Glutamate Ameliorates Experimental Vincristine Neuropathy

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ABSTRACT
The dose-limiting toxicity of the chemotherapeutic agent vincristine is peripheral neuropathy, for which there is no established therapy. The amino acid glutamate has been proposed as a neuroprotectant for vincristine, but a full preclinical evaluation of its efficacy, safety and mechanism of action has been hampered by a lack of suitable animal models. We report the development of a Dark Agouti rat model of sensorimotor peripheral neuropathy, to investigate the neurotoxicity of cytotoxic drugs. Neuropathy was manifested as gait disturbance in 100% of vincristine-treated animals (n = 12), significant elevation of the tail-flick threshold (5.1 ± 2 sec) and significantly impaired mean Rotarod times (55 ± 41 sec) developing after administration of 1.5 mg/kg vincristine over 2 weeks. Among vincristine-treated animals supplemented p.o. with sodium glutamate (500 mg/kg/day in drinking water) from 24 hr before vincristine treatment, only one (8%, P = .01) developed gait disturbance, the tail-flick threshold was not significantly different from controls and the mean Rotarod score was 188 ± 18 sec (P = .004). Glutamate thus significantly protected against both sensory and motor neuropathy. We observed no intrinsic neurotoxicity with glutamate and no interference with the cytotoxic efficacy of vincristine against a transplantable rat mammary adenocarcinoma grown s.c. in Dark Agouti rats. Our findings suggest that glutamate is likely to be a safe and effective neuroproprotectant for patients receiving vincristine, and it warrants further clinical evaluation. The mechanism of this selective neuroprotection by glutamate remains to be elucidated. Our rat model may be of use in determining whether glutamate offers protection from other neurotoxic drugs.

Vincristine is a cytotoxic drug with wide application in the treatment of malignant disease (Rowinsky and Donehower, 1991). Its efficacy relates primarily to disruption of microtubule function through binding to tubulin, causing mitotic arrest in dividing cells. The principal dose-limiting toxicity of vincristine is a sensorimotor peripheral neuropathy (Sandler et al., 1969) that is caused by microtubule dysfunction in peripheral and autonomic nerves (Schlaepfer, 1971), where the resultant interference with intracellular transport leads ultimately to axonal degeneration. The central nervous system is spared, probably because of poor drug penetration (Jackson et al., 1981). The peripheral neuropathy is variably reversible (Casey et al., 1973) and, if persistent, may lead to permanent impairment of quality of life in cancer survivors. There is no established therapy for the neuropathy other than drug withdrawal; therefore, to optimally utilize this effective drug there is a pressing need for an agent that can protect microtubules in nerves, without interfering with vincristine cytotoxicity in dividing cells.

The amino acid glutamate was proposed as a potential modifier of vincristine toxicity (Jackson et al., 1984) after the serendipitous observation that it could reduce in vitro cytotoxicity of vinblastine, a closely related drug with a shared binding site on tubulin (Johnson et al., 1960). Cutts (1964) noted a reduction in vinblastine-related mortality among glutamate-supplemented mice but also reported partial reversal of the in vivo antileukemic activity of vinblastine. Jackson et al. (1984) studied DBA/2 mice receiving 1.5 mg/kg vincristine on alternate days and reported improved survival rates in animals supplemented with 250 mg/kg/day glutamate from 24 hr before vincristine. Neurophysiological testing was not performed but a delay in onset of gait disturbance was noted, suggesting neuroprotection. In contrast to the earlier vinblastine experiments, glutamate did not appear to reverse the cytotoxicity of vincristine in leukemic mice. However, in these studies survival of the animals, rather than the direct measurement of tumor mass, was the only endpoint, which does not adequately distinguish between tumor-related and drug-related mortality.

Based on this limited preclinical evaluation, Jackson et al. (1988) went on to conduct a randomized double-blind clinical trial of p.o. glutamate (1500 mg daily) in prevention of neuropathy in 84 patients with breast cancer receiving adjuvant polychemotherapy including vincristine. Significant amelioration of neurotoxicity was demonstrated (21% vs. 43% of
patients affected, $P = .04$). The effect of glutamate on the cytotoxicity of vincristine could not be assessed in this study, because in the adjuvant setting patients do not have tumors that can be measured objectively and multiple agents were used.

In the decade since the work of Jackson and colleagues, concern over possible interference with vincristine cytotoxicity has hampered the widespread introduction of glutamate therapy. The mechanism of neuroprotection proposed by Jackson et al. (1984), i.e., direct enhancement of microtubule stability by glutamate, does not predict differences between the effects of glutamate on microtubules in nerves and malignant cells. Although purified microtubules are indeed more stable in the presence of high concentrations of glutamate, does not predict differences between the effects of glutamate on microtubules in nerves and malignant cells. Although purified microtubules are indeed more stable in the presence of high concentrations of glutamate (Hamel and Lin, 1981), this is now thought to be an electrochemical effect, unlikely to be relevant at physiological levels (Arakawa and Timasheff, 1984). Lack of an alternative hypothesis for the mechanism of neuroprotection by glutamate and difficulty establishing animal models in which to study both vincristine neuropathy and tumor response led to a reluctance by clinicians to introduce glutamate into clinical practice, despite its demonstrated efficacy, tolerability and low cost.

Recent advances, including the availability of improved methods of behavioral testing for neurotoxicity in rodents (Plummer et al., 1991; Apfel et al., 1991, 1992) and measurable tumor models (Coyle et al., 1990), have made it possible to establish more appropriate model systems. In addition, there has been an explosion of knowledge regarding the role of glutamate both as an excitatory neurotransmitter, leading to elevated intracellular calcium levels (Lipton and Rosenberg, 1994), and as an influence on neurite growth (Lipton and Kater, 1989), through its interactions with specific receptors (Pearce et al., 1987). The presence of glutamate receptors on peripheral nerves, mediating interactions with microtubules, might explain the efficacy of glutamate as a neuroprotectant. This hypothesis would predict no interference with vincristine cytotoxicity if malignant cells lack such receptors. Alternatively, recently elucidated differences in microtubule structure in nerves and dividing cells, such as tubulin isotype composition (Moskowitz et al., 1993), post-translational modifications including glutamylation (Alexander et al., 1991) and the presence of associated proteins (Pirotet et al., 1992; Esmaeli-Azad et al., 1994), might explain differential effects of glutamate on these tissues. The aims of this study were to establish a model of vincristine neuropathy in DA rats with neurophysiological tests and to determine the effects of glutamate on both neurotoxicity in this model and cytotoxicity against an implanted rat tumor responsive to vincristine.

**Methods**

**Animals**

DA rats were bred at the Gore Hill Research Laboratories, Royal North Shore Hospital (Sydney, Australia). Rats were housed six per cage from weaning and were maintained on a 12-hr light-dark cycle. All behavioral testing was performed at the same time of day, by the same investigators, who were blinded to the treatment group. Baseline measures were obtained after appropriate conditioning of the animals to the test procedures, with each animal thus serving as its own control. All i.p. injections, tumor implantations and tumor measurements were performed under inhalational anesthesia with nitrous oxide/oxygen/halothane. All methods used in these investigations were approved by the Animal Care and Ethics Committee of the Royal North Shore Hospital and met the requirements of the Australian National Health and Medical Research Council "Guidelines for the Use and Care of Laboratory Animals."

**Peripheral Neuropathy Model**

**Drug administration.** Two groups of six female DA rats (10 weeks of age, 150–200 g) were injected i.p. with vincristine (David Bull, Mulgrave, Australia) on days 1 through 5, days 8 through 12 and days 15 and 16. The vincristine was made up each day in normal saline solution at a concentration of 0.03 mg/ml, and a dose of 0.15 mg/kg was given, based on daily weights.

One of the groups received glutamate (BDH, Poole, UK) supplements in drinking water commencing on the day before chemotherapy, which was found to be the most effective schedule by Jackson et al. (1984). A concentration of 4 g/liter was prepared daily. Based on results of preliminary investigations, rats with this level of supplement drank a mean of 150 ml/kg b.wt. The average daily dose per rat approximated 500 mg/kg. All rats were given free access to the same diet throughout the treatment period. Two control groups of six rats received placebo injections of normal saline, proportionate to weight, and one of these groups also received glutamate supplements as described above, to rule out any intrinsic neurotoxicity of glutamate.

**Behavioral testing.** Sensory and motor neuropathy was assessed twice weekly in all groups and compared with base-line values for each animal. Gait disturbance was assessed independently by two observers, who were blinded to the assigned treatment. The first definitive change consisted of toe-walking with an arched back (Gottschalk et al., 1968), which was scored as a positive result. This progressed to a general paucity of motor activity and ultimately to severe hind limb weakness, which interfered with standing and grooming behavior.

Thermal thresholds were measured with a tail-flick test (Ugo Basile, Varese, Italy). After a period of acclimatization, the rat was held lightly restrained with its tail draped over a photocell, onto which a light was focused (100-W bulb). When the infrared source was activated, a timer automatically started and recorded the time taken for the rat to flick its tail out of the beam. A cut-off time of 11 sec was used to avoid tissue damage. A potentiometer knob was adjusted to obtain a rate of heating that produced base-line values between 2 and 4 sec. Even in the presence of motor weakness this test has been validated as a measure of peripheral nociceptive reflexes (Plummer et al., 1991).

Motor (Plummer et al., 1991) and proprioceptive (Apfel et al., 1992) testing was performed by using an accelerating Rotarod (Ugo Basile, Varese, Italy). A rotating drum, 7 cm in diameter and suitably roughened to allow the rats to grip it, was set in motion at 10 rpm. Rats were placed on the rod while it turned at a constant speed; after 10 sec, acceleration commenced and timers were activated. A maximum speed of 25 rpm was reached at 180 sec, and a cut-off time of 200 sec was used. When rats were no longer able to continue, they fell onto a trip switch, which recorded the time. Rats were acclimatized to the apparatus, and base-line values were tested in the light, to establish motor capability. After 5 min of rest, the test was repeated in the dark. Normal rats were as agile in the dark as in the light and, in addition, were less inclined to jump off in the dark, repeated in the dark. Normal rats were as agile in the dark as in the light and, in addition, were less inclined to jump off in the dark, leading to less variable scores. A reversal of this trend, with preserved light scores but reduced dark scores, has been used as a measure of proprioceptive abnormality (Apfel et al., 1992), equivalent to the Romberg test in clinical neurology.

**Statistics.** Each experiment was performed twice, with similar results on each occasion. For analysis of gait disturbance, group results were pooled and the incidence of abnormality on days 8 and 15 was compared by CBT ($n = 12$), with a supplementary log-rank analysis of time to onset of gait disturbance. Group mean tail-flick
times on day 15 were compared by a FLSD, and the incidence of an abnormal result (doubling of base-line values) was analyzed by CBET. Mean Rotarod times for each group were compared with controls by using a Wilcoxon rank sum test at day 15. No significant difference was observed between light and dark values for any group, and dark values are shown, because they were less subject to variability.

Tumor Growth Model

A spontaneously arising transplantable mammary adenocarcinoma of DA rats was obtained as a generous gift from Dr. Alan Rofe of the Institute of Medical and Veterinary Science (Adelaide, Australia) (Coyle et al., 1990). Implantation of $5 \times 10^6$ cells s.c. into the flank produced a palpable tumor nodule at 14 days. Intraperitoneal vincristine treatment was commenced at this time, and five daily injections of 0.2 mg/kg were administered (determined in preliminary experiments to be the maximum tolerated dose on this schedule). Tumor dimensions were measured daily with vernier calipers, and volume was calculated according to the formula $0.5 \times (\text{length} \times \text{width}^2)$ (Euhus et al., 1986). Growth was expressed as the tumor volume on each day divided by the base-line value on day 1 of chemotherapy. Group means were analyzed by one-way ANOVA.

Two groups of six rats received vincristine; one group also received glutamate p.o. at a dose of 500 mg/kg from the day before chemotherapy. An additional two groups received saline injections in a blinded fashion, and one of these control groups was supplemented with p.o. glutamate, to exclude any stimulatory effect of glutamate on DA mammary adenocarcinoma growth. The experiment was terminated when animals in any group developed limiting toxicity or tumor dimensions disturbing ambulation. Survival was not used as an endpoint, based on ethics committee requirements.

**Results**

Peripheral Neuropathy Model

Toxicity. All rats survived to day 17. Abdominal distension resulting from blood-stained ascites, presumably as a local irritant effect of the cytotoxic agent, was present in both vincristine-treated groups but not in saline-treated controls. Weight loss was observed in both the vincristine ($5.5 \pm 3.6\%$) and vincristine/glutamate ($7 \pm 3.1\%$) groups, compared with a small weight gain in both control groups (saline, $3.7 \pm 0.7\%$; glutamate, $5.6 \pm 3\%$).

Gait disturbance. Onset of gait disturbance (fig. 1B) was observed in vincristine-treated rats from day 8 (cumulative dose, $0.75$ mg/kg) and by day 15 affected $100\%$ of rats receiving vincristine alone (cumulative dose, $1.5$ mg/kg). On that day, only one glutamate-supplemented rat was affected ($8\%$, $P = .01$, CBET). By day 17 ($1.8$ mg/kg) all vincristine-treated rats were severely disabled, with mild disturbance being observed in $58\%$ of glutamate-supplemented animals (fig. 2). Log-rank analysis of time to gait disturbance revealed a significant delay in glutamate-supplemented animals, compared with vincristine alone ($P < .001$). No gait disturbance was detected in saline- or glutamate-treated controls.

Thermal thresholds. Base-line tail-flick times ranged between 2 and 4 sec (mean, $3.4 \pm 0.8$ sec). By day 15, five rats in the vincristine group ($42\%$) had more than doubled their base-line values, indicative of peripheral sensory impairment. No glutamate-supplemented rats experienced a significant change in threshold, and neither did saline- or glutamate-treated controls ($P = .039$, CBET). Group mean values for day 15 are shown in figure 3, with values for vincristine-treated animals being significantly elevated above controls

(RP = .007, FLSD). The glutamate-supplemented group was significantly lower ($P = .05$) and not different from controls ($P = .416$).

Rotarod performance. Control rats consistently improved from base-line values, presumably as a result of learning (mean, $170 \pm 30$ sec on day 15). Vincristine-treated rats deteriorated in performance from day 8, and at day 15 the mean time on the rod was significantly lower in both light and dark, indicative of motor neuropathy rather than proprioceptive abnormality (mean, $55 \pm 41$ sec; $P = .004$, Wilcoxon
Vincristine-treated rats supplemented with glutamate did not show impaired Rotarod performance in light or dark, with no detectable difference from controls (mean, 188 ± 18 sec) (fig. 4). Glutamate-supplemented controls were also unimpaired to day 15 (mean, 198 ± 6 sec).

**Discussion**

Validation of the neurotoxicity model. We have developed a model of vincristine-induced sensorimotor peripheral neuropathy in DA rats that more closely resembles the human condition than any previously described. Motor abnormalities, as measured by Rotarod performance, were pronounced, consistent with the findings of Apfel et al. (1993) in mice and Rebert et al. (1984) in Fischer 344 rats, where motor nerve conduction and other behavioral tests were used. Although a direct comparison is not possible, the Rotarod test appears to be at least as sensitive in detecting motor dysfunction as electrophysiological testing and is considerably less invasive. It is likely that the gait disturbance observed represents mixed sensory and motor abnormalities, consistent with the "high-stepping" neuropathic gait of humans with vincristine neuropathy (Sandler et al., 1969).

Induction of measurable sensory abnormalities has been a difficulty with animal models for vincristine neuropathy. Altered thermal thresholds have not been detected previously in vincristine-treated rats, perhaps because of differences in dosing schedules. Higher cumulative doses, as given here, might be expected to produce a more consistent abnormality, because effects of vincristine on DRG structure and function are known to be dose dependent in humans (Rowinsky and Donehower, 1991) and mice (Journey et al., 1969). A modified tail-flick test has been used in the study of paclitaxel-induced sensory neuropathy (similar in many respects to the clinical neuropathy from vincristine) by Apfel et al. (1991), who found it to be the most sensitive measure of DRG dysfunction in mice. Other methods of sensory testing, such as nerve conduction and measurement of DRG neuropeptides, have not been useful in vincristine-treated rodents (Rebert et al., 1984; Apfel et al., 1993) and hence were not used in this study. Histological studies of peripheral nerves and DRGs show abnormalities only at very high doses and are much less sensitive to early dynamic abnormalities (Uy et al., 1967; Journey et al., 1969). Our model is in keeping with the...
clinical neuropathy produced by vincristine, in which sensory symptoms and disturbance of function far outweigh signs and electrophysiological abnormalities (Sandler et al., 1969; Casey et al., 1973). The battery of tests used in this model could prove useful for the assessment of the neurotoxicity of other cytotoxic drugs.

**Efficacy of glutamate as a neuroprotectant.** Glutamate at a dose of approximately 500 mg/kg/day proved effective in ameliorating all manifestations of neuropathy in this model system. This confirms the work of Jackson et al. (1984) in vincristine-treated mice, where survival was significantly prolonged and a delay in the onset of toe-walking was seen, at a comparable dose (250 mg/kg/day i.p.). Oral glutamate was well tolerated in our study, with no effect on weight gain, no diarrhea, and no detectable intrinsic neurotoxicity.

These findings imply an action of glutamate on the multiplet cell types that are targets of vincristine, including DRG cells (Journey et al., 1969) and anterior horn cells (Wisniewski et al., 1968). Other agents, particularly growth factors, that have undergone preliminary testing as neuroprotectants for vincristine do not share this feature. Insulin-like growth factor shows some protection against motor abnormalities in rats (Apfel et al., 1993), and ORG 2766, an adrenocorticotropic hormone fragment, shows some activity against sensory changes (van Kooten et al., 1992). Nerve growth factor, which is active in preclinical models of cisplatin and paclitaxel neuropathy, has not been reported to be active against vincristine neuropathy, and preliminary in vitro results suggest that neurotrophin-3 does not protect the DRG from vincristine (Malgrange et al., 1994). Apart from the likely specificity of action of growth factors in only a few cell types, with a cocktail of factors being required clinically, another concern about their use in oncology as neuroprotec-
tants is their potential to stimulate tumor growth (Beech et al., 1995).

**Effect of glutamate on vincristine cytotoxicity.** Our models allow us to be confident that glutamate neither stimulates tumor growth nor interferes with vincristine cytotoxicity in the breast cancer model used. This is consistent with our previous in vitro data in human leukemic cell lines. By using a tetrazolium assay, vincristine cytotoxicity was assessed in the acute lymphoblastic leukemia cell line CCRF-CEM and the myeloid leukemic cell line K562 and was found to be unimpaired by glutamate supplementation up to 1 mM (Boyle et al., 1992). Additional testing of solid tumor cell lines is in progress to confirm that this is a general phenomenon.

There are at least two possible explanations for the apparently different effects of glutamate on microtubules in nerves and malignant cells. Firstly, microtubule structure and function are subtly different in these two settings. Neural microtubules have different tubulin isotype composition (Moskowitz et al., 1993) and are posttranslationally modified by the addition of glutamate residues (Alexander et al., 1991). They have different associated proteins, such as tau (Esmaili-Azad et al., 1994) and stable tubule-only peptide (Pirollet et al., 1992), which enhance stability. The effects of these differences on steady-state microtubule dynamics are presently being teased out, along with factors that regulate changes in the stability of microtubules in mature vs. newly forming neurites (Moskowitz et al., 1993). Alternatively, glutamate may be exerting an indirect effect on microtubules after interacting with a receptor found only on neural cells. Possible links in this pathway include local growth factor release or alteration of intracellular calmodulin, ATP or calcium concentrations (Lipton and Rosenberg, 1994), which have been shown to be regulators of microtubule formation and stability.

One of the main targets of vincristine is DRG cells (Journey et al., 1969). The ability of these cells to grow new nerve processes after explantation is dependent on an intact microtubule system and thus may be used as an index of toxic effects of antimicrotubule drugs (Malgrange et al., 1994). We are currently studying explants of rat DRG cells to determine the pathway of glutamate neuroprotection.

Such studies will pave the way for the introduction of glutamate into clinical usage in this disabling condition. It is possible that a general enhancement of repair in nerve cells is responsible, which may indicate glutamate efficacy in protection from other forms of toxic neuropathy.

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