these studies, NDV was the most effective inducer. MDBK cells were the most effective producers of IFN. It is possible that MDBK cells synthesize more IFN messenger RNA than the other bovine cell types and/or may have more receptors for the inducer molecules. It is also possible that the various cell types could have different control mechanisms which affect the production of specific repressors. It may not, however, be ruled out that the higher IFN levels obtained were the results of culture conditions including different cell densities.

When the rates of synthesis of leukocyte, fibroblast, and immune IFN were compared, the former two types of IFN reached maximum levels earlier than immune IFN which took more than 72 h. This is in agreement with other workers[5,6]. Babiuk and Rouse[5] reported that bovine immune IFN induced by IBR virus was derived from T lymphocytes and that when T lymphocytes were combined with autologous macrophages, there was a 2–10-fold increase in IFN synthesis. This was confirmed by others[7]. In the present studies, higher titers of IFN were produced by isolated lymphocytes. It is possible that certain of the leukocyte populations produced inhibitors of IFN production, i.e. prostaglandins. Another possiblity could be that the amount of IFN could relate directly to the number of T lymphocytes in a mixed population of leukocytes simply reflecting the number of cells producing IFN.

Animal IFNs have been characterized by their pH and temperature stabilities[8–10]. It is generally accepted that immune IFNs are unstable at pH 2, whereas stability at 56°C varies considerably depending on the IFN source. Observations made in regard to Bov IFN in this study generally agree with the reports of others[11–16]. Bovine leukocyte and fibroblast IFNs were both relatively stable at pH 2, whereas immune IFN was not. Leucocyte and fibroblast IFNs retained at least 40% of their original titer as compared to only 4% of immune IFN. Three IFN



a Synthesis of interferon by bovine fibroblasts. 40 HA untis of NDV/ml were used to induce IFN synthesis in MDBK cells, ○—○, EBTr cells, △–△, and BT cells, ▲–▲. Also, 40 HA units of UV irradiated NDV/ml were added to MDBK cells, ●–●. Results represent the mean of four experiments. *b* Comparison of inducers of interferon synthesis by leukocytes. Leukocytes were exposed to 10 µg of Con A/ml, ●–●, 10 µg of poly I:C/ml, △–△, and 40 HA units of NDV/ml, ○—○. Results represent the mean values of four experiments.

Table 1. Comparison of immune IFN titer* between leukocytes and isolated lymphocytes

Leukocyte	Lymphocyte
147[b] ± 62	533 ± 75

* Average of four experiments. ** Units IFN/ml.

Table 2. Stability of bovine interferons[a]

IFN type	pH 2	45 °C	56 °C	Trypsin[b]	Ribonuclease[b]
Leukocyte	65	109 ± 9	90 ± 30	2 ± 0.8	98 ± 2.3
Fibroplast	78	59 ± 16	53 ± 13	4 ± 1.2	99 ± 1.2
Immune	4	ND	15 ± 9	2 ± 0.6	97 ± 0.9

[a] Percent of the control titers. [b] 0.05 mg of trypsin/ml of sample. Incubated at 37 °C/30 min. [c] 0.10 mg of ribonuclease/ml sample. Incubated at 37 °C/30 min.

Table 3. Cross-species protection

Cell line	Relative protection by bovine IFNs[a]	
	Leukocyte	Fibroblast
MDBK[b]	100	100
EBTr[b]	43 ± 19	100 ± 7
BT[b]	80 ± 11	95 ± 8
CT[c]	78 ± 14	77 ± 17
Vero[d]	0	0
Wish[e]	5 ± 3	80 ± 9
MRC-5[e]	7 ± 2	63 ± 7
HEp-2[e]	2 ± 1	55 ± 16
L-929[f]	32 ± 14	84 ± 11

[a] Percent of titer on MDBK cells, average on three or more experiments. [b] Bovine. [c] Feline tongue cells. [d] Primate. [e] Human. [f] Murine.

types were shown to differ in their temperature stabilities. Leukocyte IFN was the most stable at 56 °C, whereas immune IFN was the least stable. Fibroblast IFN was intermediate. Instability of bovine fibroblast IFN has also been reported previously[17]. Differences were also noted regarding stability at 45 °C where fibroblast IFN lost 40% of its original titer, whereas leukocyte was completely stable. Because of the lack of specific antibodies to these IFNs, it is difficult to adequately characterize and separate the various classes.

A number of reports have described cross-species activities between mammalian IFNs[18–21]. In the present study both bovine leukocyte and fibroblast IFNs were tested for their ability to protect heterologous cells. Results indicated that bovine fibroblast IFN was more effective in protecting heterologous cells than leukocyte IFN. This was in contrast to results reported by Carter et al.[24] using porcine IFN. It is possible that this could relate to the extent of glycosylation, and/or purity, however, data in this area are not available.

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0014-4754/84/121410-03$1.50 + 0.20/0
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T cell and T cell subset determination in normal peripheral blood: comparison of the indirect immunofluorescence and lymphocytotoxicity techniques

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Summary. We compared a simple complement-dependent lymphocytotoxicity test with a widely used indirect immunofluorescence procedure to enumerate total T, T helper, and T suppressor lymphocytes in normal blood samples. Results with the two techniques were closely similar.
Key words. Blood, peripheral; T cells; immunofluorescence technique; lymphocytotoxicity technique.

Perturbations in immunoregulatory T lymphocyte dynamics have been observed in a variety of immunopathologic disorders[1], and determination of circulating T helper and T suppressor cells has become a common procedure in both clinical and research laboratories. T cell subset analysis has been most commonly performed by indirect immunofluorescence, using either manual or cytofluorographic techniques[2,3]. However, a simple manual technique which requires only a standard laboratory microscope for the visualization and enumeration of T helper and T suppressor cells would be an advantage for a small laboratory. In principle, it should be possible to use a lymphocytotoxicity test for T cell enumeration since the monoclonal anti-T cell antisera fix complement[4].

Materials and methods. We studied total T, T helper, and T suppressor cells in 14 healthy subjects (9 males, 5 females; age 27–45 years) using complement-dependent lymphocytotoxicity and the standard manual indirect immunofluorescence techniques in parallel.

Peripheral blood mononuclear cells were isolated by ficoll-hypaque density gradient centrifugation[5]. We used murine monoclonal antisera termed OKT3, OKT4, and OKT8 to characterize all T, T helper, and T suppressor cells respectively. Lymphocytotoxicity was determined by trypan blue exclusion using rabbit serum as the source of complement. 80 µl of mononuclear cell suspension (concentration 2.5×10^6 cells/ml) and 10 µl of appropriately diluted OKT3, OKT4 and OKT8 antibody, and phosphate-buffered saline (control) were placed in 12 × 75 mm round bottom glass test tubes and incubated for 5 min at 37 °C. 10 µl of rabbit serum was added to each test tube which was then further incubated for 45 min. Thereafter, 100 µl of 0.2% trypan blue solution were added and 10 min later, 1–2 drops of the cell suspension were placed in a Neubauer hemocytometer. The number of living and dead cells were counted using a standard light microscope equipped with a 40 × objective. Approximately 200 cells were counted and all determinations were performed in duplicate. Lymphocytotoxicity was expressed as the percentage of cells killed by each antiserum. The indirect immunofluorescence technique was performed according to the procedure recommended by the serum supplier (Ortho Pharmaceutical, Raritan, N.J.). Briefly, aliquots of 1×10^6 mononuclear cells in 200 µl of wash medium (RMPI-1640, 5% fetal calf serum, 25 mM hepes, Grand Island Biological, Santa Clara, Ca) were pelleted in four separate 12 × 75 mm round bottom glass test tubes, and resuspended in 5 µl of reconstituted monoclonal antibody solution, and phosphate-buffered saline (control), respectively. After 30 min incubation on ice followed by two washes with wash medium, the cells were incubated for 30 min on ice with a 1:20 dilution of a fluorescein-conjugated goat antimouse immunoglobulin (Meloy Laboratories, Springfield, Va). The cells were