

## The Antiproliferative Effects of Interferon on Murine Embryonic Cells (40385)

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Although interferon is best known for its antiviral effects, it has also been shown to inhibit the growth of a variety of primary, established, malignant and embryonic cell lines and strains (1-6). However, very little work has been done to determine when mammalian embryos and embryonic cells become sensitive to the antiproliferative effects of interferon. Consequently, the experiments to be reported here were undertaken to characterize the time at which these effects become manifest during early mouse development. We have found that the growth and development of preimplantation mouse embryos is not affected by exogenous interferon and that sensitivity to the antiproliferative effect of interferon is first detectable in cells from 8-day embryos and becomes significant in cells from 10-day embryos.

**Materials and methods.** Mouse interferon induced *in vitro* by Newcastle disease virus in C-243-3 mouse cells (7) was obtained from Litton Bionetics, Inc. (lot # 231-41-3), as was mock interferon (lot # 231-67-5) prepared from noninfected cells by the same procedure. The stated potency of the interferon preparation was  $10^6$  units per ml. However, when compared with NIH freeze-dried reference mouse interferon (G-002-904-511) by the VSV plaque reduction assay (8), 1000 units of the Litton Bionetics interferon had the same potency as 336 units of the reference standard. According to the manufacturer the protein concentration of the preparation, excluding the added fetal calf serum is 16.8 mg/ml. Therefore the specific activity of the interferon is  $2 \times 10^4$  NIH mouse reference standard units/mg protein. All results in the present study are given in terms of NIH mouse reference standard units and represent final concentrations. Mock interferon, although devoid of activity, is also quantitated in "units" to indicate the equivalence with the active interferon preparation of all con-

stituents other than interferon itself.

Preimplantation mouse embryos were obtained from superovulated Swiss-Webster mice and cultured in modified Whitten's medium under oil in 5% CO<sub>2</sub> in air at 37° (9, 10). Two-cell embryos were obtained 1.5 days after mating and 8-16 cell embryos 2.5 days after mating. Inner cell masses (ICMs) were prepared by immunosurgery (complement dependent antibody mediated lysis of trophoblast) using a rabbit anti-mouse serum and guinea pig complement (GIBCO) (11).

To determine incorporation of [<sup>3</sup>H]thymidine, the embryos of ICMs grown in the presence of interferon were washed in culture medium and incubated for 4 hours in fresh medium with 5 μCi/ml [<sup>3</sup>H]thymidine prepared by diluting stock [<sup>3</sup>H]thymidine (10 μCi/ml, 2.1 Ci/mmol) 1:1 with 2 × concentrated medium containing interferon, mock interferon, or PBS. The embryos or ICMs were then washed 10 times in medium, transferred to small tubes containing 50 μg carrier DNA in 0.5 ml water, and frozen at -70° until assayed. The DNA was precipitated with 2 ml 5% trichloroacetic acid for 30 min at 0°, the precipitates collected on glass fiber disks (Whatman GF/C) and prepared for scintillation counting as described previously (12).

Cell cultures were prepared from 7 to 17 day Swiss-Webster mouse embryos. The embryos were removed in a sterile manner, washed in PBS, and either minced with scissors or forced through the hub of a 10 ml syringe (after removal of heads and limbs, if necessary) into an extraction flask containing a Teflon-coated stirring bar. The dispersed embryos were washed twice with stirring in warm PBS for 3 min and the wash solutions decanted. They were then dissociated 3 or 4 times for 5 to 10 min each in a PBS-0.05% trypsin-0.02% EDTA solution. The separated cells were plated in 35 mm culture dishes at a density of  $5-7.5 \times 10^4$  cells per dish in 1 ml of Dulbecco's modification of Eagle's mini-

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mal essential medium with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 mg/ml), and amphotericin B (2.5 mg/ml) in 5% CO<sub>2</sub> in air at 37°. If the cells were not used immediately for assay as primary cultures, the medium was changed after 3 days. The cultures were harvested with the saline-trypsin-EDTA solution before reaching confluence and reseeded as secondary cultures in 35 mm dishes at a density of 5 × 10<sup>4</sup> cells per dish.

To assess the effects of interferon, the primary or secondary cultures were incubated for 6 hr in 1 ml Dubecco's medium with 10% fetal calf serum and 4.5 g/l glucose but without antibiotics. Appropriate dilutions of interferon or mock interferon were then added in 0.1 ml of medium, and the cells incubated for another 42–44 hr. To determine the rate of incorporation of [<sup>3</sup>H]thymidine into DNA, 1 μCi of <sup>3</sup>H-thymidine (2.1 Ci/mmol) in 0.1 ml of medium was added for the terminal two hours of the culture. The medium was decanted and the cells were harvested with 1 ml of saline-trypsin-EDTA solution (15 min at 37°), collected on a glass fiber filter (Whatman GF/A) and prepared for scintillation counting as described above. To estimate cell growth, cells were harvested as described above, and counted in a hemocytometer.

**Results.** Preimplantation ICR-Swiss embryos at the 2-cell and 8–16 cell stages were cultured *in vitro* for 69 and 45 hr, respectively, in the presence of 3.4 × 10<sup>3</sup> units/ml of mouse interferon and the number developing into

morulas and blastocysts determined. Blastulation was examined because it is a particularly sensitive indicator of embryonic development (9, 13). As a control, parallel cultures of embryos were exposed to an equivalent amount of mock mouse interferon. As is shown in Table I, neither the interferon nor the mock interferon had any effect on development of the embryos. Furthermore, the rate of incorporation of [<sup>3</sup>H]thymidine into the blastocysts which developed after being exposed to interferon during the entire period of *in vitro* culture was not significantly reduced.

Although blastocysts developing *in vitro* were not sensitive to the antiproliferative effect of interferon, it was possible that sensitivity to interferon could have developed in the inner part of the blastocyst, the inner cell mass, after its differentiation from the trophoctoderm and that the outer trophoctoderm might interfere with access of the agent. Therefore, bare inner cell masses prepared by immunosurgery were exposed to interferon for 48 hours and incorporation of [<sup>3</sup>H]thymidine then determined (Table I). Again, a concentration of 3.4 × 10<sup>3</sup> per ml units of interferon did not significantly reduce [<sup>3</sup>H]thymidine incorporation, and it is concluded that preimplantation embryos are not sensitive to this concentration of exogenous interferon.

To determine the concentration of interferon to be tested against cells derived from postimplantation embryos, a preliminary se-

TABLE I. EFFECT OF INTERFERON ON PREIMPLANTATION MOUSE EMBRYO DEVELOPMENT AND [<sup>3</sup>H]THYMIDINE INCORPORATION.

	Saline	Addition to culture Mock interferon	Interferon <sup>a</sup>
<i>2-cell (day 1) embryos</i>			
Total number of embryos	81	80	80
Morulas and blastocysts after 72 hr in culture	63 (78%)	72 (90%)	68 (85%)
Incorporation of [ <sup>3</sup> H]thymidine after 69 hours in culture <sup>b</sup>	—	60 cpm/embryo	50 cpm/embryo
<i>8–16 cell (day 2) embryos</i>			
Total number of embryos	28	30	30
Morulas after 24 hr in culture	21 (75%)	24 (80%)	29 (97%)
Blastocysts after 75 hr in culture	28 (100%)	24 (80%)	29 (97%)
Incorporation of [ <sup>3</sup> H]thymidine after 45 hr in culture <sup>b</sup>	52 cpm/embryo	46 cpm/embryo	63 cpm/embryo
<i>Isolated inner cell masses (ICM)</i>			
Incorporation of [ <sup>3</sup> H]thymidine <sup>b</sup>		53 cpm/ICM	50 cpm/ICM

<sup>a</sup> Final concentration in cultures was 3.4 × 10<sup>3</sup> NIH reference units mouse interferon/ml.

<sup>b</sup> Each value represents the mean of two experiments.

ries of experiments was carried out with secondary cell cultures derived from whole day 13 embryos. Utilizing both cell counts and [ $^3\text{H}$ ]thymidine incorporation (Table II), it was found that 34 units/ml had no antiproliferative effect, 336 units/ml gave equivocal results, and  $3.4 \times 10^3$  units/ml had a clearly inhibitory effect on both cell growth and thymidine incorporation. Therefore, the concentration of  $3.4 \times 10^3$  units/ml of interferon was used for all subsequent experiments. Human interferon (Sendai virus induced leukocyte interferon obtained from the National Institutes of Health) at a concentration of  $10^4$  NIH reference units/ml had no greater effect on either the thymidine incorporation or cell proliferation of the secondary cultures than did the mock mouse interferon preparation. This indicates that the inhibitory effect of interferon was, as would be expected, species specific.

Primary embryonic cell cultures established from embryos of various ages were exposed to interferon, mock interferon, or phosphate buffered saline, and the incorporation of [ $^3\text{H}$ ]thymidine into DNA determined (Fig. 1). Although thymidine incorporation into cells derived from 7-day embryos was not inhibited, inhibition was observed in cells from 8 and 9-day embryos and reached a maximum of about 44% (expressed relative to the mock interferon control) or 36% (expressed relative to the saline control) in cells from 10 to 12 day old embryos. The discrepancy between the two values results from the fact that the mock interferon preparation often had a stimulatory effect on cell growth. As with the secondary cultures, human interferon at  $10^4$  units/ml had no effect on the primary cells from 10- to 17-day embryos.

TABLE II. INHIBITION BY INTERFERON OF [ $^3\text{H}$ ]THYMIDINE INCORPORATION AND PROLIFERATION OF SECONDARY EMBRYONIC CELL CULTURES.

Interferon or mock interferon (units/ml)	$^3\text{H}$ -incorporation <sup>a</sup> (% inhibition) <sup>b</sup>		Cell proliferation <sup>a</sup> (% inhibition) <sup>b</sup>	
	Mock	Interferon	Mock	Interferon
34	2.4	0	—	—
336	15.6	13.8	0	7.5
$3.4 \times 10^3$	25.6	43.6	0	28.0

<sup>a</sup> Each value represents the mean of two experiments.

<sup>b</sup> Inhibition is represented as percent reduction of control values.



FIG. 1. The inhibition of incorporation of [ $^3\text{H}$ ]thymidine into the DNA of primary mouse embryo cells. The open circles represent inhibition of incorporation by  $3.4 \times 10^3$  units/ml of interferon expressed relative to the mock interferon control; the closed circles represent inhibition expressed relative to the PBS control. Each point is the mean of two experiments.

*Discussion.* Several studies have been carried out on the response of fetal cells to the antiviral effects of interferon (14–19). Comparison of chick embryos at different stages of development (16–18) demonstrated a greater sensitivity in cells from 12 to 15 day than from 6 to 8 day chick embryos, but this difference has been attributed to the elaboration of a repressor by the younger cells (18). Studies of human cell strains obtained from embryos ranging in age from eight weeks on and passaged at least 10 passages in culture, demonstrated that cells from the least mature embryos were the least sensitive to interferon (18). Similarly, cells from 18-day mouse embryos were stated to be more sensitive than are cells from 7-day embryos to the antiviral effects of interferon (14), consistent with our observations on the antiproliferative effect of interferon in embryonic mouse cells.

Two studies of the antiproliferative effects of interferon on mouse embryonic cells have been reported. Lindahl-Magnuson and collaborators (6) studied the effects of  $10^3$  units/ml of a Newcastle disease virus induced MSV-Ia cell interferon on primary C3H “fibroblasts” derived from either 12-day or 18-day embryos. After 5 days in culture, the rate of [ $^3\text{H}$ ]thymidine incorporation was inhibited 35 to 55%, although at times no effect was demonstrable until the 7th or 8th day. Despite the variability of the system, the minimum effective dose of interferon was stated to be

125–250 units/ml. In close agreement with our results, insofar as the quantity of interferon necessary, are those of Ohwaki and Kawadi (4) who found that the growth over 4 days of 5th or 6th passage fibroblasts from 14 to 15 day outbred mouse embryos was inhibited 20 to 40% by 333 NIH reference units/ml and 40–50% by  $3.3 \times 10^3$  NIH reference units/ml of partially purified L cell interferon. They also noted that the embryonic cells were only a tenth as sensitive as L cells to the antiproliferative effects of interferon. Because of the high specific activity of their material,  $3.3 \times 10^7$  NIH reference units/g protein, they argued that the growth inhibition that they observed was due to interferon itself and not to a contaminating protein.

The reasons for the changing sensitivity of embryonic cells to interferon are not known. Among the possibilities are the appearance or increase in concentration of an interferon receptor and a change in the activity of one or more components of the system which mediates the antiviral and antiproliferative effects. However, whatever the mechanism or mechanisms involved, it seems clear that significant changes do occur early in embryogenesis and that responsiveness to interferon is achieved in the mouse embryos by 10 days. Interestingly, this is the stage which corresponds developmentally to the time of maximal sensitivity to the teratogenic consequences of congenital viral infection in human embryos (14).

**Summary.** The antiproliferative effect of interferon was studied at various stages of pre- and postimplantation mouse embryo development. Although  $3.4 \times 10^3$  reference units/ml of NDV induced mouse interferon did not inhibit development of DNA synthesis in preimplantation embryos, an inhibitory effect was first detectable in primary cell cultures derived from whole 8-day embryos. This effect reached a maximum of 36 to 44% inhibition of DNA synthesis in cells cultured from 10-day embryos and remained at the same level with cells cultured from 12, 14, and 17-day embryos. These results indicate

that responsiveness to interferon is achieved in the mouse embryos by 10 days.

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1. Epstein, L. B., in "Interferons and Their Actions" (W. E. Stewart, II, ed.) p. 91. CRC Press, Cleveland (1977).
2. Gresser, I., in "Cancer, a Comprehensive Treatise" (F. F. Becker, ed.) p. 521. Plenum, New York (1977).
3. Rozee, K. R., and Lee, S. H. S., in "Interferons and Their Actions" (W. E. Stewart, II, ed.) p. 133. CRC Press, Cleveland (1977).
4. Ohwaki, M., and Kawadi, Y., *Acta Virol* **16**, 477 (1972).
5. Dahl, H., and Degre, M., *Acta Pathol. Microbiol. Scand. Sect. B* **84**, 285 (1976).
6. Lindahl-Magnusson, P., Leary, P., and Gresser, I., *Proc. Soc. Exp. Biol. Med.* **138**, 1044 (1971).
7. Oie, H., Gazdar, A., Buckler, C. E., and Baron, S., *J. Gen. Virol.* **17**, 107 (1972).
8. Epstein, L. B., in "Manual of Clinical Immunology" (N. R. Rose and H. Friedman, eds), p. 120. Amer. Soc. Microbiol. Publications, Washington (1976).
9. Golbus, M. S., and Epstein, C. J., *Differentiation* **2**, 143 (1974).
10. Epstein, C. J., *J. Biol. Chem.* **245**, 3289 (1970).
11. Solter, D., and Knowles, B., *Proc. Nat. Acad. Sci. U. S. A.* **72**, 5099 (1975).
12. Epstein, L. B., Stevens, D. A., and Merigan, T. C., *Proc. Nat. Acad. Sci. U. S. A.* **69**, 2632 (1972).
13. Golbus, M. S., Calarco, P. G., and Epstein, C. J., *J. Exp. Zool.* **186**, 207 (1973).
14. Mims, C. A., *Prog. Med. Virol.* **10**, 194 (1968).
15. Rinaldo, C. R. Jr., Isackson, D. W., Overall, J. C. Jr., Glasgow, L. A., Brown, T. T., Bistner, S. I., Gillespie, J. H., and Scott, F. W., *Infect. Immun.* **14**, 660 (1976).
16. Baron, S., and Isaacs, A., *Nature* **191**, 97 (1961).
17. Cantell, K., Valle, M., Schakier, R., Saukkonen, J. J., and Uroma, E., *Ann. Med. Exp. Fenn.* **43**, 125 (1965).
18. Grossberg, S. E., and Morahan, P. S., *Science* **171**, 77 (1971).
19. Siewers, C. M. F., John, C. E., and Medearis, D. N. Jr., *Proc. Soc. Exp. Biol. Med.* **133**, 1178 (1970).

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