Production of Crude Human Interferon with High Specific Activity (36486)

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The interferon systems of chick, mouse and rabbit have been studied extensively in the past. The choice of these animal species was conditioned mainly by the availability of efficient production and titration systems. As to human interferon, interest in good production systems stems not only from scientific motivation but also from a desire to produce an interferon which may eventually be used to treat human disease. Three methods have in the past been envisaged for mass production of human interferon: (i) infection of suspended human leucocytes with Newcastle disease virus (NDV) or Sendai virus, (3, 5–7, 12–15, 18), (ii) infection of human skin fibroblasts with NDV (10), and (iii) infection of suspended human amniotic membrane with NDV (4). From these three methods, only the leucocyte system has, until now, been used on a more or less large scale. It has served to prepare the international human reference preparations 67/87 and 69/19 (8). However, current yields are too small to permit large scale clinical trials in humans. Furthermore, in the event of generalized application, the method would require a regular and massive supply of fresh human material. Cells of different individuals would be pooled for each preparation, and would have to be monitored for the presence of different endogenous viral agents.

Both leucocytes and fibroblast monolayers infected with interferon-inducing viruses release viral antigens which are difficult to remove completely. Also the presence of bovine serum or serum albumin (6, 12) in interferon preparations is a handicap for its use in humans.

The purpose of the present study has been to carefully select cultivated human cell strains, inducers and optimal conditions for induction, in order to obviate these different drawbacks.

Materials and Methods. The continuous cell lines ACA, AV3 (both derived from amnion) and L132 (derived from embryonic lung), as well as the diploid cell lines Flow-1000 (embryonic skin and muscle) and Flow-2000 (embryonic lung) were purchased from Flow Laboratories, Irvine, Scotland. Other skin, muscle or lung cells were started from apparently normal human embryos or from skin biopsy specimens taken for cytogenetic examination. Embryonic tissues were fragmented and partially digested by trypsin. The cells harvested from the supernatant were washed and cultured in Eagle's minimum essential medium supplemented with nonessential amino acids and 10% fetal bovine serum. Cultures derived from skin biopsy specimens taken for cytogenetic examination were kindly provided by Dr. H. Van den Berghe (Center for Human Genetics, University of Leuven, Belgium). The biopsy specimens were cut in fragments of 2 mm which were fixed to the bottom of a glass Petri dish by a serum clot. As soon as cell colonies had developed, the cells were subcultured by regular trypsinization procedures. Maintenance medium contained 2% heated calf serum.

Newcastle disease virus (NDV, Komarov strain) and Sendai virus were propagated by allantoic inoculation of 11-day-old chick embryos. NDV had a chick red cell agglutinating titer of 1/10,240. The Sendai virus titer

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TABLE I. Comparison of Interferon Yields in Different Human Cell Lines Stimulated with NDV, Sendai Virus or Sindbis Virus (Induction Schedule Indicated in Materials and Methods).[a]

Human cell strains			Yield of interferon (log_{10} units/ml)		
Designation	Origin	Characteristics	NDV	Sendai	Sindbis
ACA	amnion	continuous lines;	< 1.0	< 1.0	< 1.0
AV3	amnion	epithelial	< 1.0	< 1.0	< 1.0
L132	embryonic lung		< 1.0	< 1.0	< 1.0
VGS	embryonic skin and muscle	fibroblasts	2.7	3.0	2.0
N_2S			3.1	2.6	2.6
NS			2.2	2.6	< 1.6
NM			3.1	1.8	1.8
Flow 1000			2.2	2.5	2.0
VGL	embryonic lung	fibroblasts	3.6	2.3	1.7
Flow 2000			2.3	2.4	< 1.6

[a] VGS, VGL, NM, NS, and N_2S are lines started from apparently healthy human embryos (see Materials and Methods).

was 1/1280. Sindbis virus was grown and titrated on chick embryo fibroblast cultures; the titer was $10^{8.0}$ PFU/ml. Semliki forest virus was grown on mouse embryo fibroblasts and titrated on mouse L929 cells; the titer was $10^{4.8}$ PFU/ml. Chikungunya virus was a mouse brain preparation containing $10^{7.0}$ mouse LD_{50}/ml.

Single-stranded polyriboinosinic (poly-I) and polyribocytidylic (poly-C) acids were dissolved in Dulbecco's phosphate buffered saline (PBS) at 1 mg/ml. They were annealed into double-stranded polyinosinic-cytidylic acid (poly-I:C) by combination of equal volumes, heating at 90° and slowly cooling to room temperature. Hypochromicity at 245 mμ was more than 40%.

For induction of interferon, cultures were grown in unstoppered glass cups with flat bottom (diam 27.5 mm) kept in a CO_2-incubator. When the cups were seeded with *ca.* 300,000 cells, confluent monolayers (100,000 cells/cm^2 or 600,000 cells/cup) were reached in 3–4 days. The cultures were used within 3 days after confluency. Standard conditions for induction with virus were: incubation for 1 hr with 0.25 ml of a 1/3 dilution of stock virus preparation and addition of 1 ml of maintenance medium after 1 hr. Interferon was harvested from the four cultures after 24 hr of incubation at 37°. Virus was killed in the preparations by acidification at pH 2 for 5 days at 4°. Neutralized samples were kept frozen at —20° until titrated. Conditions for induction by double-stranded RNA were: incubation for 2 hr with 1 ml of medium containing 50 μg of poly-I:C followed by three washes and addition of 1.25 ml of medium per cup. When indicated in the text, 10 μg/ml of cycloheximide (Sigma Chemical Co., St. Louis, MO) was included in the medium. Unless stated otherwise, interferon was harvested at 24 hr. When cycloheximide was present, the samples were dialyzed against PBS.

Interferon was titrated by inhibition of Sindbis virus cytopathic effect in human embryonic skin fibroblasts (VGS strain). Cells for interferon titration were grown on the bottom of unstoppered glass tubes (diam 12 mm, round bottom) in a CO_2-incubator. Duplicate monolayers were incubated for 24 hr with serial 0.5 log_{10} dilutions of interferon. The tubes were decanted and challenged with Sindbis virus at a multiplicity of infection of 10. After 1 hr of incubation, excess virus was removed by three washes and the cells were refed. Cytopathic effect was read after 24 hr. A laboratory reference preparation of human interferon was included in each titration. The preparation was assigned a number of units/ml equal to the reciprocal of its average end point dilution in a large number of

assays. This procedure permitted to express all values in terms of this standard. The 67/87 IAMS reference preparation (8), quoted to contain 100 IU of human interferon, contained 350 units when titrated by our method. The 69/19 reference preparation, quoted to contain 5,000 IU, titrated 12,500 units by our method.

Protein content of interferon samples was determined by a modified Folin reaction (9).

Results. 1. Induction of interferon in human cell monolayers by different viruses. In order to select a cell line and a viral inducer which would produce the highest possible interferon yields, three continous human cell lines, ACA, AV3 and L132, as well as seven subcultured human cell strains, prepared from embryonic skin, muscle or lung were infected under standard conditions (see Materials and Methods). Five viruses, which have been reported in literature to induce interferon in different cells were chosen: NDV, Sendai virus, Sindbis virus, Semliki Forest virus (SFV) and Chikungunya virus. Table I shows that the continuous cell lines were poor yielders, while the skin and muscle fibroblasts produced the highest amounts. In some of the cell lines high yields of interferon were induced by NDV or Sendai virus. In the experimental conditions used, Sindbis virus induced only small amounts of interferon, while neither SFV or Chikungunya virus yielded appreciable amounts of interferon in any of the cell types tested.

In order to further define conditions for optimal yields, a number of experiments were done using VGS cells and both NDV and Sendai viruses. The results of these experiments can be summarized as follows: (a) The highest possible multiplicity of infection (undiluted allantoic fluid) was needed to obtain maximal yields. The viral inoculum could either be left on the cells and diluted with added medium during production, or it could be removed after 1 hr adsorption, without decrease in interferon yield. (b) The concentration of interferon reached a maximum at 9 hr with NDV and at 24 hr for Sendai virus. (c) When no serum was present during the time interval of interferon production, the final yield was reduced to 30% of that observed with 2% calf serum, when both Sendai virus and NDV were used as inducers. (d) Preincubation of the cultures for 30 min with 1 μg/ml of actinomycin D completely blocked production of interferon while the growth of NDV or Sendai virus was enhanced by a factor of 10 and 3, respectively. This seems to rule out the possibility that activity considered as interferon was due to killed virus whose interfering activity had resisted pH 2-treatment. The interferon activity, furthermore, resisted ultracentrifugation at 100,000g for 4 hr.

2. Induction of human interferon in monolayer cultures by double-stranded RNA. Optimal time and dosage conditions for induction of interferon by poly-I:C in human skin fibroblasts (VGS line) were reported elsewhere (2). It was found that incubation of the cells for 2 hr with a concentration of 50 μg/ml of poly-I:C, followed by thorough washing and refeeding gave the best results. The interferon induced was differentiated from residual poly-I:C, or from poly-I:C released by the cells, by its trypsin sensitivity, species specificity, and lability when exposed to 56° for 1–6 hr. Furthermore it was not sedimented at 100,000g.

The stability of the material was comparable to that of Sendai virus-induced interferon. Both preparations were stable for several months when kept frozen at —20° or in lyophilized form at 4°. Solutions kept at 4° or at 20° lost about 30% of their activity in 8 days. More rapid degradation occurred at 37°; half-life times of 1 and 1.5 days were noted, respectively, for Sendai virus-induced interferon and poly-I:C-induced interferon.

The production of interferon can be enhanced in cultures by pretreatment (priming) with small amounts of poly-I:C (1, 2), or by exposure to metabolic inhibitors, such as cycloheximide (11, 16, 17). The following experiments were done to evaluate the usefulness of these procedures to increase production of interferon in human cell monolayers. Cultures were preincubated for 24 hr with 0, 0.01, 0.03, or 0.1 μg/ml of poly-

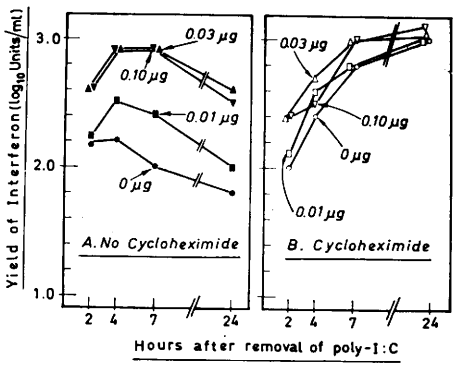

FIG. 1. Time–response curves of interferon production by human skin fibroblasts exposed to poly-I:C (50 μg, 2 hr). Combined effects of preincubation (24 hr) with small amounts of poly-I:C (indicated in figure), and of addition of cycloheximide (10 μg/ml) during production.

I:C and were subsequently exposed to 50 μg/ml for 2 hr. After three careful washes, the cultures were refed with either control medium or medium containing 10 μg/ml cycloheximide. Separate groups of cultures were harvested at different times afterward. Figure 1 illustrates the results of a representative experiment. It can been seen that priming with 0.03 or 0.1 μg/ml poly-I:C enhanced the interferon production, in the absence of cycloheximide, about six-fold. Addition of cycloheximide resulted in 24 hr yields which were substantially higher than those in cultures not treated with the inhibitor. These conclusions were substantiated by the pooled results of seven experiments, the statistical analysis of which is shown in Table II. Particularly, it can be seen that combination of priming and cycloheximide treatment resulted in interferon yields which were significantly higher than those obtained with priming alone, but not with cycloheximide treatment alone. From Fig. 1 it also appears that, in cultures not treated with cycloheximide, peak values were reached at 4–7 hr, whereafter the interferon concentration lowered. Over nine experiments this disappearance of interferon from the supernatant was found to be significant at a p-level $< .01$. Addition of cycloheximide caused an initial slight delay in production, also described in rabbit kidney cultures (16, 17), followed by rapid release of large amounts of interferon. Due to this difference in time–response curves, 24 hr yields in the presence of cycloheximide were only slightly higher than 6 hr yields in adequately primed cultures which were not exposed to the inhibitor. Since long incubation times and addition of inhibitors are liable to increase release of impurities, preference may be given to the priming procedure, which permits increase of interferon yields and harvest early after induction.

In order to compare interferon yields induced with poly-I:C and viruses, different human cell lines, derived from skin biopsy

TABLE II. Statistical Analysis of Seven Experiments in which the Effect of Priming or Treatment with Cycloheximide, or both, was Tested on the Induction of Interferon by Poly-I:C in VGS-Cells.[a]

Comparison	Average increase in interferon yield ($\Delta \log_{10}$ units/ml)	Number of degrees of freedom (ν)	p-value (t test on paired data)
Priming vs control	.56	10	< 0.001
Cycloheximide vs control	.99	6	< 0.001
Cycloheximide + priming vs priming only	.54	10	< 0.005
Cycloheximide + priming vs cycloheximide only	.06	10	> 0.70

[a] Cultures were primed for 24 hr with 0, 0.01, 0.03 or 0.10 μg of poly-I:C, carefully washed and induced for 2 hr with 50 μg/ml of poly-I:C. After removal of the inducer, cultures were replenished with medium containing either 0 or 10 μg/ml of cycloheximide. Interferon was harvested 24 hr later. Figures shown refer to differences in production rates between paired cultures.

TABLE III. Effect of Presence or Absence of Serum on Production of Interferon in Human Cultures Stimulated with Poly-I:C.

Priming[a]	6 hr Interferon production (log_{10} units/ml)	
	2% serum	No serum
+	3.6	3.2
−	3.2	2.7

[a] Priming consisted of incubation for 24 hr with 0.01 μg/ml of poly-I:C prior to induction with 50 μg/ml of poly-I:C for 24 hr.

specimens taken for cytogenetic examination, were stimulated under standard conditions (see Materials and Methods) with NDV, Sendai virus or poly-I:C. Cycloheximide (10 μg/ml) was included in the medium of cells stimulated with poly-I:C. There was no correlation between responses to the three inducers, which implies that, in the populations of cell lines that were tested, the interferon response to a certain inducer is not predictable from the response to another one. On the average, cells induced with NDV yielded $10^{2.4}$ units/ml, those induced with Sendai virus $10^{2.2}$ units/ml and those stimulated with poly-I:C in the presence of cycloheximide, $10^{2.8}$ units/ml. In conclusion, over a large number of human cell strains, average production using poly-I:C as an inducer could be considered at least as effective as that with NDV or Sendai virus.

Finally, the possibility of producing interferon in the absence of serum was evaluated. This was done in primed as well as unprimed VGS cells. Priming consisted of incubation with 0.01 μg/ml poly-I:C for 24 hr prior to induction with 50 μg/ml poly-I:C for 2 hr. The results, as shown in Table III, indicate a relative need for serum, as yields were about three times lower in serumless medium. Primed cultures could still regularly produce $10^{3.0}$ units/ml in the absence of serum. These preparations contained 0.008 mg/ml protein (sp act of interferon: $10^{5.15}$ units/mg). A saline dilution of the international standard preparation 69/19 titered $10^{2.9}$ units/ml for a protein content of 0.495 mg/ml (sp act: $10^{3.2}$ units/mg). In conclusion, VGS cells properly induced with poly-I:C could produce interferon having a titer $10^{3.0}$ units/ml and a specific activity 89 times higher than that of a potent standard leukocyte preparation.

Discussion. Different human cell lines and different induction systems were compared in order to select a suitable method of production of concentrated and relatively pure human interferon. Of five viruses tested, NDV and Sendai proved to be the best inducers. Using the VGS line of skin fibroblasts in medium containing 2% calf serum, yields of 10^3 units/ml could regularly be obtained. This is equivalent to 2 units/1000 cells. Although comparison with literature data is difficult in view of the different assay methods used by different authors, this value is in good agreement with the yields reported by Merigan *et al.* (10). Indeed, these authors produced 20,000 units on confluent Blake bottles having a growth surface area of 120 cm^2. Since skin fibroblasts reach confluency at about 100,000 cells/cm^2, this yield corresponds to 1.7 units/1000 cells.

Poor yields were obtained with Sindbis virus, SFV and Chikungunya virus. Conceivably, this may have been due to low infectivity of our strains for human cells. Adaptation of Sindbis virus by serial passage in human cells did not result in higher interferon production (unpublished results). No attempts were made to concentrate the virus stocks by physical procedures, since this would have been unpractical for large-scale production purposes.

Good yields of interferon were also obtained using VGS cells and the double-stranded RNA, poly-I:C as inducer. Either cycloheximide or priming by small amounts of poly-I:C were used to increase the production of interferon. The advantage of the second method was that interferon could be harvested early after induction, so that the risk for contamination with inactive cellular material could be reduced. It has been shown, indeed, that repeated or prolonged exposure of human cells to relatively small concentrations of poly-I:C results in

cytopathic effect (2).

In a comparative test using different cell lines, the average yield of interferon was higher when the cells were induced with poly-I:C and cycloheximide than with NDV or Sendai virus. The stability of poly-I:C-induced interferon was comparable to that of virus-induced interferon.

When serum was omitted from the medium of cultures stimulated by poly-I:C, the yield was reduced to 30% of that obtained when 2% calf serum was present. Still, in the absence of serum, preparations containing 10^3 units/ml, or 2 units/1000 cells, were regularly obtained. This value compares favorably to data reported by other authors for leukocyte interferon. From literature data the following values (units/1000 cells) can be calculated: 0.1 (13), 0.3 (15), 0.4 (3), 1.0 (5), 1.25 (7) and 2.5 (18).

The procedure of manipulating the cells with a minimum amount of trauma, and omitting serum from the medium favorably affected the specific activity of the interferon obtained. Expressed as units/mg protein, the specific activity of interferon induced by poly-I:C in primed VGS cells without serum was $10^{5.15}$ compared to $10^{3.20}$ for the potent 69/19 IAMS reference leukocyte preparation (8), and $10^{3.96}$ for a 350-fold purified preparation described by Falcoff *et al.* (3). Apart from this high specific activity, poly-I:C-induced interferon has the additional advantage over leukocyte interferon of being free of viral antigens. Also, once a certain cell strain can be found safe for production of interferon for use in humans, repeated screening, as would be necessary for the leukocyte substrate, would be superfluous.

For these various reasons poly-I:C-induced interferon represents a valuable alternative to leukocyte interferon as starting material for the preparation of purified and concentrated interferon for human use. Additional investigation is needed to further increase the interferon yields and to adapt the method for large-scale production purposes.

Summary. The production of interferon by different human cell lines after stimulation by viruses and by poly-I:C was compared. It was found that under optimal conditions, yields of interferon obtained after stimulation with poly-I:C were higher than those obtained after induction by viruses. The production of interferon in poly-I:C-stimulated skin fibroblasts could be increased by pretreatment with small amounts of poly-I:C (priming) or by inclusion of cycloheximide in the medium. Omission of serum from the medium resulted in a threefold decrease of interferon production. The interferon yield per cell and the specific activity of the interferon prepared in serum-free medium compared favorably with values reported in the literature for human leukocyte interferon.

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1. Billiau, A., J. Gen. Virol. **7**, 225 (1970).

2. Billiau, A., Van den Berghe, H., and De Somer, P., J. Gen. Virol. **74**, 25 (1972).

3. Falcoff, E., Falcoff, R., Fournier, F., and Chany, C., Ann. Inst. Pasteur, **111**, 562 (1966).

4. Fournier, F., Falcoff, E., and Chany, C., J. Immunol. **99**, 1036 (1967).

5. Gresser, I., Proc. Soc. Exp. Biol. Med. **108**, 799 (1961).

6. Hadhazy, G. Y., Strander, H., and Cantell, K., J. Gen. Virol. **5**, 351 (1969).

7. Horowitz, B., Stjernholm, B. L., and Wheelock, E. F., International Symposium on Standardization of Interferon and Interferon Inducers. London 1969. Symp. Ser. Immunobiol. Standard. **14**, 9 (1970).

8. International Symposium on Standardization of Interferon and Interferon Inducers. London 1969. Symp. Ser. Immunobiol. Standard. **14**, 326 (1970).

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

10. Merigan, T. C., Gregory, D. F., and Petralli, J. K., Virology **29**, 515 (1966).

11. Myers, M. W., and Friedman, R. M., Fed. Proc. **30**, 242 (1971).

12. Strander, H., Appl. Microbiol. **18**, 810 (1969).

13. Strander, H., and Cantell, K., Ann. Med. Exp. Fenn. **44**, 265 (1966).

14. Strander, H., and Cantell, K., Ann. Med. Exp.

Fenn. **45**, 20 (1967).

15. Valenta, J. R., DiCuollo, C. J., Pagano, J. F., Haff, R. F., and Ferlauto, R. J., Symp. Series Immunobiol. Standard. **14**, 17 (1970).

16. Vilcek, J., "Interferon," Proceedings of a Symposium sponsored by the New York Heart Association. 76 pp. Little, Brown and Co., Boston (1970).

17. Vilcek, J., Ann. N.Y. Acad Sci. **173**, 390 (1970).

18. Wheelock, E. F., J. Bacteriol. **92**, 1415 (1966).

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