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17. Note that an epilimnetic *TN*:*TP* ratio of 29:1 should not be equated with such a value in loading. The retention and recycling of nitrogen within lakes differs from that of phosphorus, and an epilimnetic *TN*:*TP* ratio > 29:1 by weight may result from much lower loading ratios (*9*). Nitrogen-loading models such as that of R. W. Bachmann [in *Restoration of Lakes and Inland Waters* (EPA-440/5-81-010, Office of Water Regulations and Standards, Washington, D.C., 1981), pp. 320–324] can be coupled with phosphorus loading models (*1*) to predict epilimnetic *TN*:*TP* ratios.
18. I thank V. J. Bierman, Jr., W. T. Edmondson, G. G. Ganf, N. P. Holm, J. Kalff, D. W. Schindler, J. Shapiro, E. B. Swain, D. Tilman, and an anonymous reviewer for help and comments, and I thank J. DePinto for the use of unpublished data. Supported by NSF grant DEB-7921755 to J. Shapiro and a NATO postdoctoral fellowship to V.H.S. Contribution 190 from the Limnological Research Center, University of Minnesota.

4 June 1982; revised 28 February 1983

Naltrexone Modulates Tumor Response in Mice with Neuroblastoma

Abstract. *Naltrexone, an opiate antagonist, had both stimulatory and inhibitory effects, depending on the dosage, on the growth of S20Y neuroblastoma in A/Jax mice. Daily injections of 0.1 milligram of naltrexone per kilogram of body weight, which blocked morphine-induced analgesia for 4 to 6 hours per day, resulted in a 33 percent tumor incidence, a 98 percent delay in the time before tumor appearance, and a 36 percent increase in survival time. Neuroblastoma-inoculated mice receiving 10 milligrams of naltrexone per kilogram, which blocked morphine-induced analgesia for 24 hours per day, had a 100 percent tumor incidence, a 27 percent reduction in the time before tumor appearance, and a 19 percent decrease in survival time. Inoculation of neuroblastoma cells in control subjects resulted in 100 percent tumor incidence within 29 days. These results show that naltrexone can modulate tumor response and suggest a role for the endorphin-opiate receptor system in neuro-oncogenic events.*

In addition to their analgesic and behavioral effects, opioid compounds are known to alter cell function and growth, particularly in developing neural systems (*1*, *2*). Zagon and McLaughlin have reported that long-term administration of heroin to mice with transplanted neuroblastoma inhibits tumor growth and prolongs survival time (*3*). These antitumor effects were blocked by concomitant administration of naloxone, an opiate antagonist. Paradoxically, in subsequent studies in which only naloxone was used and at concentrations that also interact at the opiate receptor level (*4*), this agent was found to be extremely effective in preventing or retarding tumor appearance and improving the survival of neuroblastoma-inoculated mice. The mechanisms underlying heroin's and naloxone's actions in regard to neural neoplasia are unknown but may involve the endorphin-opiate receptor system (*3*, *4*).

In view of the antitumor properties of naloxone alone, we were prompted to examine the chemotherapeutic potential of naltrexone, a narcotic antagonist that is eight times as active and three times as long-acting as naloxone (*5*). We chose the C1300 murine neuroblastoma, a well-characterized tumor that resembles human neuroblastoma in many respects (*6*), to assess naltrexone's actions. The results show that naltrexone can promote tumorigenesis at a dosage that continuously prevents morphine-induced analgesia but exerts an antineoplastic effect at a dosage that only temporarily blocks antinociception by morphine.

Male syngeneic A/Jax mice were inoculated with S20Y neuroblastoma cells and, beginning 2 days later, received daily subcutaneous injections of either 0.1, 1, or 10 mg of naltrexone per kilogram of body weight or sterile water (control). On day 29 after inoculation with tumor cells, all mice in the 10 mg/kg and control groups had measurable tumors, whereas only 75 percent of the 1 mg/kg group and no animal in the 0.1 mg/kg group had developed measurable tumors. By day 75, when every other tumor-bearing mouse had died (Fig. 1), 10 of 12 mice (83 percent) and 4 of 12 mice (33 percent) receiving 1 and 0.1 mg of naltrexone per kilogram, respectively, had developed tumors. The percentage of mice developing tumors in the latter group differed significantly from that of the controls ($P < 0.01$, chi-square test). Observations on the remaining mice for the next 25 days (that is, until 100 days after tumor cell inoculation) revealed no tumor development.

The survival time (Fig. 1) of mice receiving naltrexone (1 mg/kg) was comparable to that of control animals (mean and median life-spans = 50 days). Mice receiving naltrexone (10 mg/kg) survived for a significantly ($P < 0.02$) shorter time than control mice (mean and median life-spans 19 and 22 percent shorter, respectively, than controls). Moreover, the latency prior to tumor appearance for this group was reduced 28 percent from control values (21.25 ± 1.23 days). For those mice injected with naltrexone (0.1 mg/kg) that developed tumors, an increase in mean and median survival times of 42 and 36 percent, respectively, were recorded relative to controls, as well as a 98 percent increase in latency time prior to tumor onset.

In general, the patterns of tumor growth for mice in the control and 0.1 mg/kg groups were similar throughout

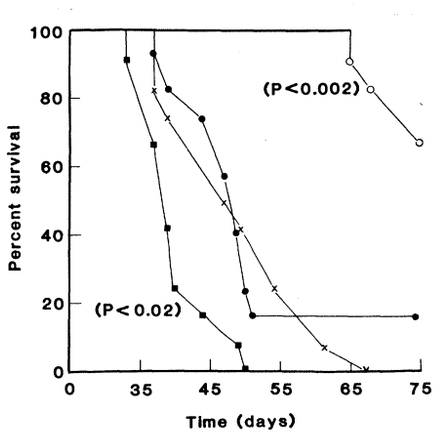


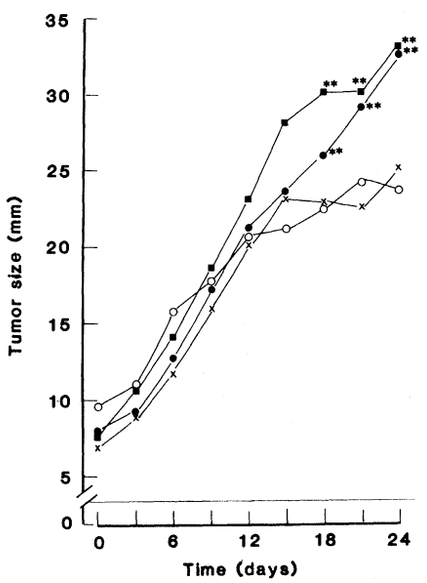
Fig. 1. Effect of daily subcutaneous injections of naltrexone (Endo Laboratories, Garden City, New York) on survival time (days after tumor cell inoculation) of mice inoculated with 10^6 S20Y neuroblastoma cells. The S20Y cells were cloned from the A/Jax mouse C1300 neuroblastoma and obtained from M. Nirenberg (National Institutes of Health, Bethesda, Maryland). Tumor cells were injected in the dorsal surface of the right shoulder. Survival curves for mice receiving naltrexone at dosages of 0.1 mg/kg (○), 1 mg/kg (●), or 10 mg/kg (■) or sterile water (×) were analyzed by the Mann-Whitney U test.

the 24-day observation period (Fig. 2). The mean tumor sizes of mice injected with naltrexone (1 and 10 mg/kg) were similar to the case for controls for the first 15 days after the initial tumor appearance. However, on days 18, 21, and 24, tumors were significantly larger in both these groups. The average tumor size on the day of death was similar in all groups, except for mice in the 10 mg/kg group, where tumor size (32.4 ± 1.8 mm) was significantly larger ($P < 0.01$) than that for controls (26.2 ± 0.8 mm).

These results demonstrate that naltrexone can markedly alter the course of neural neoplasia, with the direction and magnitude of effects dependent on dosage. Naltrexone's major effect is to modify the interval between tumor inoculation and tumor appearance. The mechanisms underlying naltrexone's actions are unknown. The naltrexone dosages (0.1 to 10 mg/kg) mediating these effects were 0.02 to 2.0 percent of the LD_{50} [dose lethal to 50 percent of the animals tested, 570 mg/kg (*5*)] and did not elicit overt toxicological symptoms such as ataxia, convulsions, or loss of body weight. As evidenced in opiate challenge experiments (Fig. 3), the dosages of naltrexone used did invoke an opiate receptor blockade that lasted 4 to 6 hours per day at a dosage of 0.1 mg/kg, 12 to 24 hours per day at a dosage of 1 mg/kg, and the entire 24-hour period at a dosage of 10 mg/kg. Long-term treatment with narcotic antagonists causes an increase in the number of opiate receptor binding sites and produces a supersensitivity to opiates (*7*). Long-term naltrexone treatment in mice also increases the plasma β-endorphin concentrations (*8*). In addition, S20Y neuroblastoma cells possess opiate receptors (*9*). The growth-inhibiting properties of opiates in cultured tissues are well known (*2*), and neuroblastoma cells grown in culture exhibit dose-dependent growth retardation in the presence of opiates (heroin) and endorphins (Met-enkephalin) that can be blocked by concomitant administration of naloxone (*10*). If we relate these observations to the results of our study, it could be postulated that naltrexone perturbs the endorphin-opiate receptor system in the host or the neuroblastoma cells or both. Continuous occupation of the opiate receptor (that is, a naltrexone dosage of 10 mg/kg) and therefore prevention of the interaction between these receptors and the endogenous opiates exacerbates tumor response. This would imply that the endorphins mediate early events in the establishment of tumors as well as tumor growth. Low naltrexone dosages (that is, 0.1 mg/kg) may produce more binding sites on the neuroblastoma cells or in the host, or both, so that a supersensitivity to basal (or even elevated) concentrations of endorphins could evoke an antitumor response in the interval (6 to 18 hours per day) when naltrexone is no longer present. This hypothesis may explain earlier results showing that exogenous opiates (heroin) have antineoplastic activity (*3*). Moreover, this hypothesis would support earlier suggestions (*4*) that naloxone works by provoking cellular supersensitivity or an overcompensating release of endorphins, or both, that may in turn act during the period (18 to 20 hours per day) when the drug is not present. Intermediate naltrexone dosages (1 mg/kg) seem to evoke characteristics that are borderline between conditions of continuous and intermittent opiate receptor blockade and that resulted in little overall effect on neoplastic response.

Although the endorphin-opiate receptor system does provide a framework for unifying many of the data to this point, narcotic antagonists may have biological actions unrelated to opiate receptor blockade (*11*). For example, naloxone can reduce resting serum prolactin and growth hormone concentrations (*12*) and decrease serum titers of interferon (*13*). Alterations in hormonal or immunological functions may, by themselves or in concert with the endorphin-opiate receptor system, be responsible for naltrexone's action.

Our results indicate that naltrexone can remarkably alter the course of murine neuroblastoma. Our evidence suggests that opiate receptors and endogenous opioid peptides play a role in neuro-oncogenesis. These findings may have important implications for the etiology and treatment of neural neoplasia in humans.



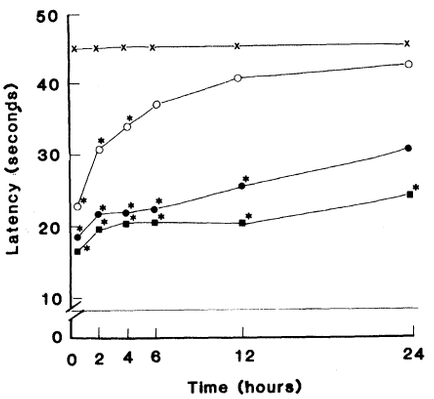


Fig. 2 (left). Effect of naltrexone treatment on tumor size of mice receiving naltrexone at dosages of 0.1 mg/kg (○), 1 mg/kg (●), or 10 mg/kg (■) or sterile water (×). Individual tumors were measured with vernier calipers (accuracy, ±0.05 mm); perpendicular dimensions (*a, b*) were recorded, and tumor size was computed as follows: $(a + b)^{1/2}$. We compared growth of tumors for each mouse beginning on the day when a measurable tumor was observed and every 3 days thereafter until day 24 after tumor appearance, using analysis of variance; subsequent comparisons were made with the Newman-Keuls procedure (differences from controls that are significant at $P < 0.01$ are indicated by **). Day 0 indicates the time when the mean tumor size measured 5 mm or larger and day 24 represents the time when at least 50 percent of all tumor-bearing mice were alive. Fig. 3 (right). The latency of response (for example, licking of paws) in mice tested on a hot plate (55°C; Analgesia Meter, Technilabs). Morphine (0.2 mg/kg) was injected 30 minutes before testing. Animals were tested only once, and they were removed from the hot plate after 45 seconds if no response was observed. Six mice in each of the groups receiving naltrexone at dosages of 0.1 mg/kg (○), 1 mg/kg (●), and 10 mg/kg (■) plus six control mice (×) were examined at 30 minutes and at 2, 4, 6, 12, and 24 hours after the daily injection of naltrexone. We analyzed latency times using analysis of variance; subsequent comparisons were made with the Newman-Keuls procedure (differences from controls that are significant at $P < 0.05$ are indicated by *).

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21 March 1983; revised 2 May 1983

Labeled Putrescine as a Probe in Brain Tumors

Abstract. *The polyamine metabolism of transplanted* N-*nitrosomethylurea–derived rat glioma was determined with radiolabeled putrescine used as a marker for malignancy. The uptake of putrescine in vivo was complete within 5 minutes and was specific for tumor tissue. The conversion of putrescine to spermine and other metabolites by the tumor was rapid, in contrast to the case for adjacent normal brain. These results suggest that putrescine labeled with carbon-11 may be used as a positron-emission tomographic tracer for the selective metabolic imaging of brain tumor and may be used in an appropriate model as a marker for tumor growth rate.*

With positron emission tomography (PET) it is now possible to determine the location of brain tumors and to distinguish between the metabolic activities of the neoplasm and the surrounding brain (*1*). Earlier strategies for the diagnosis of brain tumors have used substances that are both readily available and modeled for tomographic reconstruction, such as glucose, oxygen, amino acids, and their analogs (*2*). However, appropriate rate constants and equated steady-state conditions for the quantitation of metabolic and functional pathways remain to be determined. Were a compound to be found that is taken up and metabolized solely by the tumor, this would enhance the selectivity of the technique and facilitate the assessment of the response to treatment. Because adult brain parenchyma does not normally divide, we considered that putrescine, a polyamine and putative marker of cell division and growth, should provide a superior index of neoplastic activity. Numerous studies have confirmed the importance of polyamines in both normal and pathological states (*3*). The metabolism of these substances has been correlated with the potential for cell replication and appears to be coupled to the stages of the cell cycle that precede mitosis (*4*). In brain tumors the concentration of putrescine has been shown to correlate with the degree of malignancy (*5*), whereas in normal brain putrescine metabolism is minimal (*6*).

To test the feasibility of putrescine as a PET tracer for brain tumors, we conducted a series of experiments in which we used a transplanted rat gliosarcoma derived from the *N*-nitrosomethylurea–induced rat glioma (*7*, *8*). Using conventional autoradiographic and densitometric techniques, we found that the in vivo uptake of [^{14}C]putrescine was greater in the tumor than in the surrounding normal brain (Fig. 1). Uptake of the label was evident as early as 1 minute after injection of the putrescine into the femoral vein, and uptake was essentially complete within 5 minutes. The specific activity of the label within the tumor was 35 times greater than in normal brain. No radiodense material was observed in necrotic tissue, and the only brain structure that showed evidence of uptake within the time frame of these experiments was the choroid plexus.

Clearance of putrescine from the circulation was equally rapid (95 percent of the amount injected was cleared in less than 5 minutes). These data are consistent with published reports for both rat and human (*9*). This rapid loss of putrescine from circulation is the result of uptake by tissue and excretion in urine (Table 1). The radioactivity remaining in the tissue 2 hours after an intravenous injection of [^{3}H]putrescine accounts for 30 percent of the injected amount and is confined primarily to skeletal muscle, liver, and the blood compartment (Table 1). Furthermore, the excretion of putrescine accounted for 30 percent of the amount injected and was as rapid as its clearance from circulation. Essentially all the radioactivity recovered in urine at 2 hours occurred within the first 5 minutes after the initial injection (data not shown). The radioactivity that cannot be accounted for (approximately 40 percent) is probably lost through putrescine catabolism, since it has been reported that 43 percent of labeled putrescine injected intraperitoneally into rodents was metabolized and expired as CO_2 within 2 hours (*10*). We also observed the evolution of a tritiated compound in expired air within minutes after the injection (data not shown).

These findings demonstrate the efficacy of putrescine as a PET tracer for brain

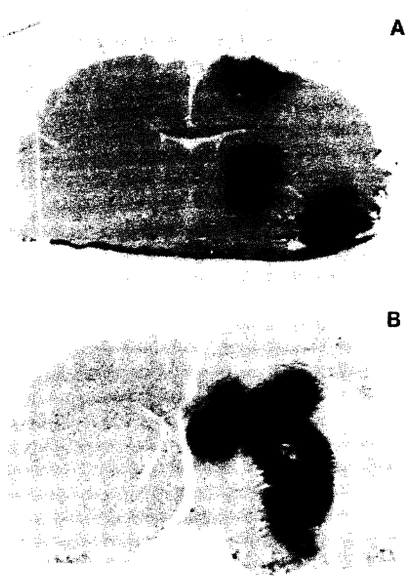

Fig. 1. Autoradiography of [^{14}C]putrescine in transplanted rat gliosarcoma. Representative overlays at (A) 1 minute and (B) 30 minutes of the hematoxylin and eosin–stained brain section on the corresponding autoradiogram. The overlay was necessary because the uptake of label by normal brain was too low for photography. Rats 6 to 8 weeks old (cesarean derived CD Fisher strain) were injected intracerebrally by stereotaxic technique with 10^6 cells as described (*8*). Twelve days after transplantation, they were anesthetized with sodium pentobarbital (25 mg/kg, intraperitoneal), the femoral vein was cannulated, and approximately 50 μCi of [^{14}C]putrescine (New England Nuclear) (107.1 mCi/mmole) was administered in 1 ml of 0.9 percent NaCl. Animals were killed (at 1, 5, 15, or 30 minutes after the administration of isotope) with 1 ml of saturated KCl, intravenous; the brain was removed and frozen in liquid Freon (−20°C). Cryostat sections were prepared for autoradiography as described (*21*), and the tissue radioactivity of the autoradiogram was determined densitometrically (*21*). The unfixed tissue sections used for autoradiography were stained by conventional hematoxylin and eosin. The specific activity of the isotope in tumor at 1 minute was 475 nCi/g (wet weight) versus 15 mCi/g in normal brain. At 5 minutes, the respective specific activities were 750 versus 20; at 15 minutes, 710 versus 26; and at 30 minutes, 800 versus 22.