# *INCREASED SURVIVAL IN MICE INOCULATED WITH TUMOR CELLS AND TREATED WITH INTERFERON PREPARATIONS*

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*Communicated by André Lwoff, March 11, 1969*

*Abstract.*—Repeated administration of potent mouse interferon preparations increased the survival of Balb/c and C 57/Bl$_6$ mice inoculated with 2,000–3,000 RC$_{19}$ and EL$_4$ tumor cells. Only 7/188 (3.7%) untreated mice (or mice treated with control preparations) survived more than 22 days after intraperitoneal inoculation of RC$_{19}$ tumor cells. None survived more than 60 days. In contrast, 101/103 (98%) interferon-treated mice survived beyond 22 days, and sixteen (15%) survived more than 60 days. None of these 16 surviving mice show any sign of tumor at present. Three mice (of the 16) from an early experiment are alive ten months after inoculation of RC$_{19}$ tumor cells.

Mouse interferon preparations derived from three different tissue sources—brain, serum, and monolayer cell cultures (with Newcastle disease virus and West Nile virus as interferon-inducing agents)—all proved effective. A *purified* preparation of mouse brain interferon was as effective as crude brain interferon. Human amniotic membrane interferon and control tissue preparations were without effect. These findings suggest that interferon itself (or a factor closely associated with interferon) is the active moiety in these preparations.

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*Introduction.*—We have previously reported that the repeated administration of interferon preparations delayed the evolution of Friend and Rauscher leukemias in mice.[1, 2] Although it seemed likely that interferon acted by repressing viral multiplication, it was emphasized that a direct action of interferon on the proliferation of viral-infected transformed cells had not been excluded.[3, 4] To explore this possibility, Balb/c or C 57/Bl$_6$ mice were inoculated with RC$_{19}$ or EL$_4$ tumor cells and treated daily with interferon preparations. We report here that this treatment resulted in a marked increase in the survival of tumor-inoculated mice.*

*Materials and Methods.—Mice:* One-month-old male and female Balb/c and C$_{57}$/Bl$_6$ mice were obtained from the breeding colony of the Institut de Recherches sur le Cancer.

There was no statistically significant difference between male and female mice in their response to inoculation of tumor cells or to interferon treatment. Accordingly, in all experiments, equal numbers of male and female Balb/c and C 57/Bl$_6$ mice were grouped for statistical analysis.

*Tumor cells:* (1) The RC$_{19}$ ascitic cell line originated from Balb/c mice inoculated with Rauscher virus (laboratory of Dr. J. P. Lévy). Electron microscopic examination of the cells reveals the presence of occasional type C virions.

(2) The EL$_4$ ascitic cell line was derived in 1945 from C 57/Bl mice inoculated with 9:10 dimethyl-1:2 benzanthracene.[5] These cells (laboratory of Dr. J. P. Lévy) have been serially transplanted in C 57/Bl$_6$ mice. Numerous intracellular type A "viral particles" are present.[6]

Numbers of cells inoculated were determined by counts at ten-fold serial dilutions, with trypan blue dye exclusion as a criterion of cell viability.

*Interferon preparations:* Interferon preparations were obtained from the brains of Swiss and Institut du Cancer (IC) mice inoculated with West Nile virus,[7] and the serum of IC mice and the nutrient medium of monolayer cultures of MSV-Ia cells inoculated with Newcastle disease virus.[7,8] Brain and cell culture interferons were concentrated tenfold as previously described.[7] Brain interferon was purified by batch chromatography on CM Sephadex $C_{50}$ and subsequent elution.[3,9] 98% of the protein in the original crude interferon preparation was eliminated by these techniques, and the specific activity rose from 3,200 interferon units/mg protein (crude preparation) to 64,000 units/mg protein in the purified interferon preparations. Human interferon was obtained from the nutrient medium of amniotic membrane inoculated with Newcastle disease virus.[10] The techniques utilized in the preparation of control material (i.e., brains and sera of uninoculated mice and cell culture nutrient medium) were identical to those used in the preparation of interferon.

The methods of assay of mouse and human interferon on monolayer cultures of L-cells and human fibroblasts inoculated with vesicular stomatitis virus have been previously described.[7] A standard reference mouse brain interferon was included in each test, and its titer (1:1600) rarely varied by more than one dilution in the different assays. *Tenfold* concentrated mouse brain interferon preparations usually titered 1:8,000–1:16,000/0.2 ml. The same interferon preparations titered 1:48,000–1:96,000/2 ml in simultaneous 50% plaque reduction assays (with L-cells and vesicular stomatitis virus).

*Absence of toxicity of interferon preparations for $RC_{19}$ and $EL_4$ cells as tested in vitro:* The concentrated preparations of mouse and human interferon (and the corresponding control materials) were incubated in equal volumes at 37°C with approximately $2 \times 10^6$ $RC_{19}$ or $EL_4$ cells. At varying intervals between 2 and 24 hr thereafter, aliquots of the cell suspension were removed and tested for cell viability (trypan blue dye exclusion). No significant difference in viability was detected between $RC_{19}$ and $EL_4$ cells incubated with interferon, control preparations, or nutrient medium.

*Statistical analysis:* Mean animal survivals were calculated using the reciprocal of the survival time in order to include survivors (for which the reciprocal of survival may be considered as zero). The results are expressed as the harmonic mean survival in days (i.e., the average of the reciprocals of survival). In the analysis of experimental results, standard tests (analysis of variance and $t$ test) were utilized.

*Results.*—(1) *The effect of interferon treatment on survival of Balb/c mice inoculated with $RC_{19}$ tumor cells:*

(*a*) *$RC_{19}$ Cells inoculated intraperitoneally—Various mouse or human interferon preparations inoculated intraperitoneally or subcutaneously:* One-month-old male and female Balb/c mice were inoculated intraperitoneally (i.p.) with approximately 2,000 $RC_{19}$ tumor cells (equivalent of $\cong$500 $LD_{50}$). Untreated mice or mice treated with control preparations died 17–24 days (mean 19 days) after tumor cell inoculation.

A significant increase in mouse survival was observed (as determined both by the number of mice surviving more than 22 days and the mean survival in days) when interferon preparations were injected i.p. (0.25 ml) 24 hours after inoculation of tumor cells and daily thereafter for 2 months (Table 1). This increased survival was noted after treatment of mice with mouse brain interferon (expts. 1–4, 6), serum interferon (expt. 1), or cell culture interferon (expt. 5). A *purified* preparation of mouse brain interferon proved as effective as a crude interferon preparation (expt. 4). Administration of human interferon (expt. 6) or control preparations did not increase the survival of tumor-bearing mice, and

in two experiments (expts. 1 and 3) inoculation of a normal brain or serum preparation shortened the survival time.

As can be seen from experiment 1 (Table 1) subcutaneous inoculation of mouse brain interferon also increased survival (mean survival, 23 days, control

TABLE 1. *The effect of continued administration of interferon preparations on the surviva of BALB/c mice inoculated with $RC_{19}$ tumor cells.*

| Expt. no. | Treatment* | No. of mice surviving >22 days/ total no. of mice | Mean harmonic survival (days) | Confidence interval (0.95) | Significance |
|---|---|---|---|---|---|
| 1. Control | Untreated | 0/15 | 19 | 18–20 | <0.05 |
| | Normal brain | 0/15 | 18 | 17–19 | N S |
| | Normal serum | 1/15 | 18 | 17–20 | |
| Interferon | Brain | 13/13 | 42 | 31–65 | <0.0001 |
| | Brain (subcutaneous route) | 8/15 | 23 | 21–24 | <0.001 |
| | Brain (initial week only) | 15/15 | 27 | 25–30 | <0.001 |
| | Brain (begun after 1 week) | 10/15 | 26 | 22–30 | <0.001 |
| | Serum | 8/15 | 24 | 21–30 | <0.001 |
| 2. Control | Untreated | 0/17 | 17 | 16–18 | |
| Interferon | Brain | 12/12 | 46 | 33–78 | <0.0001 |
| 3. Control | Untreated | 0/20 | 20 | 19–21 | <0.001 |
| | Normal brain | 0/20 | 17 | 16–18 | |
| Interferon | Brain | 19/20 | 27 | 26–29 | <0.0001 |
| 4. Control | Untreated | 1/15 | 20 | 18–21 | N S† |
| | Normal brain purified | 0/15 | 19 | 18–20 | |
| Interferon | Brain, crude | 15/15 | 34 | 28–42 | <0.0001 |
| | Brain, purified | 14/15 | 35 | 28–48 | <0.0001 |
| 5. Control | Untreated | 2/16 | 21 | 19–23 | N S |
| | Cell culture medium | 1/16 | 20 | 18–22 | |
| Interferon | Cell culture | 16/16 | 38 | 32–48 | <0.0001 |
| 6. Control | Untreated | 1/12 | 19 | 18–21 | N S |
| | Human amniotic membrane interferon | 1/12 | 20 | 19–21 | |
| Interferon | Brain | 12/12 | 47 | 33–81 | <0.0001 |

* All treatments were by the intraperitoneal route with one exception (expt. 1) in which one group of mice was inoculated subcutaneously.

All treatments were initiated 24 hr after inoculation of tumor cells and continued daily i.p. for one month unless otherwise specified as in expt. 1 (see text). Mice surviving the first month were inoculated subcutaneously for the second month.

† N.S.: Not significant.

When a significant difference in survival existed among control mice, the survival of interferon treated mice was compared to each of the control groups.

| Expt. no. | Approximate no. $RC_{19}$ cells inoculated i.p. | Units of interferon inoculated (i.p.) 0.25 ml/mouse/day | |
|---|---|---|---|
| 1 | 3,000 | Brain | 20,000 |
| | | Serum | 8,000 |
| 2 | 2,000 | Brain | 20,000 |
| 3 | 2,000 | Brain | 10,000 |
| 4 | 2,300 | Crude brain | 60,000 |
| | | Purified brain | 16,000 |
| 5 | 2,200 | Cell culture | 20,000 |
| 6 | 2,160 | Brain | 20,000 |
| | | Human amniotic Membrane | 4,000 |

TABLE 2. *Effect of mouse brain interferon preparations (i.p.) on survival of Balb/c mice inoculated (i.p.) with $RC_{19}$ tumor cells.*

| Survival | More than 22 days | | More than 60 days | |
|---|---|---|---|---|
| Controls | 3.7% | (7/188) | 0% | (0/188) |
| Interferon treated | 98% | (101/103) | 15.5% | (16/103) |

18–19 days), although the intraperitoneal route was more effective (mean survival, 42 days).

A summary of the effect of mouse brain interferon treatment on the survival of Balb/c mice inoculated (i.p.) with 2,000–3,000 $RC_{19}$ cells in these six experiments is presented in Table 2. (Only the results for treatment i.p. initiated 24 hours after inoculation of tumor cells are included.) In the control groups only 7/188 (3.7%) mice survived more than 22 days and none survived more than 60 days. In contrast, 101/103 (98%) interferon-treated mice survived beyond the 22nd day and 16 mice (15%) survived more than 60 days (to date). None of these 16 mice show any sign of tumor at present. Three mice (of the 16) from an initial experiment are alive ten months after tumor cell inoculation.

Untreated mice (or mice treated with control materials) were found at autopsy to have ascites (2–3 ml containing $10^8$ to $10^9$ tumor cells/ml) and extensive invasion of the retroperitoneal tissues by tumor. In contrast, ascites was rarely observed in interferon-treated animals, although solid tumor masses were present both intra- and extraperitoneally.

(*b*) *Effect of varying the time of initiation and duration of interferon treatment (i.p.):* In one group of mice in experiment 1 (Table 1) interferon treatment was initiated 24 hours after inoculation of tumor cells and daily for the ensuing six days. These mice were subsequently maintained without treatment for the remainder of the experiment. In a second group, interferon treatment was initiated one week after inoculation of tumor cells.

There was no significant difference in survival between these two groups of mice. Both groups survived longer (mean 27 and 26 days) than the control groups of mice (mean 18–19 days), but neither treatment schedule was as effective as an interferon treatment initiated 24 hours after inoculation of tumor cells and continued daily throughout the experiment (mean 42 days).

(*c*) *Effect of intraperitoneal interferon administration on the survival of Balb/c mice inoculated subcutaneously with $RC_{19}$ cells:* In two experiments, one-month-old male and female Balb/c mice were inoculated subcutaneously in the interscapsular region with approximately 100 $RC_{19}$ cells. In one of these experiments, daily i.p. inoculation of 20,000 units of mouse brain interferon delayed the appearance and growth of subcutaneous tumor nodules and increased survival (mean survival for control mice, 26 days; for interferon-treated mice, 36 days). In a second experiment, however, no difference could be demonstrated between untreated and interferon-treated mice.

(2) *Effect of mouse brain interferon preparations on the survival of C 57/$BL_6$ mice inoculated intraperitoneally with $EL_4$ cells:* One-month-old male and female C 57/$Bl_6$ mice were inoculated i.p. with 2,000–3,000 $EL_4$ tumor cells (equivalent of 400–600 $LD_{50}$). Untreated mice or mice treated with control preparations died 15–26 days (mean 19 days) after tumor cell inoculation.

Mouse brain interferon treatment (i.p.) initiated 24 hours after the inoculation of 2,000–3,000 $EL_4$ tumor cells and continued daily thereafter significantly increased the survival of C 57/$Bl_6$ mice (Table 3) (mean survival for control mice, 19–20 days; for interferon-treated mice, 25 or 27 days).

TABLE 3. *The effect of continued administration of mouse brain interferon on the survival of C 57/$BL_6$ mice inoculated with $EL_4$ tumor cells.*

| Expt. no. | Treatment | No. of mice surviving > 22 days/ total no. mice | Mean harmonic survival (days) | Confidence interval (0.95) | Significance* |
|---|---|---|---|---|---|
| 1. | Control: | | | | |
| | Untreated | 4/19 | 19 | 17–20 | NS |
| | Normal brain | 2/9 | 20 | 18–22 | |
| | Interferon: | | | | |
| | Brain | 17/18 | 27 | 25–28 | <0.0001 |
| 2. | Control: | | | | |
| | Untreated | 1/11 | 19 | 18–21 | NS |
| | Normal brain | 1/7 | 19 | 17–21 | |
| | Interferon: | | | | |
| | Brain | 10/12 | 25 | 23–28 | <0.0001 |

All treatments administered i.p. and initiated 24 hr after inoculation of tumor cells.
* Significance of the difference between interferon-treated and control groups of mice:

| Expt. | No. $EL_4$ cells inoculated | Units of mouse brain interferon inoculated 0.25 ml/mouse/day |
|---|---|---|
| 1 | 2,200 | 20,000 |
| 2 | 3,100 | 20,000 |

At autopsy, $EL_4$ tumors appeared more solid and invasive than $RC_{19}$ tumors in Balb/c mice and only minimal ascites was present. No significant difference was observed between control and interferon-treated mice.

*Discussion.*—The repeated administration of potent mouse interferon preparations increased the survival of Balb/c and C 57/$Bl_6$ mice inoculated intraperitoneally with 2,000–3,000 $RC_{19}$ and $EL_4$ tumor cells. Several findings suggest that interferon itself (or a factor intimately associated with interferon) was responsible for the effects observed: (1) Preparations of mouse interferon derived from brain, serum, and monolayer cultures of transformed mouse cells proved effective (interferon inducers—West Nile virus or Newcastle disease virus.) The corresponding control preparations were ineffective. (2) A *purified* preparation of mouse brain interferon proved as effective as crude brain interferon. (3) A potent preparation of human interferon (interferon inducer, Newcastle disease virus) proved ineffective, suggesting that the antitumor effect of mouse interferon preparations was species specific (as is the antiviral action of interferon).

Further support for this interpretation stems from experiments in which the daily inoculation of Newcastle disease virus and polyinosinic-polycytidilic acids (both potent inducers of endogenous interferon in mice[11, 12]) also increased the survival of tumor-inoculated Balb/c mice (unpublished observations). Levy, Law, and Rabson have reported an increased survival in mice inoculated with a variety of tumor cells and treated with poly I·poly C.[13] These investigators

suggested, however, that the antitumor effects of the synthetic polynucleotides may not have been related to the induction of interferon.

The explanation for the increased survival of interferon-treated mice inoculated with tumor cells is not apparent. Interferon preparations (or a hypothetical associated factor) may have acted on the mouse, enhancing mechanisms of tumor cell rejection, or directly on the tumor cells themselves. Several experimental observations support this latter hypothesis. Interferon treatment appeared most effective when intimate contact between cell suspensions (ascites) and interferon was maximal (i.e., $RC_{19}$ cells and interferon both inoculated intraperitoneally). The finding that solid tumor masses developed intra- and extraperitoneally in spite of interferon treatment (ascites was rarely observed in these mice) may have reflected the relatively diminished contact between individual cells and the interferon preparations.

As regards the possible direct effect of interferon preparations on cellular growth, Paucker, Cantell, and Henle[14] reported that exposure of L-cells to interferon resulted in a depression of cellular replication depending on the antiviral potency of the interferon preparations utilized and the duration of the incubation period. Paucker and Golgher recently observed similar effects with purified preparations of mouse interferon.[15] Dubbs and Scherer suggested that interferon might have been responsible for the inhibition of the growth of L-cells in cultures inoculated with Japanese encephalitis virus.[16] Baron and his co-workers could not consistently confirm these findings and attributed the occasional inhibition of L-cell growth to "noninterferon contaminants."[17] Furthermore, they found that purified chicken interferon did not inhibit the growth of chick embryo cells.[17] (An important difference may exist, however, between the response of primary and secondary cultures of "normal" chick fibroblasts to interferon preparations and that of a cell line, L-cells.)

The $RC_{19}$ cell line was derived initially from the spleen of a mouse inoculated with Rauscher virus. Type C virions are observed on electron microscopic examination, and infectious virus can be recovered from these cells. The $EL_4$ cell line was derived from a mouse exposed to a chemical carcinogen. Biologically active virus has not been recovered from $EL_4$ cells, and no known specific viral antigen has been detected.[18] Nevertheless, these cells contain numerous type A intracellular "viral particles," similar to those frequently associated with murine tumors.[19] It seems possible, therefore, that the antitumor effect of interferon in our experiments was mediated by its antiviral property, although it is difficult at present to explain the mechanism of action.

We acknowledge with pleasure the excellent technical assistance of Mlle. Sylvie Thomas, Mlle. Madeleine Breuilh, Messrs. Sylvain Gilly, and Jean-Michel Reymond. We are indebted to M. Philippe Lazar and Mme. Suzanne Guéguen of the Unité de Recherche Statistique de l'Inserm for analyzing our experimental results and aiding us in their presentation, and to Dr. David Lang for his help in the preparation of the article.

One of us (I. G.) would like to express his gratitude to Dr. Sidney Farber, Director of Children's Cancer Research Foundation, Boston, Mass., for his constant encouragement and support of these investigations.

* A preliminary report of some of this work has been published in the *Compt. Rend. Acad. Sci. (Paris)*, **268**, 994 (1969).

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