L-Dopa: Selective Toxicity for Melanoma Cells in vitro

Abstract. *In a study of the effect of L-dopa, an intermediate in the biosynthesis of the pigment melanin, on the growth of human and murine melanoma cells a highly selective inhibition of growth was observed for pigmented cell lines (S91A and human melanoma) as compared to the nonpigmented control cells (amelanotic melanoma S91B, mouse fibroblast L929, and Chinese hamster ovary). There was a correlation between toxicity and the extent of incorporation of radioactively labeled L-dopa by each line.*

Melanocytic cells possess a special biochemical mechanism for the conversion of L-3,4-dihydroxyphenylalanine (L-dopa) to the biopigment melanin (*1*). Since this reaction is mediated by the enzyme tyrosinase, which is restricted to normal and malignant melanocytes, a potential basis for a selective chemotherapeutic approach may exist.

We have previously reported the highly selective incorporation of [^{3}H]L-dopa by pigmented murine and human melanomas in vitro (*2*), and thus it was of interest to evaluate the effect of L-dopa itself on the growth of pigmented melanoma cells.

Evaluation of pharmacologic injury to cancer cells in vitro has been measured by a variety of techniques. Effects upon doubling time are, in general, dose-dependent and agree well with other methods (*3*). Figure 1 shows the dose-response curve for growth inhibition of melanoma cells 48 hours after treatment with L-dopa (*4*). A highly selective inhibition of growth of the melanotic S91A melanoma line (*4*) as compared to the nonpigmented control cells is observed. There was a 62 percent inhibition of growth of pigmented S91A and a 35 percent inhibition of human melanoma, while nonpigmented control cells including the amelanotic melanoma (S91B) were essentially unaffected. Longer exposure times resulted in increased toxicity but decreased selectivity.

The relation of the extent of incorporation of exogenous [^{3}H]L-dopa (specific activity, 21 c/mmole) to sensitivity to L-dopa, after a 60-minute incubation, is given in Table 1. Pigmented cells incorporate up to 20 times the label of control cells and display a corresponding increase in toxicity.

Melanocytic cells are unique in possessing the enzyme tyrosinase and a metabolic pathway for the conversion of L-dopa to melanin. Several agents (for example, hydroquinone) have demonstrated selective toxicity against melanocytes in vivo and are used clinically as depigmentary agents (*5*). These agents all have a quinol moiety as a common structural feature, as does the prototype compound L-dopa itself. Previous studies in which L-dopa was used for long exposure times have demonstrated a general cytotoxicity without selectivity for pigment cells (*6*). Presumably, with brief incubation periods the decomposition of L-dopa is minimized and selectivity is enhanced.

Although the mechanism of action of L-dopa is still undefined, toxicity appears to parallel the ability of the cell to incorporate [^{3}H]L-dopa, which in turn is dependent on the presence of tyrosinase activity. Melanin exists as a complex polymer of 5,6-dihydroxyindole, which can undergo reversible oxidation-reduction with formation of free radicals (*7*). Since L-dopa is an *o*-quinol and can form free radicals readily, it is possible that radicals are generated by interaction with melanin or tyrosinase (or both), which results in disruption of cellular metabolism (*8*). Alternatively, the high concentration of L-dopa made available serves to alter the biologic properties of the melanin formed, rendering it toxic (*11*).

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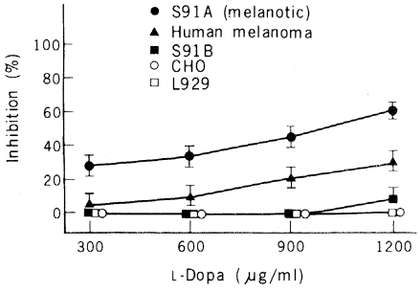

Fig. 1. Comparison of sensitivity of each cell line to L-dopa. All cell lines have been maintained in McCoy's 5A medium supplemented with 15 percent fetal calf serum, 100 units of streptomycin per milliliter, and 100 μg of penicillin per milliliter. Single cell suspensions were inoculated into 60-mm Falcon petri dishes and cells were allowed to attach for 24 hours prior to exposure. L-Dopa was freshly prepared in Hanks balanced salt solution (HBSS) at its limit of solubility and was sterilized by filtration. After washing, 1 ml of a solution containing 300, 600, 900, or 1200 μg of L-dopa per milliliter was added, and cultures were incubated at 37°C for 1 hour. Cells were harvested and counted in a model Z Coulter counter. Results are expressed as the percentage of growth inhibition according to the formula [(number of control cells − number of treated cells)/number of control cells] × 100 by comparison with parallel control cultures that were manipulated similarly except that they did not have L-dopa added; values represent mean ± standard error of the mean of four to six determinations.

Table 1. Comparison of growth inhibition by L-dopa and incorporation of [^{3}H]L-dopa. Experimental cultures were used 72 hours after plating; they were washed once with HBSS and then in 1 ml of HBSS containing unlabeled $10^{-5}M$ L-dopa and 2 μc of tritiated material. Cells were incubated at 37°C in CO_2-humidified air for 60 minutes, and the medium was removed. Cells were washed and precipitated with 10 percent trichloroacetic acid for 30 minutes. The precipitate was washed twice with normal saline, followed by addition of 1.0*N* KOH, and let stand at 4°C for 24 hours. A sample was added to scintillation fluid (Aquasol; New England Nuclear) and counted in a Beckman LS 335 scintillation counter. Quenching was corrected by addition of an internal toluene standard. Values for incorporation represent mean ± standard error of the mean for three samples and are expressed as disintegrations per minute per 10^5 cells after a 60-minute incubation. The cell lines are S91A, pigmented melanoma; human, human melanoma; S91B, amelanotic melanoma; L929, mouse fibroblast (*4*); and CHO, Chinese hamster ovary.

Cell type	Incorporation of [^{3}H]L-dopa	Percentage of inhibition (6.0 m*M* L-dopa)
S91A	1531 ± 151	62
Human	1021 ± 21	35
S91B	236 ± 21	5
L929	80 ± 15	0
CHO	92 ± 8	0

References and Notes

1. J. K. Luce *et al.*, in *Cancer Medicine*, J. F. Holland and E. Frei, III, Eds. (Lea & Febiger, Philadelphia, 1974), p. 1823.
2. M. M. Wick and E. Frei, III, *Cancer Res.*, in press.
3. G. E. Foley and H. Lazarus, *Biochem. Pharmacol.* **16**, 659 (1967); P. R. Roper and B. Drewinko, *Cancer Res.* **36**, 2182 (1976).
4. L-Dopa was a gift from Hoffmann–La Roche, Nutley, N.J., and [^{3}H]L-dopa was obtained from New England Nuclear, Boston, Mass. The cell lines used and their sources were S91A, a pigmented line, American Type Culture Collection (CCL 53.1), Rockville, Md.; S91B [described in (*9*)], a grossly amelanotic clone, gift from Dr. Jewel Cobb, Connecticut College, New London; mouse fibroblast L929 and Chinese hamster ovary, Grand Island Biological, Grand Island, N.Y.; and human melanoma [described in (*10*)], gift from Dr. John David, Harvard Medical School.
5. S. S. Bleehan, M. A. Pathak, Y. Hori, T. B. Fitzpatrick, *J. Invest. Dermatol.* **50**, 103 (1968).
6. D. Schachtschabel, R. Fisher, S. Zilliken, *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1402 (1970); J. Pawelek, G. Wong, E. M. Sanson, J. Morowitz, *Yale J. Biol. Med.* **46**, 430 (1973).
7. M. S. Blois, *J. Chem. Phys.* **23**, 1351 (1955).

8. B. Commoner, J. Townsend, G. Pake, *Nature (London)* **174**, 689 (1954).
9. J. P. Cobb and A. McGrath, *J. Natl. Cancer Inst.* **48**, 885 (1972).
10. N. Levy, *Natl Cancer Inst. Monogr. 37* (June 1973), p. 85.
11. We acknowledge Dr. Aaron B. Lerner (personal communication), who has independently demonstrated selective toxicity of L-dopa using a different technique.
12. M.W. thanks Dr. Thomas B. Fitzpatrick, who was responsible for initiating interest in the melanoma problem, for many helpful discussions. Supported in part by NIH grant CA 06516.

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Bacteriophages in Live Virus Vaccines: Lack of Evidence for Effects on the Genome of Rhesus Monkeys

Abstract. *Four juvenile rhesus monkeys were inoculated with 10^{12} plaque-forming units of the bacteriophage ϕV1 isolated from live virus vaccines. After ϕV1 had been cleared from the blood, DNA's were isolated from the livers and kidneys and analyzed for the presence of bacteriophage by plaque assays, and for the presence of ϕV1 DNA by DNA-DNA reassociation kinetics. No evidence was found for persistence of the bacteriophage or for replication of the phage genome in these rhesus monkeys.*

In 1972 Merrill reported that bovine serums contained bacteriophages as contaminants (*1*). This finding was confirmed by Chu *et al.* (*2*), and it was subsequently shown that live virus vaccines frequently contained bacteriophages, presumably due to contamination in the serums used to grow the cells in which the vaccines were produced (*3*). Further studies showed that the three bacteriophage plaque types isolated from 161 lots of vaccines were indistinguishable by physical and immunological assay (*4*). Our work was, therefore, limited to the original vaccine isolate designated ϕV1.

Previous studies with ϕX174 as a prototype bacteriophage had indicated that no biological or clinical effects were noted in mice, monkeys, or humans on exposure to high titers of the bacteriophage (*5*); however, more recent evidence has suggested that ϕX174 can replicate in monkey livers and human lymphocytes, and can become associated, perhaps transiently, with the host genome (*6*).

Because of the above findings with ϕX174, we attempted to determine whether ϕV1 injected into monkeys had any effect on the host genome or, indeed, if this bacteriophage were capable of replicating in a primate host. Our data show no evidence for replication or persistence of the ϕV1 genome by bacteriophage titration with the use of DNA's isolated from these monkeys or by nucleic acid hybridization studies. These results suggest that ϕV1 might play a useful role as a safe vector in recombinant DNA research.

Four 6- to 8-week-old rhesus monkeys were studied for presence of neutralizing antibody (*K* value) to ϕV1 as described for other bacteriophages (*7*). All were antibody-negative. One week later, a partial hepatectomy and unilateral nephrectomy were performed on each animal. These tissues served as preinoculation baseline materials. Biopsies of these organs were assayed and found to be negative for the presence of ϕV1 by means of the agar overlay technique with *Escherichia coli* C-3000 (*3*). DNA was extracted from the remaining tissue by a modification of the Marmur method (*8*). Three weeks later, after recovery from surgery, each monkey received 1×10^{12} plaque-forming units (PFU) of ϕV1 intravenously in a volume of 1 ml.

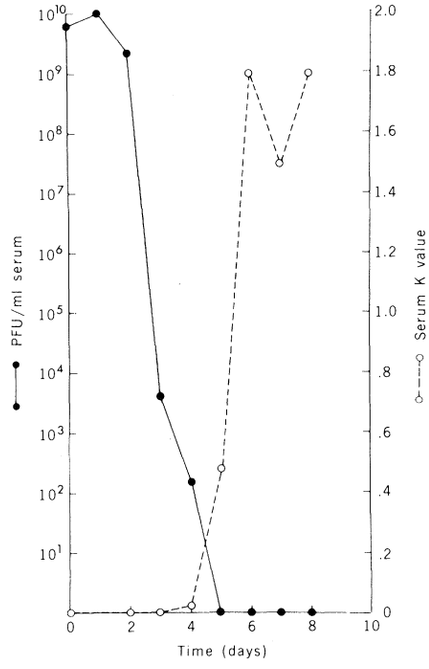

Fig. 1. Clearance of ϕV1 from the peripheral circulation (●) and antibody response (*K* value) to ϕV1 (- - ○ - -) after inoculation of 1×10^{12} PFU into a juvenile rhesus monkey. For inoculation, ϕV1 purified by CsCl banding, was grown in *E. coli* strain K12 F$^-$ (CR 63) at a multiplicity of 0.1 in a volume of 2 liters. The titer of the bacteriophage was ascertained as described (*11*).

Serum samples were obtained 15 minutes after inoculation and daily thereafter. A clearance pattern for ϕV1 typical of that seen for the monkeys in our study and the antibody response to this bacteriophage are shown in Fig. 1. The phage is cleared from the circulation by 5 days, and antibody begins to appear by 4 days after inoculation. Nine days after the phage inoculation the animals were killed. The remaining liver and kidney and the serum of each animal were assayed individually for the presence of ϕV1, and DNA was extracted from livers and kidneys.

The organ DNA's before and after inoculation were assayed by the plaque method on *E. coli* C-3000 for the presence of infectious ϕV1. No evidence of bacteriophage was found in any of the DNA's at any of the dilutions tested (undiluted to 10^{-6}). Thus, there was no intact, infectious ϕV1 present in the samples which might reanneal in the hybridization reaction mixture.

Quantitation of viral DNA copies by acceleration of reassociation had been described by Gelb *et al.* (*9*). In order to use this approach, we first examined the reassociation behavior of radioactively labeled ϕV1 DNA in the presence of an excess of unlabeled salmon sperm DNA. It reassociated with a $C_0t_{1/2}$ of 2.17×10^{-2} (C_0t is the concentration of nucleotide in moles per liter times the time in seconds) and followed typical second order kinetics [see Fig. 2 (open circles) and Fig. 3]. A reconstruction experiment to show the effect of adding copies of unlabeled ϕV1 DNA on the reassociation is illustrated in Fig. 2. The control reaction contained the equivalent of 7.64 copies per cell of ^{33}P-labeled ϕV1 DNA, calculated by the formula of Gelb *et al.* (*9*), with the use of salmon sperm DNA at absorbance A_{260} of 19 *A* units per milliliter and a ratio of 7.26×10^4 for the molecular weights of monkey DNA to ϕV1 DNA. Addition of known amounts of unlabeled ϕV1 DNA resulted in an acceleration equivalent to the number of copies of ϕV1 DNA added (Fig. 2). In the reannealing experiments reported below, ratios of unlabeled monkey cell DNA to ^{33}P-labeled ϕV1 DNA were such that we would see a 1.4-fold increase in $C_0t_{1/2}$ if there were one copy of ϕV1 DNA per cell.

We analyzed DNA from organs removed from monkeys before and after inoculation with ϕV1. Representative data are shown in Fig. 3. In no case for any of the four monkeys tested was there