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Examination of Parameters Affecting Human Interferon Production with Microcarrier-Grown Fibroblast Cells

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Various parameters were examined for their effects on interferon production with human fibroblast cells (FS-4) grown on microcarriers, using a superinduction procedure. Optimal concentrations of cycloheximide and actinomycin D during induction were 10 μg/ml and 1 μg/ml, respectively. Cells required 5 to 6 h of exposure to cycloheximide and at least 2 h of exposure to actinomycin D to achieve maximum yields. FS-4 cells were found to grow well at low serum concentrations (2.5 and 5%) and actually produced higher yields of interferon than cells grown at higher serum concentrations (10 and 20%). Kinetics of interferon production at various temperatures revealed that significantly higher yields could be obtained at 34°C than at either 37 or 30°C. Priming cells for 16 h with 50 U of interferon per ml resulted in consistently higher yields of interferon. By modifying the superinduction procedure in accordance with the above findings, it is now possible to consistently obtain interferon yields of greater than 20,000 international units per 10^6 cells.

In recent years an intensive effort has been under way to evaluate the usefulness of human interferon as a chemotherapeutic agent. Results of many clinical trials have been encouraging (1, 7, 8, 11, 12, 16), and tests are continuing. Perhaps the biggest obstacle in these efforts is the difficulty in obtaining the required amounts of interferon for proper clinical evaluation. The leukocyte/Newcastle disease virus system has been used for large-scale human interferon production (16), but the system has obvious disadvantages, including risk of contamination with viruses and a limited supply of leukocytes.

Human diploid fibroblast strains appear to be entirely suitable substrates for human interferon production (6) and do not have the above disadvantages pertaining to leukocyte cultures. The development of superinduction procedures (2, 5, 17), coupled with the recent development of an improved microcarrier system for the large-scale production of anchorage-dependent cells (9, 10), has provided the potential for a feasible means of mass-producing high-titered, low-cost human fibroblast interferon. In recent studies in our laboratories, we have demonstrated the potential usefulness of microcarrier culture for both virus production (4) and human interferon production (3). In the present study we have examined various parameters for their effects on interferon yields from diploid human fibroblasts grown on microcarriers, in an attempt to optimize conditions for large-scale interferon production.

MATERIALS AND METHODS

Cell culture. Human diploid foreskin cells (FS-4) were obtained from Jan Vilček, New York University School of Medicine, New York, and were used in all experiments. Cells were obtained at approximately the 18th population doubling. They were viably frozen in one batch for the entire series of experiments and were used between population doublings 30 and 40.

Stock cultures were maintained in Corning plastic roller bottles in a walk-in incubator at 37°C. For experimental purposes, microcarrier cultures were seeded from these stocks. Cultures were seeded in either 250-ml glass spinner bottles (Wilbur Scientific, Inc., Boston, Mass.) at 100 ml per bottle or 2-liter spinner vessels (Wheaton Scientific) at 1 liter per vessel, with a cell density that generally ranged from 3×10^5 to 4×10^5 cells per ml. A microcarrier concentration of 5 mg/ml was used, and cultures were incubated in a humidified incubator supplied with 10% CO_2.

Growth for stock cultures consisted of Dulbecco-modified Eagle medium (DMEM) (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (Sterile Systems, Inc., Logan, Utah). For microcarrier growth the serum concentration was reduced to 5%. Antibiotics used were penicillin (100 U/ml) and streptomycin (100 μg/ml), obtained from Sigma Chemical Co., St. Louis, Mo. Cultures were split using a trypsin-ethylenediaminetetraacetic acid solution (GIBCO Laboratories, Grand Island, N.Y.).

Microcarrier preparation and initiation. The procedure for microcarrier preparation has been described previously (10). The initiation of microcarrier cultures has also been previously described, and essentially consisted of the following: microcarriers were suspended in phosphate-buffered saline at a concen-

tration of 10 mg/ml and were sterilized in glass bottles by autoclaving. Microcarriers were then dispensed into spinner flasks containing growth medium to give a final concentration of 5 mg/ml.

Interferon production. The superinduction procedure used was a modification of the method reported by Havell and Vilček (5). Cells were grown to confluency on microcarriers in volumes from 100 to 1,000 ml to a density $\simeq 10^6$ cells per ml and induced between days 6 and 8 of growth. The induction and production procedure was always carried out at 10^6 cells per ml in a 50-ml volume. Cells were first washed twice with DMEM containing no serum, after which 50 ml of DMEM containing 50 μg of polyinosinic acid·polycytidylic acid [poly(I)·poly(C); PL Biochemical Co., Milwaukee, Wis.] and 50 μg of cycloheximide (Sigma) were added per ml. After incubation for 4 h at 37°C, actinomycin D (Sigma) was added to give a concentration of 1 μg/ml. After 1 h more of incubation, the medium was removed, and cells were washed twice with DMEM. DMEM containing 0.5% human plasma protein (Plasmanate; Daly Hospital Supply, Lynnfield, Mass.) was then added, and cultures were incubated overnight (20 to 24 h) at 37°C. All media, including washes, were prewarmed to 37°C. Culture fluids were then collected, clarified by centrifugation at 2,000 rpm for 10 min, and either assayed immediately or frozen at −70°C until assayed. This method was considered our standard procedure but was gradually modified during the course of our studies.

Interferon assay. The interferon assay, described by Havell and Vilček (5), consisted of the following: samples were assayed in duplicate in Costar 96-well culture dishes (Microbiological Associates, Walkersville, Md.). Growth medium (100 μl) was added to each well, and twofold dilutions of each sample were made in duplicate using a P200 Gilson Pipetman (Rochester Scientific, Rochester, N.Y.) or a Titertek Multipipetter (Flow Laboratories). Each well was seeded with 50 × 10^3 FS-4 cells in 100 μl of growth medium, and dishes were incubated for 20 to 24 h in a humidified incubator at 37°C supplied with 10% CO_2. Cells were then challenged with 1,000 plaque-forming units of vesicular stomatitis virus (Indiana strain, obtained from David Baltimore, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge) per well. Several wells in each dish served as cell and virus controls. Dishes were incubated at 37°C and scored microscopically after 48 to 72 h. The highest dilution of the sample showing 50% destruction of cells was considered the endpoint. An internal standard calibrated against the international standard B69/19 (obtained from the National Institutes of Health, Bethesda, Md.) was included with each assay.

Cell counts. Cells in microcarrier cultures were enumerated by counting nuclei with a modification of the method of Sanford et al. (14) as described by Van Wezel (18). Roller-bottle cell counts were made by dispersion with a trypsin-ethylenediaminetetraacetic acid solution, followed by counting with a hemacytometer.

Quality control. Cultures were thoroughly screened for the presence of mycoplasma using the culture method (isolation of mycoplasma colonies on artificial media), the uridine-uracil assay described by Schneider et al. (15), and the deoxyribonucleic acid staining method reported by Russell et al. (13). All results were negative.

RESULTS

Antimetabolite concentration. The effects of varying the concentrations of cycloheximide and actinomycin D during induction were investigated in an attempt to establish optimum levels. The results of the cycloheximide studies (shown in Table 1) indicate that a four- to eightfold reduction in the concentration of cycloheximide (from 50 to 12.5 or 6.25 μg/ml) resulted in approximately a twofold increase in interferon yields. Yields obtained at concentrations from 18 to 100 μg/ml were not significantly different. As a result of these studies, the standard concentration for cycloheximide was changed from 50 μg/ml to 10 μg/ml.

Table 2 gives the results of varying the concentration of actinomycin D from 0 to 2 μg/ml.

TABLE 1. *Effect of cycloheximide concentration on interferon yields*[a]

Cycloheximide concn (μg/ml)	Interferon yield[b] (U/ml)
0	750
6.25	22,000
12.5	24,000
18	9,000
25	13,000
50	11,000
100	11,000

[a] Induction was carried out under standard conditions described in the text. Cells were grown on microcarriers in 250-ml spinner flasks.

[b] Results represent an average of two separate experiments. Figures also refer to the number of units per 10^6 cells.

TABLE 2. *Effect of actinomycin D concentration on interferon yields*[a]

Actinomycin D conc (μg/ml)	Interferon yield[b] (U/ml)
2.0	6000
1.0	6800
0.5	6400
0.25	5100
0.125	2600
0	2100

[a] Induction was carried out under standard conditions described in the text except that the cycloheximide concentration was reduced from 50 to 10 μg/ml. Cells were grown on microcarriers in 250-ml spinner flasks.

[b] Results represent an average of two separate experiments. Figures also represent the number of units per 10^6 cells.

There appeared to be no significant differences in interferon yields resulting from varying the actinomycin D concentration between 0.25 and 2.0 μg/ml. Lowering the concentration to 0.125 μg/ml resulted in approximately a twofold drop in interferon yield.

Effect of time of exposure to cycloheximide and actinomycin D. It was shown by Havell and Vilček (5) that the addition of cycloheximide later than 3 h after stimulation with poly(I)·poly(C) significantly decreased interferon yields. These experiments were done in small plastic dishes. In separate experiments, we decided to test the effect of varying (i) the time of addition of cycloheximide relative to poly(I)·poly(C) and actinomycin D, and (ii) the time of exposure of cells to actinomycin D upon the interferon yields in microcarrier culture. Figure 1A shows the results of varying the time of addition of cycloheximide. Cycloheximide was added 1 h before and 1 to 4 h after the addition of poly(I)·poly(C). Actinomycin D was added at the standard time of 4 h after addition of poly(I)·poly(C) and left on for 1 h. Thus, the total length of exposure to cycloheximide ranged from 1 to 6 h. Interferon titers increased significantly with increased time of exposure of cells to cycloheximide. A plateau was observed at around 5 to 6 h of exposure when cycloheximide was added either with poly(I)·poly(C) (5 h) or 1 h before poly(I)·poly(C) (6 h). The data appear to indicate that for optimum interferon yields at least a 5-h exposure to cycloheximide is required.

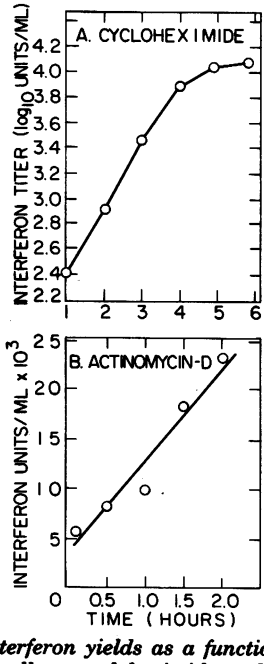

FIG. 1. *Interferon yields as a function of time of exposure of cells to cycloheximide and actinomycin D. FS-4 cells grown in 1-liter cultures were used to set up 50-ml cultures at 10^5 cells per ml for induction. (A) Cycloheximide exposure: poly(I)·poly(C) was added 4 h before actinomycin D in all samples, and actinomycin D was left on for 1 h. Cycloheximide (10 μg/ml) addition ranged from 1 h before to 4 h after addition of inducer, i.e., total time of exposure ranged from 1 to 6 h. (B) Actinomycin D exposure: actinomycin D was added 4 h after the addition of poly(I)·poly(C) and cycloheximide and left on the cells for the times indicated (10 min to 2 h).*

Figure 1B shows the effect of varying the time of exposure of cells to actinomycin D. In this experiment, poly(I)·poly(C) was added with cycloheximide, and actinomycin D was added 4 h later. Cultures were then exposed to actinomycin D for from 10 min to 2 h. Significant levels of interferon were obtained after only 10 min of exposure to actinomycin D. Furthermore, it appeared that increasing the exposure time to more than the standard 60 min, i.e., to 90 or 120 min, could result in significantly higher yields.

Effect of serum concentration on cell growth and interferon yields. We have generally observed over a period of time that a fetal bovine serum concentration of less than 10% is required for growth of FS-4 cells on microcarriers. To examine the effect of serum concentration (during the growth phase) on both growth rate (and final cell density) and interferon yields, FS-4 cells were grown on microcarriers at various serum concentrations and then induced for interferon production. Figure 2 gives growth curves of the FS-4 cells at serum concentrations ranging from 2.5 to 20%. The growth rates and maximum cell densities observed at 2.5, 5.0, and 10% were essentially the same. A lag time of 2 days was seen, after which the cells entered log phase and grew to a density of slightly more than 10^6 cells per ml. At a 20% serum concentration, a 3-day lag period was seen, and the cells grew to slightly under 10^6 cells per ml. Morphologically, the cells appeared much healthier and less cell detachment was observed at the lower serum concentrations (2.5 and 5.0%).

Table 3 presents interferon yields obtained from cultures grown at the various serum concentrations. In this experiment, induction and production were carried out using standard procedures described above (except that the cycloheximide concentration was 10 μg/ml) in the absence of any serum. As indicated in the table, highest yields were obtained when cells were grown at low serum concentrations. An extremely low yield was obtained when a serum concentration of 20% was used. Microscopic examination showed that a significantly greater amount of cell detachment occurred during induction with cultures grown at serum concentra-

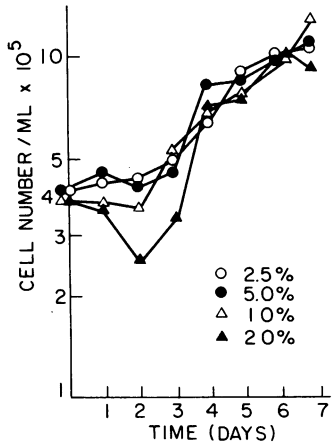

FIG. 2. *Growth of FS-4 cells on microcarriers at various serum concentrations. Cells from confluent monolayers grown in roller bottles were pooled and used to seed 100-ml microcarrier cultures at various concentrations of fetal bovine serum in DMEM. All cultures were seeded in duplicate at 4 × 10⁵ cells per ml. At specified times during incubation, samples were taken and cells were counted as described in the text.*

TABLE 3. *Effect of serum concentration during cell growth on interferon yields obtained with FS-4 cells*[a]

Serum conc (%)	Interferon yield (U/ml)[b]
2.5	11,000
5.0	14,000
10.0	6,600
20.0	310

[a] After growth at different serum concentrations, cells were induced under identical conditions using 50 μg of poly (I)·poly (C) per ml, 10 μg of cycloheximide (added with inducer) per ml, and 1.0 μg of actinomycin D per ml. Serum was used only during cell growth and not during induction or production phases. Results represent an average value obtained from two experiments.

[b] Figures also represent the number of units per 10^6 cells.

tions of 10 and 20% than at the lower serum concentrations.

Cell growth at different inoculum densities. Growth curves of FS-4 cells seeded at various densities are shown in Fig. 3. These curves show that an initial cell inoculum of at least 3 × 10^5 cells per ml was required for adequate cell growth. Results were essentially the same with an inoculum of either 3 × 10^5 or 4 × 10^5 cells per ml, i.e., slightly more than 10^6 cells per ml, final density. At a seed density of 2 × 10^5 cells per ml, a significant amount of growth occurred; however, the final density over a 7-day period was considerably less than 10^6 cells per ml. A seed density of 10^5 cells per ml resulted in no significant cell growth over a 7-day period.

Variation in interferon yields with different lots of poly(I)·poly(C). Four different lots of poly(I)·poly(C), all obtained from PL Biochemical Co. in the lyophilized sodium salt form, were tested to determine whether a significant variation in yield might be observed from one lot of inducer to another. Table 4 gives the results, showing that one lot of the four (no. 647232) gave a considerably lower yield than the other lots. In repeated experiments this lot has been shown to be less effective as an inducer than others tested.

Kinetics of interferon production at various temperatures. Interferon induction and production were controlled at three separate

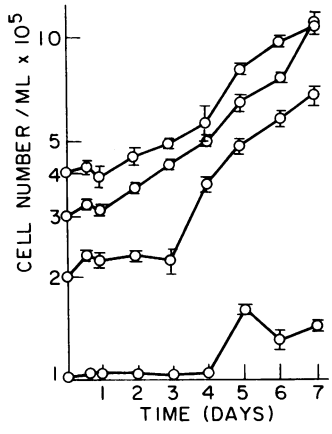

FIG. 3. *Growth of FS-4 cells on microcarriers at various inoculum densities. FS-4 cells from eight roller bottles were pooled and used to seed cultures, in duplicate, at the cell densities indicated. At the times indicated, samples were taken and cells were counted as described in the text.*

TABLE 4. *Interferon yields using different lots of poly (I)·poly (C)*[a]

Lot no.	Interferon yield (U/ml)[b]
747232	16,000
447231	16,000
847122	11,000
647232	4,800

[a] All lots (in the sodium salt, lyophilized form) were purchased from PL Biochemical Co. Induction was carried out using standard procedures described in the text except that cycloheximide was used at a concentration of 10.0 μg/ml.

[b] Figures also represent the number of units per 10^6 cells.

temperatures (30, 34, and 37°C) in an effort to determine the effect of temperature on interferon yields. Figure 4 clearly shows that a significant increase in interferon yields could be obtained by lowering the temperature during induction and production phases from 37 to 34°C (no attempt was made to determine the effect of temperature in one phase versus the other). At 34°C, interferon production increased from a rate of 300 U/ml per h during the first hour to slightly under 4,000 U/ml per h around h 6. The rate decreased significantly between 6 and 16, after which essentially no further interferon production occurred.

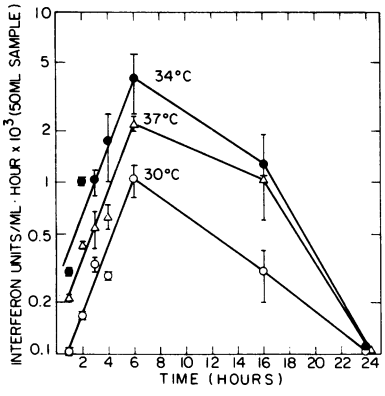

FIG. 4. *Kinetics of interferon production at various temperatures. Duplicate cultures of FS-4 cells grown to confluency on microcarriers were set up at a density of 10^6 cells per ml in 50-ml volumes and maintained at three different temperatures throughout induction and production. Induction was carried out as described in the text except that 10 μg of cycloheximide per ml was used. At the indicated times after addition of DMEM + Plasmanate, fluids were collected and frozen for assay, cells were washed with DMEM, and fresh DMEM + Plasmanate was added. (Note that each point in the graph represents the amount of interferon produced per hour during the respective time period and does not refer to accumulated totals.)*

Rates were significantly less at 37°C and even lower at 30°C. The total amounts of interferon accumulated at 24 h at 34, 37, and 30°C were 22,500, 14,000, and 5,600 U/ml, respectively.

Comparison of interferon yields using standard and modified superinduction procedures and the effect of priming. A series of experiments were carried out to compare the standard and modified induction procedures and to determine the effect of priming on interferon yields (Table 5). The titers obtained using the modified superinduction procedure were considerably higher than those obtained with the standard procedure. However, the yields obtained with the modified procedure were lower and less consistent than expected (mean ± standard deviation = 5,400 ± 5,200 U/ml). The effect of priming the cells with 50 U of interferon per ml had the striking effect of giving higher and much more consistent yields (mean ± standard deviation = 23,000 ± 4,100 U/ml).

DISCUSSION

In a previous report (3), the microcarrier system was shown to be a potentially useful system for the large-scale production of human fibroblast interferon. However, it was pointed out that one of the problems which needed to be resolved was an inconsistency in yields obtained, which varied from 1,000 to approximately 10,000 U/ml. In this study, we have examined a number of parameters which we felt might be important in an effort to obtain consistently high yields.

The rationale for experimenting with a range of concentrations of antimetabolites was that a different concentration (preferably a lower concentration which might result in a lower level of

TABLE 5. *Comparison of interferon yields using standard and modified superinduction procedures*

Treatment	Interferon yield (U/ml)[a] in expt no.:			
	1	2	3	4
Poly (I)·poly (C) only	<100	ND	ND	ND
Superinduction (standard)[b]	1,000	ND	ND	ND
Superinduction (modified)[c]	3,400	13,000	1,000	4,300
Superinduction (modified) + priming[d]	25,000	17,500	22,000	27,000

[a] Since production was carried out at 10^6 cells per ml, the figures in this table also refer to the number of units per 10^6 cells. The first experiment was carried out to compare yields obtained under a variety of different conditions. The next three experiments were done to determine the effect of priming cultures. ND, Not done.

[b] Standard conditions are described in the text.

[c] The standard procedure was modified as follows: cycloheximide concentration was 10 μg/ml; actinomycin D was left on cultures for 2 h, and the temperature for the experiments was maintained at 34°C.

[d] Priming consisted of exposing the cells to 50 U of interferon per ml for 16 h before induction on day 6. Cells were washed twice with DMEM, after which DMEM + 0.5% Plasmanate + interferon was added.

toxicity) might result in higher yields. The discovery that a lower level of cycloheximide (approximately 10 μg/ml) is optimal for interferon production differs from results reported by Havell and Vilček (5), who found that 50 μg/ml gave best results. However, it is in good agreement with the findings of Billiau et al. (2), who also found 10 μg/ml to be optimal.

The effect of varying the time of exposure of cells to cycloheximide (Fig. 1A) is in good agreement with Havell and Vilček (5) in showing that 4 to 6 h of exposure is required to give optimal interferon levels. Increasing the time of exposure to actinomycin D from 1 to 2 h (Fig. 1B) gave a significant increase in interferon yield. Further experimentation is under way to determine whether even higher yields can be obtained by further extending the time of exposure to actinomycin D.

It was of interest to determine the effect of serum concentration on the growth of FS-4 cells and interferon yields resulting from these cultures. As shown in Fig. 2, cells grew to approximately the same density regardless of serum concentration. It is worth noting, however, that morphologically the cells appeared to be in a much healthier state at the lower serum concentrations (2.5 and 5%), i.e., cells tended to be more spread out in a typical criss-cross fibroblast pattern and showed no evidence of detachment. In contrast, cells grown at the higher serum concentrations (10 and 20%, vol/vol) tended to round up significantly after growing to confluence (generally by day 5 or 6) and would frequently show some detachment. More importantly, these same cells (those grown at the higher serum concentrations) showed significant shedding from the microcarrier during the induction procedure. We believe this could account, by in large, for the reduced interferon yields of those cells grown at higher serum concentrations.

The reduced serum requirement for FS-4 cells grown on microcarriers coincides with earlier work (4) which showed that chicken embryo fibroblasts required fourfold less serum for growth on microcarriers than in roller bottles. As pointed out in that report, this finding may result simply from a greater efficiency in a stirred-tank configuration as compared to a stationary culture system, or it may reflect real differences at the microenvironmental level.

The results of the experiment examining the inoculation of FS-4 cells on microcarriers at different densities (Fig. 3) are interesting in that a greater cell density per unit volume ($\simeq 3 \times 10^5$ cells per ml) was required for satisfactory growth than one would expect based on experience with other types on monolayer cultures. As an example, roller bottles (Corning Plastic, 500 cm^2) are normally seeded with FS-4 cells at a concentration of about 6×10^4 cells per ml, and satisfactory growth can be obtained using one half this amount. On a per-unit-area basis, the normal seed density for both microcarriers and roller bottles is approximately 10^4 cells per cm^2. From these data it seems reasonable to conclude that the high per-unit-volume cell density requirement is due to the large surface area provided by the microcarriers, i.e., there is obviously a requirement for a minimum number of cells per microcarrier or per unit area of microcarrier surface.

The data on the kinetics of interferon production at different temperatures (Fig. 4) clearly show that significantly higher yields of interferon can be obtained by lowering the temperature from 37 to 34°C during induction and production phases. Assuming that the hypothesis involving the existence of a repressor which exerts a negative control over interferon synthesis is correct, a number of explanations for the increased yield at 34°C are possible: (i) continued rapid turnover of inhibitor messenger ribonucleic acid, coupled with a slower decay rate of interferon messenger ribonucleic acid during the induction phase, resulting in a greater amount of interferon messenger ribonucleic acid available at the start of the production phase (immediately after removal of actinomycin D); (ii) an increased stability of interferon messenger ribonucleic acid during the early critical stages of the production period; or (iii) a combination of (i) and (ii) above. The low yields at 30°C probably reflect a low rate of cell metabolism, which one would expect at this temperature. Experiments attempting to determine the importance of temperature control during each separate phase (induction and production) are currently in progress.

During the course of these experiments (in spite of a number of findings which tended to enhance interferon yields), the consistency of the yields from one experiment to the next was not as good as was hoped for. The average of all maximum interferon titers in those experiments presented so far in this paper is 17,100 ± 6,300 (mean ± standard deviation), which means that the yield for any given experiment or production run would be expected to range from approximately 27,400 to 6,800 U/ml (90% confidence level).

We decided to compare the standard and modified superinduction procedures and, in a continuous series of experiments, to measure the effect of priming cells (with interferon) in conjunction with the modified superinduction procedure (Table 5). Experiments were conducted (data not shown) which revealed the optimal concentration of interferon for priming to be approxi-

mately 50 U/ml. Titers obtained with the modified superinduction procedure were lower and less consistent than expected (mean ± standard deviation = 5,400 ± 5,200). However, the addition of priming to the procedure had the effect of giving consistently high yields (mean ± standard deviation = 23,000 ± 4,100). Under these conditions, the yield for any given experiment would be expected to fall between 29,700 and 16,300 (90% confidence level). The overall effect of priming in this series of experiments was a greater than fourfold increase in the amount of interferon produced. However, since yields in excess of 20,000 U/ml have been achieved many times without priming, the actual effect of priming probably had more to do with somehow giving consistency to interferon production with microcarrier-grown cells (perhaps by sensitizing cells to the induction procedure) than to imparting to cells the ability to produce increased amounts of interferon. Regardless of the mechanism involved in the priming procedure, priming appears to be of critical importance for obtaining consistently high interferon yields with microcarrier-grown cells when a superinduction procedure is used. A number of reports (2, 5) indicate that this is not the case for cells grown on other surfaces, e.g., plastic dishes and roller bottles.

With the superinduction procedure described, it is now possible to obtain interferon yields with normal human diploid fibroblasts of greater than 20,000 U/ml (or per 10^6 cells) with high consistency. High interferon yields, coupled with the many advantages of growing cells in suspension culture, appear to provide a highly efficient system for the large-scale production of human fibroblast interferon.

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ADDENDUM

Repeat experiments measuring the effect of increased time of exposure to actinomycin D have confirmed that maximal yields are obtained when actinomycin D is in contact with cells for a period of approximately 2.5 h.

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