PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 809–815 (1974)

A Method for the Large Scale Production of Potent Interferon Preparations (38196)

MICHAEL G. TOVEY, JACQUELINE BEGON-LOURS, AND ION GRESSER
Institut de Recherches Scientifiques sur le Cancer, 94800-Villejuif, France

Interferon is usually produced by the induction of animal cells cultivated in monolayer cell cultures, a system not readily adapted to the large scale production of interferon. Animal cells, however, can be cultivated in stirred suspension culture in a single vessel, and in virtually limitless quantity. The oxygenation, pH, and nutrient supply can be automatically controlled to yield higher cell populations with a more efficient utilization of nutrient medium than can be obtained in monolayer cultures. In spite of these advantages, it has heretofore not been practical to use this system for the production of interferon since only low yields of interferon have been obtained after induction of cells in suspension culture (1–3). Attempts have been made to circumvent this difficulty by cultivating cells in suspension culture and then seeding the cells in monolayer culture prior to interferon induction (4, 5), but in so doing many of the advantages conferred by the use of suspension culture are lost.

We report herein, an induction system which results in the production of levels of interferon in suspension culture as high as those produced by the same cells cultivated in monolayer culture. Thus cells can be cultivated in stirred suspension culture in a large vessel and then be induced to produce interferon in the same vessel. Although, we describe this method as applied to mouse sarcoma virus transformed C243-3 cells (6), these same procedures are applicable to other cells which multiply in suspension culture and have been used in our laboratory for the production of human interferon (unpublished results).

Materials and Methods. Cells and media. The C243-3 cells were a gift from Dr. S. Baron (NIH, Bethesda, USA). The cells were cultivated in monolayer culture in Eagle's minimal essential medium (MEM) supplemented with 10% calf serum, and in suspension culture in Eagle's MEM (Joklik modified) with 10% calf serum. All media and sera were purchased from GIBCO (New York, USA).

For suspension cultures the cells were cultivated in either 1.0 or 10 liter magnetically stirred (50 rpm) glass culture vessels (Fig. 1).

Viruses. Newcastle Disease Virus (NDV), Hertz strain, was propagated in 10 day old embryonated chicken eggs. After ultracentrifugation of the allantoic fluid (100,000*g* for 1 hr) the virus pellet was resuspended in Eagle's MEM at the required concentration. Multiplicity of infection was calculated on the basis of NDV infectivity in $TCID_{50}$/ml in primary chick embryo fibroblast cultures.

Interferon assay. The antiviral substance obtained fulfilled the criteria proposed by Lockart for interferon (7).

Interferon was assayed on L-cells inoculated with vesicular stomatitis virus (8) and titers are expressed in terms of mouse interferon reference units/ml.

Standard induction procedure. A 48 hr culture of C243-3 cells was primed by the addition of 10 units/ml of interferon with 0.5 mg/ml of L-glutamine and incubated with stirring for 16 hr. The culture was then centrifuged (800*g* for 15 min) and the cells resuspended in a volume of NDV equal to 1/100 of the original culture vol-

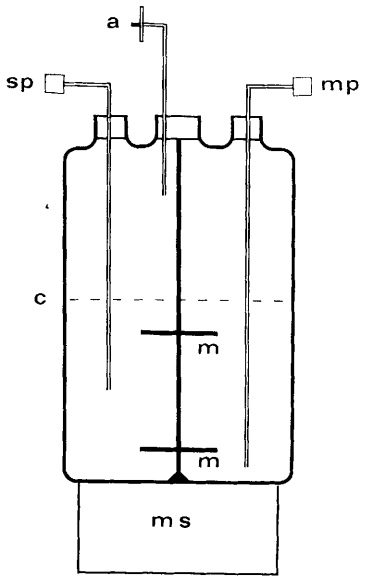

FIG. 1. Apparatus for the cultivation of animal cells and the production of interferon in suspension cultures. a—Air filter. sp—Aseptic sampling port. mp—Aseptic port for the addition and withdrawal of nutrient medium and cells. c—Level of the nutrient medium in the vessel. m—Magnets mounted on stirring assembly. ms—Magnetic stirrer.

ume to give a multiplicity of infection of 1. After a 1.0 hr adsorption period the cells were removed by centrifugation (800*g* for 15 min) and resuspended in $\frac{1}{10}$ of the original culture volume of medium with 2% calf serum and 10 μg/ml of cycloheximide. After the cells had been incubated for 3 hr in stirred suspension culture 3 μg/ml of actinomycin D was added and the culture incubated with stirring for a further 2.0 hr. The antimetabolites were then removed by centrifugation (800*g* for 15 min) and the cells were resuspended in a volume of nutrient medium, without serum, equal to the original culture volume and incubated for a further 18 hr. All the above steps were carried out at 37°. The culture supernatant was harvested by centrifugation and adjusted to pH 2.0 for 6 days prior to interferon assay.

Protein estimation. Protein content of interferon samples was estimated by the method of Lowry *et al.* with crystalline bovine plasma albumin as a standard (9).

Results. Cell cultivation and priming. Mouse C243-3 cells were adapted to growth in suspension culture in our laboratory. They were cultivated in mechanically stirred 10 liter culture vessels. (Fig. 1). (The size of the vessel is limited only by the facilities available). The C243-3 cells were seeded at a concentration of 2×10^5 cells/ml. Under optimal conditions saturation density (1.5×10^6 cells/ml) was attained after 48 hr. The addition of a small dose of interferon prior to induction has been shown to increase the subsequent production of interferon (10, 11). Priming of suspension cultures of C243-3 cells with 10 units/ml of interferon for 14–16 hr resulted in a 2–4-fold increase in interferon titer compared to unprimed cultures.

To obtain maximal interferon titers it was necessary to resuspend C243-3 cells at

TABLE I. Culture Conditions During Priming.[a]

Culture conditions	Interferon titer reference units/ml	
	Experiment I	Experiment II
Cells resuspended in fresh medium containing 5% calf serum prior to priming	2.56×10^5	1.28×10^5
No fresh medium	0.64×10^5	0.32×10^5
No fresh medium but L-glutamine added (0.5 mg/ml)	2.56×10^5	1.28×10^5

[a] Three 500 ml cultures (1.0×10^6 cells/ml) were primed with 10 units/ml of interferon for 16 hr under various cultural conditions. After the priming period each culture was treated in an identical manner as described for "standard induction" in "Materials and Methods."

saturation density in fresh medium prior to priming. It was found, however, that if 0.5 mg/ml of L-glutamine were added to cells at saturation density it was no longer necessary to resuspend the cells in fresh medium (Table I).

Interferon induction. After the priming period, the cells were removed from the depleted medium either by centrifugation or by stopping the stirring of the culture and allowing the cells to sediment under gravity for the final 6 hr of the priming period. Sedimentation of cells under gravity resulted in a 2–4-fold loss of interferon titer compared with centrifugation, but had the advantage of circumventing the centrifugation of large quantities of cell suspension. Ninety to 95% of the depleted nutrient medium was then aspirated under vacuum. The residual medium containing the cells was then centrifuged.

The cell pellet was resuspended in a concentrated suspension of live Newcastle Disease Virus (NDV),[1] Hertz strain, to give a multiplicity of infection of 1 in a volume approximately 1/100 of the original culture volume. [Higher multiplicities of infection did not result in a further increase in interferon titer (Table II).]

Resuspension of the cell pellet in a concentrated suspension of viral inducer presented 2 advantages. First—a 2-fold increase in interferon titer was obtained compared to that produced when NDV was added at the same multiplicity to the original culture volume. Secondly—and this is the principal advantage of reducing the induction volume, the inducing virus could be easily recovered at the end of the adsorption period and reutilized. The same virus suspension has in fact been used seven times without any loss of its interferon inducing capacity (Table III).

Treatment with metabolic inhibitors. It has been reported that under certain conditions treatment of cells with antimetabolites increases the production of interferon (12–14).

TABLE II. Multiplicity of Infection during Interferon Induction.[a]

Multiplicity of infection	Interferon titer reference units/ml
0.1	3.2×10^5
0.5	3.2×10^5
1	6.4×10^5
2	6.4×10^5
5	6.4×10^5
10	6.4×10^5
25	3.2×10^5

[a] A 48 hr culture of C243-3 cells (10^6 cells/ml) was primed for 16 hr in the standard manner (see Materials and Methods). 100 ml samples of this culture were then centrifuged (800*g* for 10 min) and the cells resuspended in 5 ml of NDV diluted to give the required multiplicity of infection. After a 1.0 hr adsorption period the cultures were treated in an identical manner, see Materials and Methods.

After a 1 hr virus adsorption period the cells were sedimented by centrifugation and resuspended at a concentration of 10^7 cells/ml in nutrient medium supplemented with 2% calf serum and containing 10 μg/ml of cycloheximide. The cell concentration was not critical and could be increased to 5×10^7 cells/ml. The cells were incubated in stirred suspension culture for 3 hr at which time 3 μg/ml of actinomycin D was added. These concentrations and times of addition of inhibitors were found to be optimal. After a further 2 hr of incubation the inhibitors were removed by centrifugation. Washing of the cell pellet was not necessary.

Using NDV as an inducer, treatment of primed monolayer cultures of C243-3 cells with cycloheximide and actinomycin D, under optimal conditions, resulted in only a 2–4-fold increase in interferon titer compared to untreated cultures induced with NDV (Table IV). However, under the same conditions a ten fold increase in interferon titer was obtained with C243-3 cells in suspension culture compared to untreated cultures induced with NDV alone (Table IV).

[1] In this system live NDV was found to be a more efficient inducer of interferon than UV-irradiated NDV, and the Hertz strain of NDV a more efficient inducer of interferon than either the Komarov strain of NDV or the MM strain of encephalomyocarditis virus.

TABLE III. Reutilization of NDV in the Induction of Mouse Interferon in C243-3 Cells.

Batch of NDV	Number of times used	Interferon titer reference units/ml
1	1	1.28×10^5
	2	1.28×10^5
	3	0.64×10^5
	4	NA[a]
	5	0.64×10^5
	6	1.28×10^5
2	1	0.64×10^5
	2	1.28×10^5
	3	5.12×10^5
	4	NA
	5	5.12×10^5
3	1	5.12×10^5
	2	NA
	3	6.4×10^5
	4	6.4×10^5
	5	NA
	6	NA
	7	5.12×10^5

[a] NA: Different induction conditions were used so that the interferon titers obtained were not applicable.

Each batch of NDV was used for a number of separate inductions using the standard induction procedure (see Materials and Methods). After a 1.0 hr adsorption period, the cells were removed by centrifugation (800*g* for 10 min) and the virus inducer was recovered and stored at −70° until reutilization.

Interferon production. After treatment with metabolic inhibitors the cells were resuspended in a volume of nutrient medium without serum equal to the original culture volume (cell concentration $1–2 \times 10^6$ cells/ml) and incubated for 18 hr in stirred suspension culture. (In the absence of stirring lower titers of interferon were obtained.) The amount of interferon produced was directly proportional to the cell concentration up to 4.0×10^6 cells/ml. At higher cell concentrations no further increase in interferon production was obtained (Fig. 2).

Interferon was rapidly released into the culture supernatant and maximum titers were obtained between 12 and 24 hr after the start of induction. Interferon was harvested at 24 hr post induction either by centrifugation or by sedimentation of the cells under gravity for a further 6 hr. The interferon containing supernatant could then be aspirated by mechanical means.

Using the procedures outlined in the above paragraphs consistently high titers of interferon (mean titer: 4×10^5 reference units/ml, variance: 0.64×10^5–1.0×10^6) have been obtained in a total of 51 inductions over a period of one year. [High passage (50–60) suspension cultures were found to produce the same amount of interferon as low passage (1–5) cultures.] In 16 of these inductions we have produced

TABLE IV. Interferon Production in Monolayer and Suspension Cultures of C243-3 Cells.

Culture conditions of C243-3 cells	Priming (10 U/ml)	NDV multiplicity of infection = 1.0	Cycloheximide (10 μg/ml)	Actinomycin D (3 μg/ml)	Interferon titer reference units/ml
Monolayer cultures[a]	—	+	—	—	0.51×10^5
	—	+	+	—	0.8×10^5
	—	+	+	+	1.0×10^5
	+	+	+	+	1.28×10^5
Suspension cultures[b]	—	+	—	—	0.2×10^5
	—	+	+	—	0.8×10^5
	—	+	+	+	1.0×10^5
	+	+	+	+	2.56×10^5

[a] C243-3 cells were seeded in plastic petri dishes (20 cm) so as to give approximately 1×10^6 cells/ml at 48 hr. These cultures were treated to the same induction procedures as described for suspension cultures of C243-3 cells.

[b] 50 ml suspension cultures of C243-3 cells 1.0×10^6 cells/ml were induced using the standard induction (see Materials and Methods) as indicated in the table.

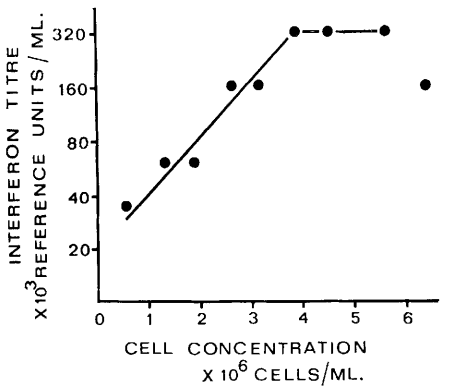

FIG. 2. The relationship between cell concentration and interferon production. A 5 liter culture of C243-3 cells was induced to produce interferon using the standard induction procedure (see Materials and Methods). After treatment with antimetabolites the cells were resuspended at the stated cell concentrations in 50 ml volumes of nutrient medium without serum and incubated for 18 hr in stirred suspension culture. The culture supernatants were harvested by centrifugation and assayed for interferon content after treatment at pH 2.0 for 6 days.

from 2 to 10 liters of interferon per induction (mean titer: 2.56×10^5 reference units/ml). The interferon produced was also of relatively high specific activity [10^6 reference units/mg of protein after removal by centrifugation of contaminating protein precipitated during treatment at pH 2.0 and dialysis against phosphate buffered saline (pH 7.4)].

These interferon preparations were concentrated by ammonium sulphate fractionation at pH 2.0 (15, E. Knight, personal communication) and dialysed against phosphate buffered saline (pH 7.4). This procedure yielded (with 100% recovery and 5 fold purification) interferon preparations with a potency in excess of 10^7 reference units/ml and with a specific activity of 5×10^6 reference units/mg of protein.

Discussion. Studies in this laboratory on the *in vivo* action of interferon (16–19) were limited by the quantity and potency of the interferon preparations available. This necessitated the development of a method capable of producing large quantities of potent interferon. We were of the opinion that the only system capable of fulfilling these requirements was one based on the cultivation and induction of animal cells in suspension culture, since monolayer cultures have an inherent size limitation and are not readily adapted to large scale production of interferon.

However, attempts to induce interferon in suspension cultures have resulted in the production of interferon of low titer (1–3). Similarly, we found that induction of suspension cultures of C243-3 cells with NDV alone produced interferon of lower titer than produced, under the same conditions, by monolayer cultures of C243-3 cells (Table IV). However, the relative efficiency of interferon production in suspension cultures could be increased by the use of metabolic inhibitors. When NDV was used as an inducer, treatment of monolayer cultures of C243-3 cells with metabolic inhibitors resulted in only a 2–4-fold increase in interferon titer, whereas under the same conditions a tenfold increase in interferon titer was obtained in suspension cultures of C-243-3 cells.

The culture conditions during various stages of the induction procedure also affected the production of interferon in suspension culture. In general, those conditions which favored cell multiplication also favoured interferon production. Optimal results were obtained when the culture was stirred during the periods of priming, contact with the metabolic inhibitors, and production of interferon. To obtain maximal interferon titers it was necessary to resuspend the C243-3 cells at saturation density in fresh medium for the priming period. This laborious step could be obviated, without loss of interferon titer, by the addition of L-glutamine to the culture medium at cell saturation density (Table I).

For the maximal production of interferon it was necessary to remove the cells from the priming medium by centrifugation and to resuspend the cell pellet in a concentrated suspension of virus inducer. This procedure also permitted recovery of the inducing virus at the end of the adsorption period. It was found that the recovered inducing virus could be reutilized at least

seven times without any loss of its interferon inducing capacity (Table III). We emphasize that, for the large scale production of interferon, this simple procedure results in a tremendous saving of the virus inducer.

Using these procedures we have consistently obtained large quantities of interferon of titer as high or higher than that produced by the same cells cultivated in monolayer culture (mean titer: 4×10^5 reference units/ml), and of relatively high specific activity (10^6 reference units/mg of protein). We reiterate that the advantage of this system is that large quantities of high titer interferon can be produced with relative ease since the cells are cultivated and induced to produce interferon in a single large vessel. The quantity of interferon produced is limited only by the capacity of the equipment available. By the use of *in situ* continuous centrifuges, or by allowing the cells to sediment under gravity and then aspirating the depleted medium, all the manipulations involved in the production of interferon can be carried out automatically thereby minimizing the risk of contamination and obviating the necessity for highly skilled operatives.

Although we have described this procedure as applied to C243-3 cells for the production of mouse interferon, this procedure is applicable to other cell lines which multiply in suspension culture, and has been used in our laboratory for the production of human interferon (unpublished results). We feel that this process applied either to the production of human interferon, or interferon of certain animal species (for use in veterinary medicine), would greatly facilitate the production of interferon on an industrial scale.

Summary. A method has been described for the large scale production of potent mouse interferon preparations based on the cultivation and induction of cells in suspension culture. By the selective use of metabolic inhibitors and the choice of optimal culture conditions, large quantities of interferon titering 4×10^5 mouse reference units/ml (specific activity 10^6 reference units/mg of protein), were consistently obtained. It was found that the inducing virus could be recovered at the end of the adsorption period and reutilized at least seven times without loss of its interferon inducing capacity. Although this method has been described for the production of mouse interferon using C243-3 cells it is applicable to the production of human or other interferons from cells which multiply in suspension culture.

We are indebted to Mrs. J. Buywid and Mrs. M. T. Bandu for their skilled technical assistance, and to Dr. S. Baron, NIH, Bethesda, for the gift of the C243-3 cells. M.G.T. is indebted to the Fondation Philippe and to Mr. Pierre Philippe for their continued interest and support. This work was supported in part by grants from l'Institut National de la Santé et de la Recherche Médicale, ATP 2-73-23 (contract no. 8).

1. Cantell, K., and Paucker, K., Virology **21**, 11 (1963).

2. Mogensen, K. E., Tovey, M. G., Pirt, S. J., and Mathison, G. E., J. Gen. Virol. **16**, 111 (1972).

3. Tovey, M. G., Mathison, G. E., and Pirt, S. J., J. Gen. Virol. **20**, 29 (1973).

4. Graff, S., Kassel, R., and Kastner, O., Trans. N. Y. Acad. Sci. **32**, 545 (1970).

5. Paucker, K., Berman, B. J., Golgher, R. R., and Stancek, D., J. Virol. **5**, 145 (1970).

6. Oie, H. K., Gazdar, A. F., Buckler, C. E., and Baron, S., J. Gen. Virol. **17**, 107 (1972).

7. Lockart, R. Z., Jr., *In* "Interferons" (N. B. Finter, ed.), p. 3. North-Holland Publishing Co., Amsterdam (1966).

8. Gresser, I., Fontaine-Brouty-Boye, D., Bourali, C., and Thomas, M. T., Proc. Soc. Exp. Biol. Med. **130**, 236 (1969).

9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

10. Stewart, W. E., II, Gosser, L. B., and Lockart, R. Z., Jr., J. Virol. **7**, 792 (1971).

11. Stewart, W. E., II, Gosser, L. B., and Lockart, R. Z., Jr., J. Gen. Virol. **15**, 85 (1972).

12. Tan, Y. H., Armstrong, J. A., Ke, Y. H., and Ho, M., Proc. Nat. Acad. Sci. USA **67**, 464 (1970).

13. Vilcek, J., and Ng, M. H., J. Virol. **7**, 588 (1971).

14. Ho, M., Tan, Y. H., and Armstrong, J. A., Proc. Soc. Exp. Biol. Med. **139**, 259 (1972).

15. Vilcek, J., Tomisova, J., Sokol, F., and Hana, L., Acta Virol. **8**, 76 (1964).

16. Gresser, I., Bourali, C., Levy, J. P., Fontaine-Brouty-Boye, D., and Thomas, M. T., Proc. Nat. Acad. Sci. USA **63**, 51 (1969).

17. Gresser, I., and Bourali, C., J. Nat. Cancer Inst. **45**, 365 (1970).

18. Gresser, I., and Bourali-Maury, C., Nature New Biol. **236**, 78 (1972).

19. Gresser, I., and Bourali, C., Eur. J. Cancer **6**, 553 (1970).

Received Mar. 1, 1974. P.S.E.B.M., 1974, Vol. 146.