ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1977, p. 11-15
Vol. 12, No. 1
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Printed in U.S.A.

Human Interferon: Mass Production in a Newly Established Cell Line, MG-63

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Received for publication 19 April 1977

MG-63 cells, a line derived from an osteosarcoma, produced high yields of interferon after superinduction with polyinosinic acid·polycytidylic acid, cycloheximide, and actinomycin D. Advantages of MG-63 cells over diploid fibroblasts as a substrate are: no requirement for aging between confluency and induction, no requirement for priming, and 3.7-fold higher yields per square centimeter of culture surface. Physicochemically and biologically, MG-63 cell interferon resembles fibroblast rather than leukocyte interferon.

Two methods are currently in use for mass production of human interferon: induction of fresh human leukocytes in suspension with Sendai virus (3), and induction of cultured human embryonal fibroblasts, using a carefully selected regimen of treatments with double-stranded ribonucleic acid, actinomycin D, and cycloheximide, called superinduction (2, 6, 10, 14). Nearly all clinical trials on human volunteers have been done with leukocyte interferon (11). Only recently has enough fibroblast interferon become available to initiate trials with systemic administration (4). With current yields of ca. 30 reference units per 1,000 cells, the production costs of both leukocyte and fibroblast interferons have so far hampered or prohibited large-scale trials especially in diseases that would seem to require long-term administration of high doses. The leukocyte system is limited, furthermore, by the availability of blood donors: one buffy coat yields about 2×10^6 reference units, i.e., approximately 1 man-day dose as administered in recent clinical trials.

The most frequently suggested possibilities for improving the production technology are: (i) development of automated mass culture systems, allowing for reduction in manpower involved in cell propagation; (ii) improvement of the interferon yield per cell by modifying the induction schedule or by (iii) selection of cell variants capable of higher production levels.

Many fibroblastoid cell strains with a diploid or anomalous karyotype have been compared by us (6) and other authors (10). Few strains were found to be low producers; most strains produced yields of approximately 20 to 30 units per 1,000 cells. So far, there are no reports that a diploid cell strain has been isolated that is capable of producing exceptionally high levels of interferon, using present induction and superinduction techniques. Continuous human cell lines were also screened (6) and were invariably found to be low producers. A single clone of simian virus 40-transformed human embryonic fibroblasts (Y. H. Tan, personal communication) has been reported to produce exceptionally high yields. We report here on a new human cell line, MG-63, which possesses both excellent interferon production potential and growth capacity.

MATERIALS AND METHODS

Cell cultures. Cultures were grown in Eagle minimum essential medium supplemented with 10% heated (56°C, 30 min) fetal bovine serum, nonessential amino acids, penicillin (200 U/ml), and streptomycin (100 μg/ml). All reagents were purchased from Flow Laboratories, Irvine, Scotland, or Grand Island Biological Co., Grand Island, N.Y.

Diploid human cells (E_1SM strain) were established from a 17-week-old male embryo obtained from an induced abortion. MG-63 cells were derived from an osteogenic sarcoma. The characteristics of the cell line are described in a separate paper (A. Billiau et al., manuscript in preparation). Briefly, the cells are karyotypically anomalous, with multiple triploidy and some constant markers. Doubling time (30 h) and saturation density (60,000 cells/cm²) are only slightly higher than those of diploid cells. Clones were isolated that grow in 1% serum. The following continuous cell lines were obtained from the American Type Culture Collection, Rockville, Md.: L-132, KB, HEP-2, RPMI-2650, HT-1080, and Detroit-562. Cell lines obtained through H. Strander, Karolinska Sjukhuset, Stockholm, Sweden, were: SAOS-2, SI-I, SI-II, TE-85, 393-T, 20-S, and RPMI-0041. RD and A-204 cells were obtained from R. E. Gallagher, National Cancer Institute, Be-

thesda, Md. SH3 and G-cells were provided, respectively, by G. Seman and E. Hersch, M. D. Anderson Hospital and Tumor Institute, Houston, Tex. MG-34 cells were derived in our laboratory (1).

Screening procedure for interferon production. For screening purposes, cells were grown to confluency in 20-cm² petri dishes. One set of plates was induced with Newcastle disease virus. Growth medium was replaced by 1 ml of Newcastle disease virus (Komarow strain, 10^9 mean egg infectious doses per ml). After 1 h of adsorption, 4 ml of medium containing 2% bovine serum was added, and the plates were incubated for 24 h. The medium was harvested, acidified to pH 2 with HCl, and left at +4°C for 5 days. The samples were neutralized, clarified (100,000 × g, 30 min), and stored at −20°C until titration. Separate sets were primed and superinduced: the growth medium was replaced by medium containing interferon (0, 10, or 100 reference units per ml). After overnight incubation, the medium was removed, and the cultures were exposed for 1 h to polyinosinic acid·polycytidylic acid [poly(I)·poly(C)] (50 μg/ml). Fresh medium was added with cycloheximide (10 μg/ml). After another 3.5 h, actinomycin D (1 μg/ml) was also added. After 2 h of incubation, all inhibitors were washed out and fresh medium was added. Interferon was harvested after overnight incubation.

Production of interferon in roller bottles. For large-scale production of interferon, cells were propagated in roller bottles incubated on a Rollacell apparatus (New Brunswick Scientific Co., New Brunswick, N.J.). The bottles were either glass (600 cm², New Brunswick Scientific Co., New Brunswick, N.J.) or plastic (490 cm², Corning Glass Works, Corning, N.Y.). Diploid cells were split at a 1:2 or 1:4 ratio and aged for 10 to 14 days postconfluency. The cultures were then drained and refed with 50 ml of medium containing 10% (vol/vol) human plasma protein fraction (National Blood Transfusion Service, Belgian Red Cross; 28 g of albumin per liter) and 100 reference units of interferon per ml. The cultures were incubated overnight and superinduced. The growth medium was decanted, and 20 ml of poly(I)·poly(C) (50 μg/ml; P-L Biochemicals, Milwaukee, Wis.) and cycloheximide (10 μg/ml) was added. Four hours later, actinomycin D was added to a 1-μg/ml final concentration. Six hours after the addition of poly(I)·poly(C), the cultures were washed and refed with 20 ml of medium containing 1% (vol/vol) human plasma protein fraction. Interferon was harvested after overnight incubation. MG-63 cells were split at a 1:4 ratio and used for induction on day 5 after seeding. Priming with interferon was omitted.

Interferon titrations. Routine assays for interferon activity were done in HEp-2 cells by a microtiter assay. Serial threefold dilutions (100 μl/well) were made in duplicate in flat-bottom microtiter plates (Falcon Plastics, Oxnard, Calif.). A laboratory standard was included on each plate. To each well 100 μl of cell suspension (6×10^5 cells per ml) was added. After an 8-h incubation period, cultures were challenged with 50 μl of a suspension containing vesicular stomatitis virus at $10^{7.7}$ plaque-forming units per ml on L-929 cells. The cytopathic effect was complete in virus control wells after 48 h of incubation. At this time, the cells were washed with Dulbecco phosphate-buffered saline and stained with a 0.5% crystal violet solution (Merck, Darmstadt, West Germany; in a mixture of formaline-ethanol-0.25% saline, 1:10:20, vol/vol). All results were expressed as reference units per milliliter in terms of the National Institutes of Health reference preparation 69/19.

Comparative titrations on cat (cat lung cell line from Flow Laboratories, Irvine, Scotland) and human diploid cells (NS strain) were done by a vesicular stomatitis virus plaque reduction assay. Briefly, cultures in 20-cm² petri dishes were incubated overnight with 5 ml of serial 0.5 $\log_{10}$ dilutions of interferon. The fluids were decanted, and the plates were challenged with 0.2 ml of vesicular stomatitis virus suspension containing 100 plaque-forming units. After 1 h, an agarose overlay was applied. Plaques were counted on day 2 after staining with neutral red. A 50% plaque reduction end point was calculated graphically.

Antibody neutralization. Antibody neutralization assays were performed as described by Havell et al. (9). Rabbit antiserum to human fibroblast interferon (code 4-478) was a gift of J. Vilcek and E. A. Havell, New York University School of Medicine, New York, and was quoted as being capable of neutralizing 20 U of human fibroblast interferon when diluted 1:400. Rabbit antiserum to human leukocyte interferon (code BK-11), containing 30,000 neutralizing units per ml when assayed by the method of Mogensen et al. (12), was kindly supplied by K. Cantell, Central Public Health Laboratory, Helsinki, Finland. K. Cantell also supplied the leukocyte interferon used in these experiments.

Purification of interferon. MG-63 interferon was purified by a batch modification (V. G. Edy et al., manuscript in preparation) of the adsorption chromatography technique described by Edy et al. (7).

RESULTS

Screening for production of interferon by solid tumor-derived cell lines. Nineteen continuous cell lines, 18 of them derived from solid human tumors and 1 from normal tissue, were screened, by a standard procedure, for their capacity to produce interferon (Table 1). The screening comprised: (i) induction with Newcastle disease virus; (ii) superinduction, using poly(I)·poly(C), cycloheximide, and actinomycin D; and (iii) a combination of priming (10 or 100 reference units per ml of interferon) and superinduction. As with diploid cells, Newcastle disease virus generally induced lower levels (average of $10^{1.8}$ reference units per ml) than superinduction (average of $10^{2.5}$ reference units per ml). In contrast to diploid cells (5), pretreatment with interferon generally lowered production (average of $10^{2.32}$ reference units per ml). Only one cell line, MG-63, produced greater

TABLE 1. *Screening of continuous human cell lines for production of interferon, using Newcastle disease virus (NDV) or superinduction with poly(I)·poly(C)[a]*

Cell line		Interferon yield (log_{10} reference units/ml) after:			
		NDV	Superinduction after priming with interferon		
Designation	Origin		0[b]	10	100
L-132	Normal human embryonic lung	NT[c]	<0	<0	<0
KB	Epidermoid carcinoma	1.2	2.8	<1.8	<1.8
HEp-2	Epidermoid carcinoma	1.4	<0	0.3	1.8
RPMI-2650	Squamous cell carcinoma	0.8	1.2	1.5	0.8
SH-3	Breast carcinoma	1.8	2.1	2.4	3.0
Detroit-562	Carcinoma	<1.5	3.5	3.2	3.2
HT-1080	Fibrosarcoma	2.3	3.3	3.2	2.9
RD	Rhabdomyosarcoma	1.4	1.4	1.5	1.5
A-204	Rhabdomyosarcoma	2.7	2.5	2.6	2.4
G	Melanoma	<1.5	1.5	1.5	1.5
TE-85	Osteosarcoma	2.5	3.2	3.0	2.5
SI-I	Osteosarcoma	1.5	1.5	1.5	1.5
SI-II	Osteosarcoma	2.0	2.8	2.2	2.2
SAOS-2	Osteosarcoma	<1.5	2.0	2.0	1.5
393-T	Osteosarcoma	2.2	3.5	3.5	3.5
RPMI-0041	Osteosarcoma	2.0	2.0	2.0	2.0
20-S	Osteosarcoma	<1.5	2.2	2.2	2.2
MG-63	Osteosarcoma	1.3	4.5	4.6	4.5
MG-34	Leiomyoma	<0.9	NT	NT	2.5

[a] Standard induction schedule: 20-cm^2 petri dishes induced with either NDV (10^9 mean egg infectious doses per culture) or priming (24 h with indicated concentration of interferon) plus superinduction with poly(I)·poly(C) (50 μg/ml from time 0 to 1 h) followed by the inhibitor cycloheximide (10 μg/ml, from 6 to 6.5 h). Total volume: 5 ml per petri dish.

[b] Reference units per milliliter.

[c] NT, Not tested.

than $10^{4.0}$ reference units per ml after superinduction.

The results of an experiment to compare superinducibility in diploid cells, MG-63 cells, and another tumor cell line, HT-1080, are shown in Table 2. In MG-63 cells, superinduction resulted in about 30 times more interferon than induction with poly(I)·poly(C) alone. This increase is comparable to that observed in fibroblasts. In HT-1080 cells, the effect of superinduction was present but less pronounced. Priming did not cause a significant increase in interferon production in MG-63 cells, whereas it did in diploid cells. Even so, the best yields from the fibroblasts were threefold lower than with MG-63 cells. These results prompted a large-scale production experiment.

Large-scale production of interferon: Comparison of MG-63 cells with diploid skin fibroblasts. Since early 1975 a pilot plant for production of human interferon on diploid cells has been operative in our laboratory. Briefly, diploid human embryonic cells are cultivated in roller bottles. Ten to 14 days after seeding at a 1:2 or 1:4 split ratio, i.e., 6 to 10 days after confluence, the bottles are primed and superinduced as outlined in Materials and Methods. At maximum capacity, 150 bottles can be induced per week. On a practical basis, the plant runs routinely at two-thirds this capacity for about 40 weeks per year. To conduct this experiment, production from diploid cells was completely halted for 5 weeks, and the plant was devoted

TABLE 2. *Interferon production in human tumor cell lines and in diploid fibroblasts induced with poly(I)·poly(C)*

Induction schedule[a]	Cell line or strain	Yield (log_{10} reference units/ml)	
		Not primed	Primed
Poly(I)·poly(C) only	HT-1080	1.70	2.20
	MG-63	3.30	3.20
Poly(I)·poly(C) + inhibitors	HT-1080	2.20	2.40
	MG-63	4.68	4.74
	Diploid (E_1S)	3.77	4.20

[a] Petri dish (20 cm^2) cultures induced by poly(I)·poly(C) (50 μg/ml, time 0 to 1 h) followed by inhibitors: cycloheximide (10 μg/ml from 1 to 6.5 h) and actinomycin D (1 μg/ml from 4.5 to 6.5 h). Priming: 24-h treatment with 30 reference units of interferon per ml prior to induction. Total volume: 5 ml/petri dish.

to the cultivation and induction of MG-63 cells. During the first 2 weeks, a cell stock was built up so that only a few bottles could be induced. Over the 5 weeks, 364 bottles were induced. The results of this experiment are summarized in Table 3. MG-63 cells can be split at a 1:4 ratio; they reach confluency in about 4 days and do not need further aging or priming for optimal inducibility. Thus, a given number of bottles can be processed in less time and with less incubator and roller space than with the E_1S diploid cells. Table 3 compares the production on MG-63 cells with that on diploid cells. MG-63 cells are a more efficient substrate by a factor of 3.75 on the basis of culture surface area and by a factor of 3.17 on the basis of cell number.

Characterization of MG-63 interferon. Human fibroblast and leukocyte interferons are antigenically distinct, although partially purified leukocyte interferon preparations contain a small amount of fibroblast interferon-like activity. Therefore, antibody neutralization was used to determine whether the interferon produced from MG-63 cells was more closely related to human fibroblast or leukocyte interferon, or was antigenically distinct. The results (Table 4) show that MG-63 interferon is more closely related to human fibroblast interferon than to leukocyte interferon.

Another characteristic in which fibroblast and leukocyte interferons differ is in their activity on heterologous cells. In particular, human leukocyte interferon has been found to have high activity on porcine, bovine, and feline cells, whereas fibroblast interferon is relatively inactive on such cells (5, 8). When MG-63 interferon was compared with fibroblast and leukocyte interferons on cat cells (Table 4), it was found to be rather inactive, as would be expected if it resembled fibroblast and not leukocyte interferons.

Finally, fibroblast interferon can be adsorbed to and eluted from Controlled Pore Glass (7), whereas concentrated leukocyte interferon cannot. Again, MG-63 interferon could be shown to behave like fibroblast interferon. In particular, by a single-step batch purification technique on Controlled Pore Glass, MG-63 interferon could be purified 53-fold to a specific activity of 3,000,000 reference units per mg of protein. The interferon was concentrated 27-fold and the yield was 32%.

DISCUSSION

Tumor cell lines can often be propagated more easily, and usually grow to higher cell density, than diploid cells. This is an advantage for the production of interferon, which has been limited due to a technology that does not permit adequately large cell production. Furthermore, the immortality of cell lines is an additional advantage for interferon production, which, in contrast to vaccines, requires vast amounts of cells. Accordingly, interferon for administration in animal experiments is usually made on continuous cell lines. The possible use of interferon made on continuous cell lines for administration to human patients has been dismissed on numerous occasions on the basis that it can never be proven to be completely devoid of oncogenic material. At best, one could verify that no oncogenic viruses or viral nucleic acids are detectable by presently available techniques.

TABLE 4. *Comparison of leukocyte, E_1S fibroblast, and MG-63 interferon: neutralization with antisera and activity on cat cells*

Interferon type	Neutralization titer[a] with antiserum raised against:		Antiviral titer[b] in plaque reduction assay on:	
	Leukocyte interferon	Fibroblast interferon	Cat cells	Human cells
Leukocyte	4.4	1.3	5.1	4.3
Fibroblast	2.3	2.5	2.6	4.4
MG-63	2.3	2.1	2.4	4.4

[a] Expressed as $\log_{10}$ of the highest antiserum dilution giving complete neutralization of 10 reference units of each interferon per ml.

[b] Expressed as $\log_{10}$ of interferon dilution reducing vesicular stomatitis virus plaque number to 50% of the control value.

TABLE 3. *Comparison of large-scale interferon production in diploid cells and in MG-63 tumor cells*

Cell line	Roller bottles induced (no.)[a]	Total culture surface area induced (cm^2)	Avg cell density (cells/cm^2)	Total cells induced (no.)	Interferon yield (reference units)			
					Total	Per bottle	Per cm^2	Per 10^3 cells
MG-63	364	218,400	62,000	13×10^9	1.41×10^9	3.87×10^6	6.46×10^3	104
Diploid (E_1S)	355	185,250	53,000	10.3×10^9	0.34×10^9	0.95×10^6	1.72×10^3	33

[a] Roller bottles (glass, 600 cm^2; or polystyrene, 490 cm^2) induced with poly(I)·poly(C) (50 μg/ml, from time 0 to 4 h) and inhibitors: cycloheximide (10 μg/ml, from time 0 to 4 h) and actinomycin D (1 μg/ml, from 4 to 6 h). Diploid cells were primed with 100 reference units of interferon per ml 24 h prior to induction; MG-63 cells were not primed.

However, these techniques may be too insensitive. Furthermore, unknown or undefined oncogenic materials may be present. Strictly taken, the same considerations hold for interferon prepared on diploid embryonic fibroblasts or on fresh leukocytes. But here, at least, the likelihood that undefined oncogenic material is present would seem to be less. Also, one can rely on past experience with diploid strains in the field of viral vaccine production.

So far, the objections against human tumor cell interferon have had no practical importance, because human cell lines with good interferon production potential have simply not been available. Many cell lines have been tested, but all have been found to be low producers. This is reflected in our study where 19 continuous cell lines were screened by standard induction schedules. Only one cell line, MG-63, developed in our laboratory from an osteosarcoma, proved to be an excellent producer. Ninety-five clones of the MG-63 cell line were screened for interferon production. None exceeded the original line in production capacity and many were inferior. It is possible that, under different superinduction conditions, some of the cell lines might produce greater amounts of interferon. A number of different schedules were tested on HT-1080 cells (data not shown). It was found that the standard procedure for diploid cells is also optimal for these tumor cells.

The involvement of chromosomes 2 and 5 in the production of human interferon has already been shown (15). Karyotypic analysis of the MG-63 clones may provide additional information as to the chromosomal control of interferon production.

Recently, some attention has also been given to lymphoblastoid cell lines as a possible substrate for interferon production (13). They can easily be cultivated in large-suspension culture vessels. Although the yield of interferon is rather low (about 1 to 4 U per 1,000 cells, against 20 to 30 in diploid fibroblasts and 120 in MG-63 cells), this would be compensated for by the fact that these lymphoblasts reach a 10-fold higher cell density (2×10^6 cells/ml of culture medium) than cells grown on a solid substrate. Lymphoblast interferon could probably substitute for leukocyte but not for fibroblast interferon from which it differs by serological and biophysical characteristics. MG-63 interferon, by the same criteria, resembles fibroblast interferon and might, therefore, be considered as a less expensive and more readily available substitute for this type of interferon. It may be useful for laboratory studies and for administration to patients with neoplasia or possibly other life-threatening diseases.

ACKNOWLEDGMENTS

This study was supported by a grant from the Cancer Foundation of the Belgian A. S. L. K. (General Savings and Retirement Fund).

We thank our technical and editorial staff for excellent help.

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