J Neurosurg 82:481–486, 1995

Effectiveness of controlled release of a cyclophosphamide derivative with polymers against rat gliomas

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Most malignant gliomas grow despite treatment by standard chemotherapeutic agents. The authors explored the use of an innovative drug, 4-hydroperoxycyclophosphamide (4HC), delivered via a controlled-release biodegradable polymer to determine whether local delivery would enhance efficacy. This drug is an alkylator-type chemotherapeutic agent derived from cyclophosphamide. Unlike the parent drug, which requires activation by hepatic microsomes, 4HC is active *in vitro*. Two rat glioma cell lines, 9L and F98, were treated in cell culture with medium containing 4HC. Both cell lines were more sensitive to 4HC than to a nitrosourea, BCNU, an agent of established value in the local therapy of gliomas.

Ninety Fischer 344 rats implanted with 9L or F98 gliomas were treated with an intracranial polymer implant containing 0% to 50% loaded 4HC in the polymer, and it was found that 20% 4HC–loaded polymers caused minimum local brain toxicity and maximum survival. These polymers were then used to compare the *in vivo* efficacy of 4HC to BCNU in rats implanted with 9L glioma. Animals with brain tumors treated with 4HC had a median survival span of 77 days compared to the median survival of 21 days in BCNU-treated animals and median survival of 14 days in untreated animals. Long-term survival for more than 80 days was 40% in the 4HC-treated rats versus 30% in the BCNU-treated rats.

The polymer carrier used in this study was a copolyanhydride of dimer erucic acid and sebacic acid 1:1, which was able to maintain the hydrolytically unstable 4HC in a stable state for local delivery. Thus, it is concluded that 4HC-impregnated polymers provide an effective and safe local treatment for rat glioma.

KEY WORDS • chemotherapy • brain neoplasm • glioma • 4HC • drug delivery • biodegradable polymer

MALIGNANT gliomas have a high rate of local recurrence;[22] therefore, to treat these tumors, we developed a novel means to deliver chemotherapeutic agents via biodegradable sustained-release polymers.[5] This local drug-delivery system provides sustained high drug levels in brain tissue.[19,33,36]

We had previously evaluated 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), delivered via polymers, in the laboratory and clinic as a treatment for brain tumors. The agent BCNU, a low-molecular-weight, lipid-soluble nitrosourea, was chosen because of its demonstrated efficacy in animals[29,32] and its well-studied clinical usage.[5,26,34,35] Rats with intracranial 9L gliosarcoma treated with BCNU in polymer demonstrated a cure rate of up to 47% compared to a 0% cure rate in rats treated with systemic BCNU.[32]

A Phase I–II study in humans, using BCNU in polymer to treat recurrent malignant gliomas, showed that these patients had an average survival of 48 weeks following reoperation and placement of the polymer containing BCNU.[5] This finding compares favorably to studies of individuals following reoperation for recurrent malignant gliomas in which the median survival is 36 weeks.[3,21] Phase III placebo-controlled studies have demonstrated a similar prolongation in survival.

The promise of this approach is apparent in its ability to deliver agents that otherwise could not adequately cross the blood-brain barrier. Cyclophosphamide, a water-soluble chemotherapeutic agent, has been used widely outside the central nervous system (CNS), but its value for CNS tumors is limited because of the toxicity of the large doses required to achieve adequate CNS levels when the drug is

given systemically.[1,2,13,16,25] Cyclophosphamide itself is not active as an alkylating agent, but must be metabolized in the liver by the P450 microsomal enzyme system to 4-hydroxycyclophosphamide and phosphoramide mustard, which are the most active metabolites.[9,10]

A cyclophosphamide derivative, 4-hydroperoxycyclophosphamide (4HC), which is metabolically active, was used for local delivery of drugs in this study. This derivative is hydrolytically unstable in the available biodegradable polymer, poly-bis-[p-carboxyphenoxy]-propane anhydride (PCPP):sebacic acid (SA). A new polymer was developed to accommodate the water-soluble drug and protect it from degradation in the interstitial space of the brain. This fatty acid dimer (FAD) polymer derived from naturally occurring oleic acid and SA (poly[FAD:SA]) was able to maintain the 4HC in a stable state for delivery.[4,11]

Materials and Methods

Glioma Cell Culture

The 9L gliosarcoma and the F98 glioma cell lines were maintained in tissue culture in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), streptomycin, penicillin, and 1% L-glutamine.* The cells were grown to confluence in the flask, and then harvested with trypsin.

Preparation of Polymer Implants

The FAD polymer (poly(dimer erucic acid-co-SA) 1:1) was synthesized as described by Domb and Maniar.[12] Disk-shaped polymeric implants (2 mm in diameter and 1 mm thick) were prepared by melt-mixing the 4HC into the melted polymer at 65°C for 10 seconds and casting the uniform mixture into a 1-mm thick film. The film was cut into 2-mm disks by means of a bore of the appropriate size. *In vitro* drug release from these disks was described by Buahin, *et al.*[6]

The PCPP-SA polymer, formulated in a 20:80 ratio, was prepared as described, and BCNU was incorporated by melt mixing.[7] This polymer was then cut into 3 × 1-mm disks for implantation.

In Vitro *Screening*

The 9L and F98 cell lines were plated at a density of 400 cells per dish in 35-mm × 10-mm Falcon dishes in 5 × Improved MEM Zinc Option† with 20% FBS. Six dishes for each cell line were used as controls and three dishes were plated for each drug dilution. The cells were grown for 24 hours, after which the medium was removed and replaced with medium containing increasing concentrations of 4HC or BCNU, but no FBS. After 1 hour the drug-containing medium was removed, the dish was washed with RPMI 1640, and fresh medium without drug was added. The dishes were incubated for 7 to 9 days, and the cells were then stained and counted for colony growth. Only colonies with more than 50 cells were counted.

Dose Escalation Study

Ninety Fischer 344 adult male rates, each weighing 175 to 325 g, were anesthetized with 2.5 to 3 ml/kg of a stock solution containing 25 ml of ketamine hydrochloride (100 mg/ml), 2.5 ml xylazine (100 mg/ml), 14 ml of 100% ethanol, and 58 ml of 0.9% NaCl. The rats' heads were shaved and prepared, first with 70% ethanol, then with prepodyne. Under aseptic conditions and with the use of an operating microscope, a midline scalp incision was made and a 3- to 4-mm craniectomy was performed 3.5 mm to the left of the sagittal suture and 5 mm posterior to the coronal suture. The animals were then placed in a stereotactic frame and 10 μl of medium containing 100,000 cells of 9L gliosarcoma or F98 glioma were injected to a depth of 3.5 mm via a Hamilton syringe with a 26-gauge needle. The needle was slowly withdrawn to prevent extrusion of the tumor cells, and the scalp was closed with clips. The animals were returned to their cages and given free access to food and water.

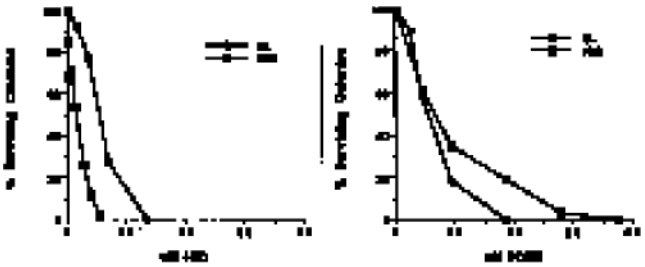

FIG. 1. Graphs comparing *in vitro* sensitivity of 9L and F98 glioma cell lines to 4-hydroperoxycyclophosphamide (4HC) *(left)* and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) *(right)*. See Materials and Methods for details.

Polymer disks (3 mm in diameter and 1 mm thick) weighing 9 to 11 mg were sterilized by placement under ultraviolet light for 60 minutes. The polymers contained 0%, 10%, 20%, 30%, 40%, and 50% 4HC in a weight/weight loading. Both the 9L- and the F98-injected rats were divided into six groups, one for each polymer loading. On postoperative Day 3 for 9L tumor–bearing rats and Day 5 for F98 tumor–bearing rats, the animals were anesthetized, their scalp incisions were reopened under aseptic conditions, and the craniectomy site was exposed. A microscalpel‡ was used to make cortical incisions 1 mm deep and 3 mm long. The polymers were placed in the tumor beds and the scalps were closed with clips. The animals were returned to their cages and given free access to food and water.

The rats were observed daily for evidence of neurological compromise, such as inability to right themselves or care for themselves, paraplegia, hemiplegia, or death. Animals with serious neurological compromise were sacrificed because they would not have survived more than a few hours. The brains were harvested at the time of death. Representative specimens from each loading-dose group were sectioned for hematoxylin and eosin staining. The brains were also examined for gross evidence of hydrocephalus, abscess, and encephalomalacia.

Comparison Study of 4HC and BCNU

To compare BCNU with 4HC, implanted tumor pieces rather than the stereotactic injection technique described above were used because BCNU had been previously studied with the tumor implantation technique.[32] The 9L gliosarcoma was maintained in a rat flank by subcutaneous injection of the tumor cells. The flank tumors were harvested when they grew to approximately 2 to 3 cm in diameter. This solid tumor was then cut into 1-mm^3 pieces.

Thirty Fischer 344 rats were anesthetized and underwent craniectomy as described above. A small portion of the left occipital lobe was aspirated and hemostasis was achieved with saline irrigation. A tumor cube was then placed in the defect, and the scalp was closed with rodent clips. On the 3rd postoperative day, the craniectomy was reopened under aseptic conditions and either empty polymer, polymer loaded with 3.8% BCNU, or polymer loaded with 20% 4HC was implanted at the tumor bed. The established tumor was not resected. The animals were then returned to their cages and given free access to food and water. They were observed daily for evi-

* Rat glioma cell lines 9L and F98 gliosarcoma, MEM, fetal bovine serum, streptomycin, penicillin, and L-glutamine supplied by GIBCO BRL, Gaithersburg, Maryland.

† Improved MEM Zinc Option supplied by GIBCO BRL, Gaithersburg, Maryland.

‡ Microscalpel obtained from Xomedtreace, Jacksonville, Florida.

TABLE 1

Histological toxicity of increasing doses of 4HC in polymer in rat brain tissue

4HC Load (%)	Abnormal Tissue* (mm)	Tumor Necrosis† (%)	Brain Necrosis‡ (mm)	Neutrophil Infiltrate§	Mean Survival (days)	
					9L Gliosarcoma	F98 Glioma
0	5–6	0–30	0	No	12	21
10	4–6	10–100	0.5–1	Yes	14	27
20	3–7	90–100	0.5–2	Yes	24	34
30	3–10	80–100	0	Yes	15	26
40	7–10	20–100	2–5	No	19	24
50	6–10	60–99	5–7	No	8	26

*This zone of abnormal tissue represents a combination of scar tissue and tumor and/or brain tissue that developed nonspecific histological changes due to the polymer therapy. This abnormal tissue was measured at its widest diameter.

†Tumor necrosis was measured as a percentage of identifiable tumor mass.

‡Brain necrosis was measured at its widest diameter.

§The significance of neutrophil infiltration has not been determined.

dence of neurological compromise and evaluated in the same way as described for 4HC treatment.

Results

In Vitro *Evaluation*

We began our investigation into the efficacy of 4HC by performing an *in vitro* evaluation of the survival of 9L gliosarcoma and F98 glioma cell lines in medium with either 4HC or BCNU. The 9L gliosarcoma and F98 glioma cell lines had no surviving colonies with medium concentrations of 27 μM and 10 μM, respectively, for 4HC while the same tumor cell lines required medium concentrations of 38 μM and 75 μM, respectively, for BCNU to prevent colony survival (Fig. 1 *left* and *right*). The *in vitro* screening of 4HC showed that it has potent activity at lower concentrations than BCNU. It was believed, therefore, that 4HC might be a more effective agent against these two tumor cell lines and that comparison of the efficacy of the two agents loaded in polymer would be of value.

Dose Escalation Study

First, an assessment was made of the effects of increasing doses of 4HC loaded in polymer on rats implanted with 9L gliosarcoma or F98 glioma. Rats implanted with either of the two tumors were treated with polymer loaded at 0%, 10%, 20%, 30%, 40%, and 50% with 4HC (Table 1). Rats given empty polymer died at a predictable and uniform rate from tumor progression: 9L gliosarcoma–bearing rats died between postimplant Days 9 and 13 and F98 glioma–bearing rats died between postimplant Days 18 and 23. The rats treated with polymer loaded with 10% 4HC showed increased survival rates over the control animals, but progression of the tumor eventually led to the death of the animal. Doses of 4HC of 30% to 50% in the polymer resulted in progressive decrease in length of survival as the dose was increased. The rats implanted with 9L gliosarcoma treated with 50% loaded polymer had a shorter survival span than did control rats, indicating that these animals died of direct drug toxicity rather than tumor progression, as discussed below. We found that loading the polymer with 20% 4HC provided the longest survival time for both tumor cell lines and the least amount of abnormal tissue and brain necrosis. The 4HC dose of 20% in the polymer was chosen as the optimum dose for further trials.

Examination for Toxicity

The brains of all animals were examined for both gross and microscopic evidence of toxicity. All animals treated with empty polymer showed large hemispheric tumors. The 10% and 20% 4HC-loaded polymer-treated animals had much less tumor, occasional hydrocephalus, no encephalomalacic cysts, and evidence of subarachnoid spread of tumor. The 30% to 50% 4HC-loaded polymer-treated animals had residual tumor, higher frequency of hydrocephalus than the 0% to 20% 4HC-loaded polymer-treated animals, subarachnoid spread of tumor, and encephalomalacic cysts increasing in size with increased loading.

Although stereotactic placement of tumor cells can be done rapidly, which enables the investigator to implant cells in large numbers of animals in a short time, it became apparent that there was subarachnoid spread of tumor from leakage of the tumor-cell suspension into the cerebrospinal fluid. Because of this problem, although stereotactic injection permitted screening for an optimum dose of 4HC, we then used implantation of tumor pieces to compare the efficacy of BCNU and 4HC. Implantation of tumor pieces had also been used for the preclinical evaluation of efficacy of BCNU in polymer.[32]

In some cases, on histological examination of the brain tissue, it was not possible to distinguish brain necrosis from tumor necrosis. Therefore a measurement of total necrosis or cyst formation was devised that was defined as a zone of abnormal tissue. This zone progressively increased, parallel with the weight-to-weight loading of the polymer with 4HC (Table 1). The percent of tumor necrosis in the region of the polymer was observed most consistently, and was maximum, in the 20% loaded group. In brains in which nonneoplastic tissue necrosis could be distinguished from tumor necrosis, brain necrosis increased with increased loading of the polymer (Fig. 2). By contrast, the 30% loaded group did not demonstrate unequivocal brain necrosis. A neutrophilic infiltrate was evident in the 10% to 30% loaded groups (Fig. 3). The

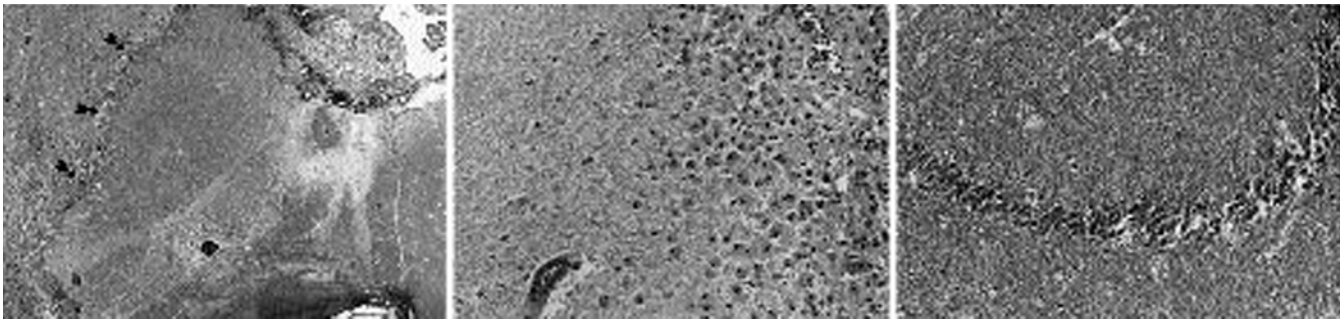

FIG. 2. Photomicrographs showing 9L gliosarcoma treated with 50% 4HC in fatty acid dimer polymer in a rat model. H & E. *Left:* Low magnification reveals extensive necrosis in the region of the implanted polymer. *Arrows* indicate interface between the brain and necrosis. Original magnification × 35. *Center:* High magnification shows area of necrosis with necrotic debris and numerous foamy macrophages. Whether this represents necrotic tumor or necrotic brain cannot be determined here. Original magnification × 235. *Right:* High magnification of another area demonstrates recognizably necrotic brain. Note the necrotic astrocytes and rim of neurons. Original magnification × 235.

20%-loading dose afforded minimum brain injury, maximum tumor necrosis, and longest survival; therefore it was selected as the optimum dose to be evaluated further and its efficacy compared with that of the BCNU polymer.

Representative tumor-bearing animals with the two highest polymer loadings, 40% and 50%, were examined for evidence of systemic toxicity. Tissue from the heart, lung, kidney, liver, spleen, intestine, colon, lymph node, and testicle was all normal, except that one animal had a minute focus of epicardial fibrosis.

Comparison of Efficacy of 4HC and BCNU

The most effective and least toxic dose of 4HC, 20% loading, was compared to the clinically used dose of BCNU, 3.8% loading,[5] to see if there was a significant improvement in survival with the 4HC therapy (Fig. 4). Control animals given empty polymers had a median survival of 14 days; all the animals had died by Day 19 after implantation. The 4HC polymer–treated animals had a significantly improved median survival (77 days; $p = 0.004$) as did the BCNU polymer–treated animals (21 days; $p = 0.007$) when compared to the control animals treated with empty polymer.

The increased survival of the 4HC-treated rats did not achieve statistical significance when compared to BCNU-treated rats. The 4HC polymer–treated animals not only had a significantly improved median survival rate compared with the BCNU polymer–treated animals, but also had a 40% long-term survival span greater than 80 days, compared with 30% in the BCNU polymer–treated group. These long-term survivors may well represent cures, as histological examination of animals in this group that survived more than 160 days showed no evidence of tumor (Fig. 3).

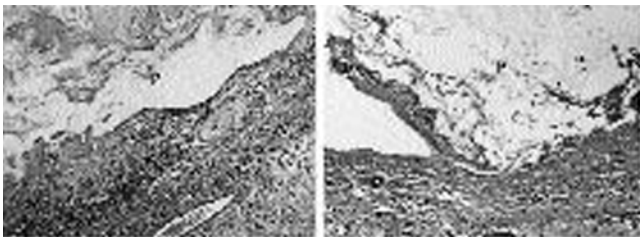

FIG. 3. Photomicrographs of 20% 4-hydroperoxycyclophosphamide fatty acid dimer polymer–treated 9L gliosarcoma showing well-delineated polymer implantation site (P). These rats were long-term survivors sacrificed at more than 200 days. There is no evidence of residual tumor. *Left:* Chronic inflammation and fibrosis surround the implantation site. *Right:* Minimal gliosis and mild lymphocytic infiltrate surround this implantation site. H & E, original magnification × 135.

Discussion

Efficacy of 4HC Therapy

Cyclophosphamide is an alkylating drug that has been used successfully to treat primary brain tumors in children, with beneficial response rates of up to 89% when used as a single agent.[1,2,13,16,17,20,28] Combination therapy consisting of cyclophosphamide and 1-methyl-1-nitrosourea in the treatment of metastatic brain tumors has a response rate of 48%.[23] Recurrent gliomas have been treated with cyclophosphamide as a single agent[1] and in combination with other agents,[25,27,28] with a median survival of 9 to 37 months.

The doses of cyclophosphamide required to treat glioma are quite high, in the range of 250 to 1000 mg/m^2 body surface area[1,25,28] and are associated with hematological, urological, and gastrointestinal toxicity; however, these high doses are required because of the inability of the active form of cyclophosphamide to enter the brain.[8] A metabolically active derivative of cyclophosphamide, 4HC, has been used clinically to purge tumor cells from bone marrow harvested for transplants.[30,31] In the laboratory, use of 4HC shows efficacy against both experimental carcinomatous meningitis[18] and a human medulloblastoma cell line.[14]

In Vitro *and* In Vivo *Efficacy*

The *in vitro* study accurately predicted that 4HC would demonstrate efficacy against 9L gliosarcoma and F98 glioma cell lines *in vivo*. We were thus able to establish a dose–response curve with 4HC-loaded polymer against

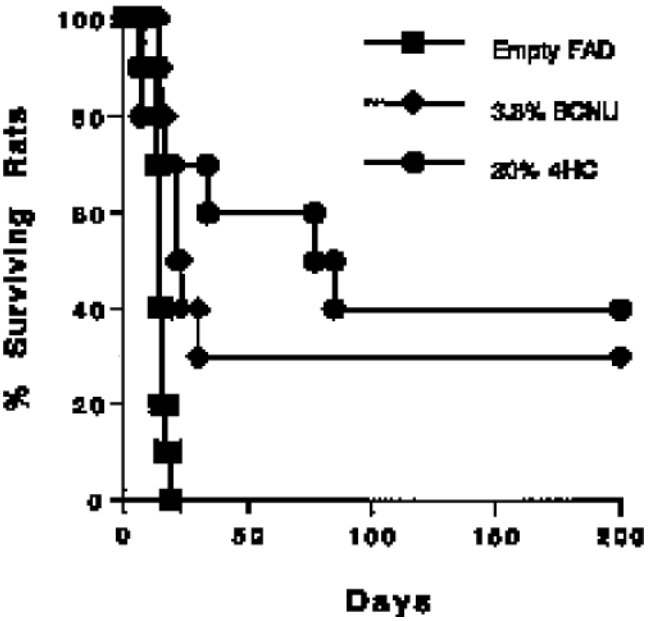

FIG. 4. Graph comparing median survival rates for rats implanted with 1 mm^3 cube of solid 9L glioma then treated with either 4-hydroperoxycyclophosphamide (4HC)– or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)–loaded polymers. FAD = Fatty acid dimer.

intracranially implanted tumors. The 20% 4HC–loaded polymer afforded minimum brain injury, maximum tumor necrosis, and longest survival and thus was selected as the optimum dose for further study. The polymers with higher loaded doses (30% to 50%) showed an increase in local toxicity to the brain. Indeed, 50% loading of 4HC was so toxic in the 9L gliosarcoma–implanted rats that survival of these animals was shorter than that of those implanted with tumor and empty polymer.

The 4HC-treated animals had a median survival of 77 days compared to the animals treated with empty polymer (14 days) or BCNU-loaded polymer (21 days). The 4HC animals had a 40% survival rate beyond 80 days, which represents an effective cure, as no tumor was seen in the animals sacrificed at 6 months. This cure rate is better than the BCNU-treated animals, which had a 30% survival beyond 30 days.

Polymer Technology

The FAD polymer delivered an unstable water-soluble agent to the brain in an effective manner. It had been previously established that this new polymer is biocompatible with brain tissue.[4] Polymer technology has been shown to deliver sustained high levels of BCNU and dexamethasone to the brain.[19,33,36] Reduction in the potentially life-threatening toxicities of chemotherapeutic agents is another benefit of local drug delivery via polymers, because there is minimum systemic absorption of the drug.[36]

Conclusions

The initial clinical experience with interstitial chemotherapy with BCNU polymer implants resulted in increased survival, from 36 weeks[3,21] to 48 weeks,[5] in reoperated recurrent gliomas. This gave an overall mean survival span of 94 weeks from the initial surgery in patients treated with intracranial BCNU in polymer; these individuals had no signs of systemic toxicity from the BCNU. Based on the results presented in this paper, 4HC polymer therapy should undergo similar clinical testing.

Although there was no statistical significance between the 4HC and the BCNU, the 4HC polymer also shows promise in the local treatment of brain glioma and will contribute to multiagent therapy in this tumor known for its heterogeneous cell population.

There is evidence that multiagent therapy is superior to BCNU single-agent therapy for malignant gliomas.[24] Malignant gliomas are noted for their cellular pleomorphism, so these cells may have independent responses to each chemotherapeutic agent, which accounts for the improved response to multiagent therapy. The ability to deliver multiple agents via polymer implants has been demonstrated. As new drugs with clinical efficacy are developed, they can be evaluated individually and in combination so that drug therapies may be tailored for each tumor type.

Acknowledgments

The authors wish to thank Michael Pinn, Betty Tyler, and Danit Ron for their technical assistance; Dr. Pamela Talalay for her review of the manuscript; and Drs. Steven Piantadosi and Eric Sipos for statistical evaluation.

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Manuscript received November 12, 1993.

Accepted in final form May 9, 1994.

This work was supported by the National Institutes of Health Grant NCDDG UO1 CA52857, the Dana Foundation, and the Johns Hopkins Clinician Scientist Program.

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