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Reduction of vincristine toxicity by Cronassial

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Summary. A mixture of gangliosides (Cronassial) protects against the chronic but not the acute lethal toxicity of vincristine in mice and affords some protection against the acute lethal toxicity of vincristine in chicks. The protective effect of Cronassial appears to be greatest near the LD_{50} of vincristine and then diminishes as the dose of vincristine increases further. Cronassial does not, however, reduce the vincristine antitumour activity, so that the net effect is an improvement in the vincristine therapeutic index. Clinical trials are underway to examine the effect of the combination on the development of neurotoxicity in cancer patients.

Introduction

The vinca alkaloids vinblastine and more particularly vincristine are important therapeutic agents in the chemotherapy of cancer. Their importance can be gauged from the fact that one or other of them is an indispensable part of each of the standard chemotherapy regimens that are used as first-line treatment in at least four of the few malignancies that can be cured by chemotherapy.

On the other hand, the dose-limiting neurotoxicity of vincristine, although only rarely presenting acute clinical problems, can be severe at therapeutic doses and life-threatening at higher doses. At the same time it is unknown whether antitumour responses to vincristine at doses in excess of those normally employed would be greatly improved, since there is at present no substance or method (except dose limitation) which prevents or reverses vincristine neurotoxicity. Claims that folinic acid might prevent vincristine neurotoxicity have not been substantiated [5], and reports that glutamic acid appears promising [4] require confirmation. Thiamine, B12 [7] and pyridoxine [6] have not been effective in preventing vincristine neurotoxicity.

The problem of finding a substance that will prevent or reverse vincristine neurotoxicity in man is compounded by the absence of a suitable animal model for human vinca neurotoxicity. Only the cat and the chicken appear to be similarly affected, but the mouse has been used, taking prevention of lethal vincristine effects as an indication of a protective effect. Whether prevention of lethal effect is also indicative of prevention of neurotoxic effect is not clear.

Peripheral nerve repair through facilitation of nerve sprouting can be enhanced by means of gangliosides extracted from bovine brain [8]. The nerve sprouting promoting activity has been observed both in an in vitro neuronal tissue culture model and in some in vivo situations such as crush denervation of the sciatic nerve and in diabetics, as well as toxic neuropathies. As a result the effects of a highly defined mixture of four gangliosides (Cronassial) was examined in two exploratory clinical trials to see if it affected vincristine-induced neuropathies [1, 2]. Both investigations found that patients receiving Cronassial for periods of between 20 days and 6 weeks in addition to their vincristine had a significant reduction of vincristine-induced neuropathies, or that such neuropathies were largely prevented.

Although both these studies indicated that administration of Cronassial with vincristine conferred substantial benefit in terms of reduction of vincristine toxicity, a more detailed study is desirable since much of the evaluation was subjective and therefore difficult to assess. Furthermore, it was not at all clear from the two studies whether or not Cronassial was affecting the antitumour activity of vincristine. A pilot clinical study to examine these questions was therefore set up and will be the subject of another report. In the present paper we described the preclinical studies undertaken to see if any experimental basis existed for the assumption that Cronassial could protect against vincristine neuropathy.

Materials and methods

Animals

Acute and chronic toxicity. Male Swiss Schneider mice varying between 25 and 30 g in weight were used for these experiments. Mice were usually kept in groups of not more than eight in standard cages. Food and water were supplied ad libitum. The animals were kept in experimental rooms under closely controlled conditions. They were weighed daily. For study of acute vincristine toxicity in chicks, 48-h-old SPF chicks were used (Wickham Laboratories Ltd.).

Antitumour effects. The antitumour effect of combining Cronassial with vincristine was assessed against three dif-

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ferent malignancies, each transplanted into a different strain of mouse. Male Swiss Schneider mice of 27 g weight were used for sarcoma Sl80 experiments. Female C57Bl mice of 20 g weight were used for Bl6 melanoma experiments. Male BDF_1 mice of 30 g weight were used for L1210 leukaemia experiments.

Tumours

Sarcoma Sl80. This tumour has been transplanted in the same strain of mouse in this laboratory for more than 20 years. Transplantation was done by s.c. inoculation of 0.1 ml of a tumour mash made by finely mincing viable tumour tissue, passed repeatedly through a 26-gauge needle into a sterile Petri dish. Quantities of 0.1 ml penicillin (20000 U/ml) and streptomycin (20000 U/ml) mixture were added to the mash. Also added was Neomycin (5 mg).

Melanoma Bl6. This tumour was prepared for inoculation in a manner identical to that used for the Sl80 sarcoma.

Ll2l0. Spleens were removed from animals 7 days after inoculation of Ll210 cells and these spleens were finely minced with 1:100 isotonic saline. A quantity of 0.1 ml of this spleen and leukaemia L1210 cell suspension was then injected subcutaneously into flank of each BDF_1 mouse.

Drugs

Vincristine. A standard vial of Oncovin (Eli Lilly & Co.) containing 1 mg vincristine was used. It was made up with the appropriate volume of diluting fluid to give the final concentration required to inject 0.1 ml/10 g body weight.

Cronassial. The active substance of Cronassial is a mixture of gangliosides extracted and purified from bovine brain. The molecular structure is characterised by the presence of a hydrophilic portion consisting of an oligosaccharide chain (glucose, galactose, *N*-acetyl-galactosamine, galactose in sequence) that binds a different number of sialic acids on galactose, and by a hydrophobic portion represented by an amino alcohol, sphingosine, which in turn binds a fatty acid residue (usually stearic acid). The two portions are linked by a glycosidic bond between the glucose and the sphingosine, while the sialic acid is linked to galactose moieties. The ganglioside mixture consists mainly of four fractions, named according to Svennerholm's nomenclature [9], with an average content of monosialotetrahexosylganglioside (GM_1) 21%: disialotetrahexosylganglioside (GD_{16} 40% and GD_{16} 16%); trisialotetrahexosylanglioside (GT_{16}) 19%.

A solution of this substance was prepared by addition of a volume of sterile distilled water sufficient to give a concentration of 200 mg/kg in a volume of 0.2 ml. This dose was given to mice of approximately 20 g weight.

Results

Acute toxicity

Mice. For acute toxicity protection experiments in mice we decided to employ the LD_{75} of vincristine. Previous tests employing a variety of vincristine doses showed that one dose of 3.0 mg/kg given i.v. was fatal in 75% of male Swiss Schneider mice by day 30. We therefore decided to employ this dose to test ths acute toxicity protection by Cronassial. The dose of Cronassial employed was 200 mg/kg because this dose by itself had shown no toxicity of any kind and we felt it to be the maximum that we could reasonably employ. The Cronassial (200 mg/kg) was given 6 h before vincristine and resulted in 87.6% deaths by day 30. It was apparent therefore that Cronassial under these conditions gave no protection against the acute toxicity of the LD_{75} of vincristine.

Chicks. Acute toxicity experiments using 48-h-old chicks (Table 3) gave no convincing evidence of acute neurotoxic signs even with high doses of i.p. vincristine (6 mg/kg). During the first 4 h after administration of vincristine only one chick of four became ataxic. Another one had preterminal convulsions at 24 h. All the chicks were dead after 24 h. In contrast, of those five chicks that had 200 mg/kg Cronassial i.p. at the same time as the vincristine, only two had died at 24 h, but only one remained alive at 28 h.

With 4 mg/kg vincristine, four of five chicks survived for 24 h, but only one of five survived for 28 h. Of the five chicks that received 200 mg/kg Cronassial in addition, three survived to 28 h.

With 2 mg/kg vincristine there was little toxicity, and this was not changed by giving Cronassial as well.

It seemed, therefore – though large numbers would be required for statistical verification – that acute lethal vincristine toxicity could be reduced by Cronassial in large doses. Since, however, even the largest doses of vincristine had produced no uniform signs of neurotoxicity, it was not possible to judge whether Cronassial affected this toxicity in any way.

Chronic toxicity

For these experiments we wanted to use the LD_{50} of vincristine. This had been determined in previous experiments to be 0.8 mg/kg when given i.p. on 5 successive days to male Swiss Schneider mice. By giving 200 mg/kg Cronassial i.p. at the same time as the vincristine, the number of survivors at 30 days was increased from 62.5% to 100%. Almost identical results were obtained by using 1 mg/kg vincristine daily for 4 days (Table 1). The cumulative dosage of vincristine administered in these two experiments was the same. However, when the cumulative dose of vincristine was increased from 4 to 5 mg/kg. Cronassial had no protective effect, with mortality of the unprotected group being 100% as compared with 80% in the combined vincristine + Cronassial group. With this overwhelming toxicity, then, there was little or no protection offered by the Cronassial.

Antitumour activity of vincristine with Cronassial

Vincristine with Cronassial was tested against the solid tumours S180 and B16 and the leukaemia L1210. Results showed that Cronassial did not interfere with the antitumour activity of vincristine against S180 and B16 tumour growth (Table 2).

Moreover, the results against leukaemia L1210 show that, as is usual and well known, vincristine has no effect on this leukaemia, but when given with Cronassial there is a 30% increase in median survival time, 10.3 vs 7–8 days. This difference is statistically significant.

Table 1. Effect of Cronassial on the acute and chronic toxicity of vincristine in mice

Toxicity	Host strain	Drug	Dose (mg/kg)	Days	Route	Timing	Survival at 30 days	
							n	%
Chronic	SN	VCR	0.8	1–5	i.p.		5/8	62.5
Chronic	SN	VCR CRON	0.8 200	1–5 1–5	i.p. i.p.	6 h before VCR	7/7	100
Chronic	SN	VCR	1.0	1–4	i.p.		3/8	37.5
Chronic	SN	VCR CRON	1.0 200	1–4 1–4	i.p. i.p.	6 h before VCR	6/8	75
Chronic	SN	VCR	1.0	1–5	i.p.		0/6	0
Chronic	SN	VCR CRON	1.0 200	1–5 1–5	i.p. i.p.	6 h before VCR	1/5	20
Acute	SN	VCR	3.0	1	i.v.		2/8	25
Acute	SN	VCR CRON	3.0 200	1 1	i.v. i.p.	6 h before VCR	1/8	12.5

SN, Swiss Schneider mice; VCR, vincristine; CRON, Cronassial

Table 2. Effect of Cronassial on antitumour effect of vincristine

Tumour	Host strain	Drug	Dose (mg/kg)	Days	Route	Timing	Mean tumour weight (g)
S180	SN	VCR	0.5	1–6	i.p.		0.64
S180	SN	VCR CRON	0.5 200	1–6 1–6	i.p. i.p.	6 h before VCR	0.69
S180	SN	CMC, controls					1.05
B16	C57B1	VCR	0.5	1–4, 7–11	i.p.		1.46
B16	C57B1	VCR CRON	0.5 200	1–4 7–11	i.p.	6 h before VCR	1.66
B16	C57B1	CMC, controls					2.33
							Median survival time (days)
L1210	BDF_1	VCR	1.0	1–3	i.p.		7.8
L1210	BDF_1	VCR CRON	1.0 200	1–3 1–3	i.p.	Same time as VCR	10.3
L1210	BDF_1	CMC, controls					8.0

SN, Swiss Schneider mice; VCR, vincristine; CRON, Cronassial; CMC = Carboxymethylcellulose

Table 3. Effect of Cronassial on vincristine toxicity in the chick

Toxicity	Host	Drug	Dose (mg/kg)	Days	Route	Timing	Survivors at		
							24 h	28 h	52 h
Acute	SPF 48 h chick	VCR	6.0	1	i.p.		0/4	–	–
Acute	SPF 48 h chick	VCR CRON	6.0 200	1	i.p.	Same time	3/5	1/5	0/5
Acute	SPF 48 h chick	VCR	4.0	1	i.p.		4/5	1/5	1/5
Acute	SPF 48 h chick	VCR CRON	4.0 200	1	i.p.	Same time	5/5	3/5	1/5
Acute	SPF 48 h chick	VCR	2.0	1	i.p.		2/2	2/2	1/2
Acute	SPF 48 h chick	VCR CRON	2.0 200	1	i.p.	Same time	2/2	1/2	0/2

VCR, vincristine; CRON, Cronassial

Analysis of mechanism of vincristine toxicity reduction by Cronassial

The three major systems affected by vincristine are the haematological, the gastrointestinal and, in some species, the neurological.

Neurotoxicity. It has not been found possible to reproduce experimentally in mice the vincristine neurotoxicity seen in the clinic. The present experiments therefore focussed on the lethal toxicity of vincristine, even though this is usually though to be due to severe myelosuppression. Vincristine neurotoxicity is usually slow to develop and follows a chronic pattern. It is moderately encouraging, therefore, that the protective action by Cronassial against vincristine lethal toxicity was on the slowly evolving chronic toxicity, but whether the process involved in the development of chronic neurotoxicity and that involved in the development of lethal toxicity are in any way related can only be speculative. A histopathological study was therefore undertaken to see if the lethal toxicity could be related to any specific organ toxicity.

Histopathology. Three groups of C57B1 mice of 10 animals were each either treated with vincristine 1 mg/kg daily for 4 days or with vincristine 1 mg/kg plus Cronassial 200 mg/kg for the same 4 days; the third group was left untreated. All injections were given i.p.

Two mice from each group were killed at days 7, 10, 14, 21 and 28. The heart, thymus, liver, spleen, kidney, duodenum, ileum, jejunum and colon were removed at autopsy and placed into formol saline. Samples from these tissues were blocked in wax, cut at 5 μm thickness and stained with hematoxylin and eosin.

Examination of the sections at various times from day 7 to day 28 was essentially unremarkable and indistinguishable from normal except for the appearance of the jejunum. In the jejunum of the vincristine-treated as well as the vincristine + Cronassial-treated animals, there was very marked mitotic arrest with associated epithelial hypertrophy of crypts on day 7 (in one animal there was superfical enterocyte degeneration). These changes were still present on day 10, very minimal on day 14 and 21 and had reverted to normal by day 28. In association with the maturation arrest there was also Paneth cell depletion or diminution.

In addition the thymus of vincristine-treated and of vincristine + Cronassial-treated animals showed marked involutionary changes at day 7 which persisted until day 14 and then appeared to revert to normal. Seven of ten spleens of both vincristine-treated and vincristine + Cronassial-treated groups showed lymphoid depletion by day 7, and three of seven spleens showed lymphoid depletion by day 10.

No other histological abnormalities were identified in any of the remaining tissues.

It appears, therefore, that histological examination as carried out in the present experiments did not contribute to the identification of the acute or subacute lethal effects of vincristine treatment and of the protection against the subacute lethal effect of vincristine exerted by Cronassial.

Haematology. Three groups of 10 Swiss Schneider mice were set up. One group received 5 mg/kg vincristine, another 5 mg/kg vincristine plus 200 mg/kg Cronassial, and the third remained as controls. Injections were given i.v. on one day only. Thereafter 0.15–0.2 ml blood was taken from two mice per group by ventricular puncture on days 5 and 8 after administration of the drugs. The blood taken was placed separately into paediatric haematology tubes containing potassium EDTA. Full blood counts were done on a Coulter counter.

The dose proved to be lethal after day 8. Therefore blood counts are only available for days 5 and 8. The red cells and platelets were normal throughout, but the white cell count dropped from a mean of $7.8 \times 10^3/mm^3$ in the controls on day 5 to a mean of $4.6 \times 10^3/mm^3$ in the vincristine-treated animals and to a mean of $1.6 \times 10^3/mm^3$ in the animals treated with vincristine and Cronassial. By day 8, however, the vincristine-treated animals had a mean white cell count of $5.3 \times 10^3/mm^3$, compared with a mean of $4.4 \times 10^3/mm^3$ in the animals treated with vincristine and Cronassial.

It seems evident, therefore, that Cronassial does not protect against the acute drop in white cell count in the vincristine-treated animals.

Discussion

Attempts to reverse or prevent vincristine neurotoxicity have in the past been singularly unsuccessful, but the apparent benefit of a mixture of gangliosides (Cronassial) on the development of diabetic and other neuropathies [3] has led to an examination of the possibility of using the same mixture of gangliosides to prevent vincristine neuropathy [2, 3]. Although there was in those preliminary clinical studies a suggestion of some benefit, the results of a full assessment have not yet been published.

Because similar attempts to prevent or reverse vincristine neurotoxicity in laboratory animals present insuperable problems, we studied the lethal toxicity of vincristine in mice to see if this could be altered in any way by Cronassial administration.

In the event it became clear that although the chronic toxicity was beneficially influenced, the same was not true of the acute toxicity. It is unknown, but doubtful, whether vincristine lethal toxicity in mice can be correlated with neurotoxicity in man. However, since the chicken displays neurotoxic symptoms as a result of vincristine administration it seemed appropriate to use 24 (or 48)-h-old chicks, and in these, although no neurological abnormalities were observed, there were signs of protection against the acute lethal effects of vincristine by Cronassial. It may be, however, that the vincristine doses used were too high to enable Cronassial to effect any reversal.

Attempts to discover the cause of death by haematological and histopathological examination following lethal doses of vincristine have not been successful, and it is not possible, therefore, as yet to define the system, protection of which by Cronassial may have been responsible for reduction of the vincristine lethality.

At the doses of Cronassial used for vincristine toxicity protection, there was certainly no evidence of any interference with what small antitumour activity vincristine had on the three tumour systems in which the combination was compared with vincristine alone. On the contrary, definite augmentation of activity was observed against the L1210 leukaemia.

Clinical trials now in progress to assess whether Cronassial can prevent the development of vincristine neurotoxicity may give some guidance as to whether the approach employed in the present experiments is worth further development.

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