

## Rapid Production of Interferon in Bovine Leucocyte Cultures. (31689)

YUJI KONO (Introduced by Monto Ho)

*Equine Infectious Anemia Research Division, National Institute of Animal Health, Tokyo*

The reticuloendothelial system (RES) has been shown to participate in the clearing of virus particles from the circulation during the course of viremia(1,2). It would appear reasonable, therefore, to postulate that leucocyte contributes to a rapid formation of interferon (IF) in the body after exposure to virus. This postulate is supported by the fact that IF is produced rapidly in high titer by organs rich in phagocytic elements (*i.e.*, the RES) *in vivo* and *in vitro*(3). If the same mode of IF production were found in leucocyte cultures, a new definition would be given to the activity of leucocytes. Experiments herein reported were performed to determine whether this presumption was true. An attempt was made to develop a method for obtaining rapid production of IF by bovine leucocytes.

*Materials and methods. Viruses.* The TCND strain of Newcastle disease virus (NDV) was grown in 10-day-old embryonated eggs and partially purified by ultracentrifugation. The titer of the stock virus was  $10^{9.5}$  TCD<sub>50</sub> per ml in chick embryo cell culture. BF<sub>1</sub> virus(4) was used for IF assay. The virus was propagated in bovine kidney (BK) culture.

*Antiserum.* NDV antiserum was prepared in rabbits. The serum was inactivated at 56°C for 30 minutes before use. One-twentieth of 1 ml neutralized  $10^{7.5}$  TCD<sub>50</sub> of NDV.

*Tissue culture.* For cultivation of bovine leucocytes (BL), bovine blood was obtained from a healthy donor, defibrinated, and centrifuged at 3,000 rpm for 30 minutes. The resultant buffy coat was washed once in Hanks' solution and trypsinized for 30 to 60 minutes. The washed leucocytes obtained were suspended in calf serum at a concentration of  $2 \times 10^7$  cells per ml. Four ml of the suspension was placed into a 50 ml bottle. The medium was changed after 1 and 7 days of incubation. BK culture was prepared after trypsinization of kidney minces and al-

lowed to grow in 50 ml bottles for IF production or in tubes ( $1.2 \times 10$  cm) for IF assay.

*Interferon production.* Duplicate BK cultures or BL cultures were inoculated with 2 ml of  $10^{-1}$  NDV and incubated. After 1 hour the inoculum was removed and replaced by 4 ml of medium (Hanks' lactalbumin hydrolysate solution containing 10% calf serum). At each time indicated after the NDV inoculation, 1 ml of culture fluid was removed and replaced by the same volume of medium. In the case of BL cultures, NDV was inoculated on day 0 at the designated input multiplicity in 2 ml of suspension. One-hour material was harvested by low-speed centrifugation (500 rpm for 5 minutes). The resultant packed cells were resuspended in 4 ml of new medium and incubated further. At each of the indicated time periods, 1 ml of suspension was collected and centrifuged. The resulting supernatant was harvested. The sedimented cells were restored in the original culture and replaced by 1 ml of medium. Incubation of all cultures after NDV inoculation was carried out first in a water bath at 37°C for 6 hours and then in an incubator in the conventional manner.

*Interferon assay.* An IF material was mixed with an equal volume of diluted immune serum (1:20). The mixture was diluted in medium containing 1:40 of the antiserum and allowed to stand at 37°C for 1 hour to inactivate NDV. Then 0.3 ml of the mixture was inoculated in duplicate BK tube cultures and incubated for 18 hours. These cultures were challenged with 1,000 TCD<sub>50</sub> each of BF<sub>1</sub> virus after the IF material had been aspirated. The IF effect was expressed by the highest dilution in which 50% of the cultures showed inhibition of the cytopathogenicity of challenge virus.

*Results. Cultivation of bovine leucocytes.* The differential leucocyte count before incubation showed that the BL suspension con-

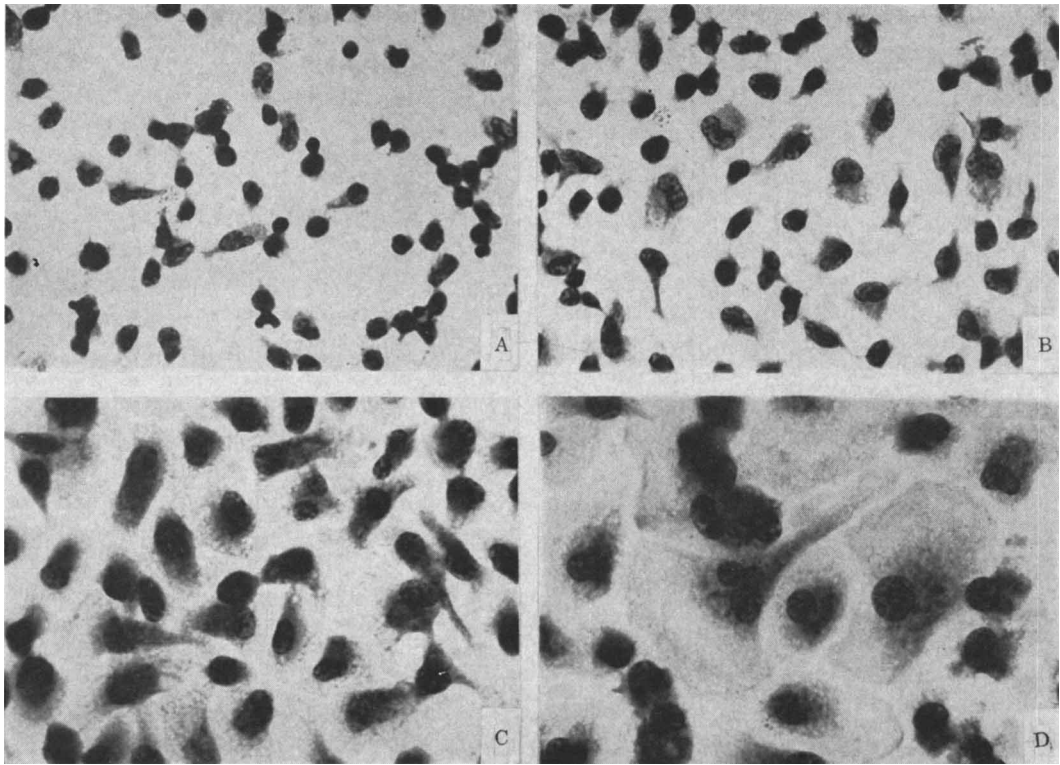


FIG. 1. Features of bovine leucocyte cultures. Remarkable increase in size of nucleus and in amount of cytoplasm are shown. Photographs are demonstrated in a same magnification ( $500\times$ ). A) Day 1 culture. B) Day 3 culture. C) Day 5 culture. D) Day 7 culture.

tained, on the average, 30% polymorphonuclear cells and 70% mononuclear cells. After one day of incubation, 95% of the cells attached to the glass wall were mononuclear cells. Further incubation increased the proportion of mononuclear cells to 100%. The size of the nucleus and cytoplasm increased remarkably up to the 7th day of cultivation. This remained stable until the 12th day. There was no change in total cell count during the 12 days of incubation. Photographs of the cells are shown in Fig. 1. These cells proved to have phagocytic activity against killed *Staphylococcus aureus* during all the stages observed (Fig. 2). They also took large granules of neutral red as demonstrated by the Unna-Pappenheim staining.

*Production of IF in BL and BK cultures.* In Table I, the IF production in BL and BK cultures over a period of 12 days of cultivation is summarized. IF was produced in day 0 BL culture as early as 2.5 hours

after inoculation of NDV. In this culture the titer reached 1:128 and 1:512 at 6 and 24 hours, respectively. In day 1 culture, the titer was 1:8 at 6 hours and then increased to  $>1:512$  at 24 hours. Older BL cultures produced more IF earlier. In day 12 BL cultures, IF could already be detected at 1:2 dilution in 1 hour and reached a peak titer of  $>1:512$  at 4 hours. On the other hand, day 7 and day 12 BK cultures failed to produce IF in 6 hours and showed lower titers of IF (1:64 to 1:128) than BL cultures at 24 hours. The results of another experiment presented in Table II show that IF appeared in BK culture at 12 hours and reached a peak titer at 20 hours. The number of cells required to produce 1 unit of IF, based on the IF titer at 24 hours, was in the range of  $3.1$  to  $4.9 \times 10^4$  in day 0 BL culture and BK cultures. In day 3 to day 12 BL cultures it was in the range of 2.5 to  $4.1 \times 10^3$  (Table I).

TABLE I. Effect of Days of Cultivation on Interferon Production in Bovine Leucocyte Cultures and in Bovine Kidney Cultures.

Culture	Bovine leucocyte					Bovine kidney	
	0*	1	3	5	7	12	12
Days of cultivation							
No. of cells per bottle	$2.5 \times 10^7$	$5.1 \times 10^6$	$2.1 \times 10^6$	$2.0 \times 10^6$	$2.4 \times 10^6$	$1.3 \times 10^6$	$4.1 \times 10^6$
m.o.i.†	127	124	248	250	242	269	250
Hours after viral inoculation							
1.0	<2†	<2	<2	<2	<2	2	<2
2.5	8	<2	2	2	32	64	<2
4.0	8	2	8	8	256	>512	<2
6.0	128	8	128	64	512	>512	<2
24.0	512	>512	512	>512	512	>512	64
No. of cells required to produce 1 unit of IF	$4.9 \times 10^4$	$<1.0 \times 10^4$	$4.1 \times 10^3$	$<3.9 \times 10^3$	$4.7 \times 10^3$	$<2.5 \times 10^3$	$3.1 \times 10^4$

\* Immediately after preparation of bovine leucocyte suspension.

† Input multiplicity of infection.

‡ Reciprocal of IF titer.

*IF production and viral growth.* IF production and viral growth were investigated in the presence of actinomycin D and in control cultures. Day 7 BL cultures were inoculated with  $10^{-1}$  of NDV (m.o.i. = 100) and incubated at 37°C for 1 hour. Then they were washed twice with medium, and further incubated for 30 minutes with 2 ml of medium containing 1:20 of NDV antiserum. After that, they were washed 3 times and incubated further in 4 ml of the medium. After the incubation, 1 ml of the material to be tested was harvested and replaced by the same amount of the medium. In the test with actinomycin D, given at a concentration of 0.1 µg in 2 ml of medium, no remarkable cytotoxicity was observed. It was incorporated 1 hour before NDV inoculation to the end of the experimental period. All samples for IF assay were dialyzed in Hanks' solution for 24 hours to eliminate the inhibitory effect of actinomycin D on IF action(5).

As shown in Table II, in the control group, using BL cultures, IF was detectable (1:32) 3 hours after NDV inoculation, and attained a maximum titer (1:1024) in 5 hours. On the other hand, actinomycin D significantly suppressed the IF production. The titer was 1:4 at 3 hours and reached a peak of 1:8 at 5 hours. There was no difference in NDV growth between the actinomycin D-treated and the control cells. Growth occurred between 3 and 5 hours after NDV inoculation and reached a peak between 5 and 7 hours, showing a virus titer of  $10^4$  TCD<sub>50</sub>. In the BK cultures, IF was not detectable until after 8 hours, and was found in 1:2 dilutions at 12 hours. Then it increased gradually until the 20th hour, when it reached the highest titer (1:32). The virus showed a sharp increase between 3 and 5 hours. After that, the titer increased gradually until 16 hours. The maximum virus titer measured was  $10^{7.0}$  TCD<sub>50</sub>.

*Properties of IF.* The IF obtained from BL cultures was examined for properties in the conventional manner. It was found that it did not inactivate BF<sub>1</sub> virus directly, that it did not inhibit the propagation and CPE of western equine encephalomyelitis virus in chick embryo fibroblast cultures or in rabbit

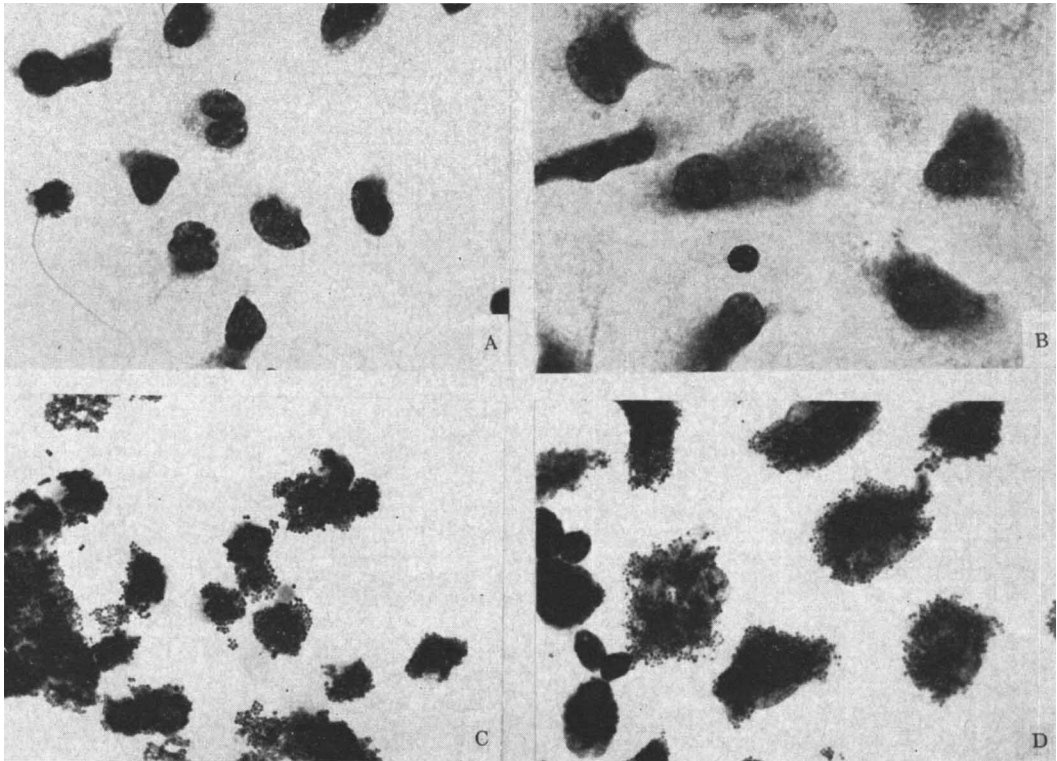


FIG. 2. Phagocytosis by cultivated bovine leucocytes. Many *Staph. aureus* were shown in cytoplasm of leucocytes. A) Normal day 1 leucocytes. B) Normal day 7 leucocytes. C.) Phagocytosis by day 1 leucocytes. D) Phagocytosis by day 7 leucocytes.

kidney cell cultures, that it was not precipitated by centrifugation at 100,000 g for 3 hours, that it was inactivated by trypsin, and that it was inactivated by

heating at 56°C for 30 minutes (the titer dropped from 1:512 to 1:32). Unlike other types of IF, it was inactivated by acidification at pH 2.0 at 4°C for 18 hours (the titer being

TABLE II. NDV Growth and Interferon Production in Bovine Leucocyte Cultures and Bovine Kidney Cultures, and Effect of Actinomycin D on These.

Culture	Treatment	NDV (upper line) and IF (lower line) titers									
		Hours after inoculation—									
		1.5*	3	5	7	8	10	12	16	20	24
Bovine kidney	None	<0†	.5	4.5	—	5.5	—	6.5	7.0	7.0	7.0
		<2‡	<2	<2	—	<2	—	2	8	32	32
Bovine leucocyte	None	.5	0	3.0	4.0	—	4.5	—	—	—	4.0
		<2	32	1024	1024	—	1024	—	—	—	1024
	Actinomycin D†	0	.5	4.0	4.0	—	4.0	—	—	—	4.0
		<2	4	8	8	—	8	—	—	—	8

After adsorption with 2 ml of  $10^{-1}$  NDV for 1 hour, the cells were washed twice, mixed with 2 ml of anti-NDV serum (1:20), and incubated again at 37°C for 30 min. After that, they were washed 3 times and incubated with 4 ml of medium.

\* The first harvest of the fluid (1.5 hr after NDV inoculation) was collected immediately after addition of medium following the final washing.

† Used at 0.1  $\mu$ g in 2 ml per bottle.

‡ Virus titer in  $\log_{10}$ .

§ IF titer expressed in reciprocal of dilution.

|| Not tested.

reduced from 1:512 to 1:32).

*Discussion.* One of the characteristic features of IF formation *in vivo* is the rapid response to stimulation(3,6), as compared with IF formation *in vitro*. In our previous paper(3), we reported on the role of RES cells in the rapid formation of IF in rabbits. It has been further demonstrated that leucocytes, macrophages, or cells belonging to RES contribute to IF production(6-9). Rapid IF production in such cells *in vitro*, however, has been reported to be incidental by a few investigators(3,7). The present study revealed that the IF titer in leucocyte culture reached a peak by 4-6 hours after virus inoculation. On the other hand, the production attained in BK cultures did not reach a peak until after 20 hours of incubation. Moreover, the cultivated BL cultures produced 10 times more IF than the cultivated BK cells. The differences in IF production between these 2 kinds of cells are very similar to those observed between the production of circulating interferon in rabbits and in rabbit kidney cell cultures after the inoculation of Sindbis virus(3). It is possible, therefore, to postulate that cells derived from leucocytes or from the same blast forms may function to bring about the appearance of IF in the circulation *in vivo*. Such cells seemed to be a type of cells belonging to the RES, since cultivated bovine mononuclear leucocytes were phagocytic against bacteria and took up neutral red, as large granules, in their body. Most of these mononuclear cells seemed to develop into macrophages, as described by Carrel *et al*(20) and Dunne *et al*(22).

The reason why BL of different ages of cultivation had a different ability to produce IF remains obscure. It is supposed, however, that after the transformation of mononuclear cells into macrophages(20-22), or after transformation of lymphocytes(11), the transformed cells may acquire a property to produce IF rapidly. This difference in cell response by culture age was not caused by the increase in phagocytic activity which might facilitate the virus to penetrate into cells(12, 13,14) since the cells of all stages of cultivation possessed equal phagocytic activity.

The relationship between IF formation and viral growth in a cell is a particularly interesting theme from the viewpoint of analysis of the defense mechanism of the body. In this experiment it is emphasized that viral propagation was shown in both BL cultures (7-12 days) and BK cultures 5 hours after NDV inoculation. Besides, IF production was found in BL cultures at 3 hours and in BK cultures at 12 hours. This relationship between IF and virus production in BK culture agreed well with that observed generally in tissue culture systems. The rapid onset of interferon production in BL culture, however, was not found in any tissue culture system. The role of phagocytic cells in viral infection *in vivo* has not been understood well. Many investigators have reported the failure of viral reproduction in the cells *in vitro* and *in vivo*(10,12,14-19). On the other hand, virus has been shown to be associated with leucocytes in the viremia of several diseases(23-27). Therefore, leucocytes work as a barrier against infection in some cases, and may have a function to transport viruses in other cases. From the viewpoint of IF production in leucocytes, however, phagocytic cells may have supported viral growth in the present experiment and also have produced IF in high titer earlier than the viral propagation. Therefore, it would be possible that IF could interfere with viral reproduction in target cells when leucocytes had disseminated virus and supported viral multiplication.

*Summary.* Rapid production of IF has been demonstrated in bovine leucocyte cultures. Such production was also greater after 7 to 12 days of cultivation. Under the experimental conditions employed IF was formed before NDV multiplication. From these data obtained, the role of phagocytic cells *in vivo* was discussed.

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