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Levamisole plus 5-Fluorouracil Inhibits the Growth of Human Colorectal Xenografts in Nude Mice

Robert Van Ginckel, Wim Distelmans, Marc De Brabander, Myriam Callens, Boudewijn Janssens, Els Jagers, Luc Wouters, Roland De Coster and Paul A.J. Janssen

Fragments of human colorectal adenocarcinomas were inserted under the renal capsule of nude mice. The growth of these tumour grafts was significantly inhibited by the combination of 5-fluorouracil (5-FU) and levamisole. An alternating regimen of levamisole 2.5 mg/kg and 5-FU 20 mg/kg decreased the size of tumour implants by 33–59% and/or increased the number of macroscopically disappeared fragments in the combined group compared with ineffective monotherapy with saline, levamisole or 5-FU. This model could be valuable for investigating the mechanism of action of levamisole and to evaluate the effects of this adjuvant therapy in other oncological settings.
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INTRODUCTION

THE IMMUNOMODULATING properties of levamisole have been extensively investigated in neoplastic disease [1–4]. In several studies, the compound was shown to be effective on slowly growing tumours particularly in combination with cytoreductive therapies (surgery, radiotherapy or chemotherapy) and to exert antimetastatic activities. The critical dose-dependency and timing of levamisole administration frequently hampered the beneficial outcome [5–9]. Due to the difficulties in establishing efficacious levamisole treatment, together with reported granulocytopenia [4], the possible benefit of immunoadjuvant therapy with levamisole was regarded with scepticism.

Recently, a revival of interest in the compound was triggered by a large scale intergroup clinical trial, which showed that adjuvant treatment with levamisole and 5-fluorouracil (5-FU) after surgery is beneficial (30% increase in 5-year survival) in patients with node-positive colon cancer [10]. Here we describe the results with levamisole, 5-FU and their combination on the growth of human colorectal adenocarcinoma grafted under the renal capsule of nude mice.

Correspondence to R. Van Ginckel.
R. Van Ginckel, M. Callens, B. Janssens, L. Wouters, R. De Coster and P.A.J. Janssen are at the Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse; W. Distelmans is at the Free University Brussels, Academic Hospital, Oncologic Center, B-1090 Jette; and M. De Brabander and E. Jagers are at the Clinical Research Unit, St. Bartholomeus, AZ Jan Palfijn, B-2170 Merksem, Belgium.
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MATERIALS AND METHODS

Colorectal carcinomas of untreated patients, obtained under sterile conditions at surgery, were immersed in minimal essential medium (MEM–REGA) supplemented with 2 μmol/l glutamine

(Gibco) and transported to the laboratory at ambient temperature. The tumours were cut into pieces of approximately 1 mm^3.

Athymic mice (Charles River) were anaesthetised with an intraperitoneal injection of 2.5% 2,2,2-tribromoethanol (Aldrich) in sterile saline. An incision was made in the dorsal flank and the tumour fragments inserted under the renal capsule. The initial size of the grafts was measured with a micrometer in the eyepiece of a macroscope. The skin was closed with wound clips and the mice allowed to recover. The mice were then randomised in four groups of 8–13 mice per group. Mortality in the different groups was due to technical difficulties and was evenly dispersed among both treated and control groups (control 16%; levamisole 17%; 5-FU 20%; levamisole + 5-FU 11% mortality).

Intraperitoneal treatment with 20 mg/kg 5-FU (Hoffmann-La Roche) was begun 24 h later for 4 days, followed by intraperitoneal injections of 2.5 mg/kg levamisole (Janssen) for the next 3 days. This alternating regimen was repeated three times in the first two trials and twice in all subsequent experiments. Control animals received sterile saline. Mice were handled and treated using aseptic conditions throughout the whole procedure.

At the end of the experiments, mice were killed and the final size of the tumours measured. The growth was expressed as a percentage of the initial size at day 0, the latter taken as 100%.

Results were analysed using analysis of variance for a randomised complete block design on the log $(x + 100)$ transformed data [11].

RESULTS

Table 1 shows an overview of the patients' tumour characteristics. Tumour resections from 9 different patients were used. All tumours were well to moderately differentiated (grade I or II) adenocarcinomas of colon, rectum or sigmoid origin. Staging according to Dukes' classification revealed 7 stage B and 2 stage A tumours.

Distinct tumour growth was observed in six out of nine experiments (resections from patients 3–7 and 9). No relation could be established between the differentiation grade and/or Dukes' staging and the growth rate of the tumours. Also the localisation of the patients' tumour was not predictive for growth in nude mice.

Table 1. Tumour characteristics (all were adenocarcinomas) from each resection.

Patient	Staging Dukes	Localisation	Differentiation grade	Growth inhibition (%) (median)*
1	B	Rectum	II	7
2	B	Left colon	II	100
3	B	Sigmoid	I	45
4	B	Sigmoid	II	44
5	B	Right colon	ND	59
6	B	Rectosigmoid	II	35
7	A	Rectum	II	33
8	B	Rectum	II	−8
9	A	Sigmoid	I	52

ND = not determined.

* Percentage growth inhibition obtained in levamisole/5-FU group.

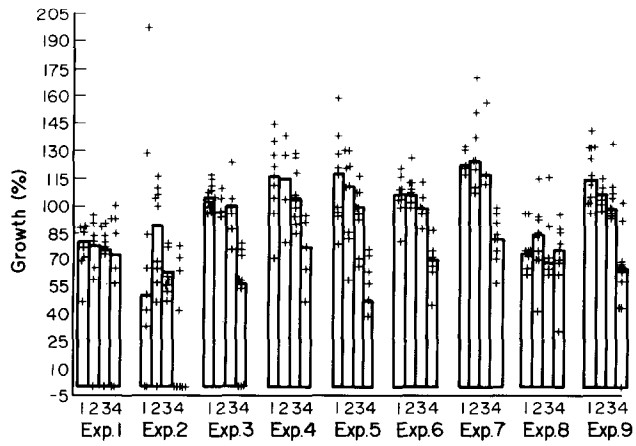

Fig. 1. Effect of levamisole, 5-FU and the combination on nine different human colorectal adenocarcinomas grafted in athymic mice. Each point = percentage growth of individual graft. Top of column = median of each treatment group: 1 = saline, 2 = levamisole 2.5 mg/kg, 3 = 5-FU 20 mg/kg, 4 = 5-FU 20 mg/kg + levamisole 2.5 mg/kg.

The individual results obtained in all nine experiments are depicted in Fig. 1. In seven out of nine experiments, the combination therapy with levamisole and 5-FU induced regression of the tumour transplants. Either compound used as monotherapy displayed no relevant growth inhibitory effects. The alternating regimen with levamisole and 5-FU was efficacious in all 6 experiments where the control fragments did grow (median growth inhibition 33–59%). In one trial (resection from patient 2) with regressive control grafts, the number of macroscopically disappeared fragments (six out of ten) in the group receiving both drugs was about 3-fold higher than in the saline (two out of nine) or levamisole (1 out of 10) treated groups. In two trials (resections from patients 1 and 8), control fragments failed to grow and neither therapy had any additional effect.

Analysis of variance indicated a highly significant superadditive effect for the combined use of both drugs ($P < 0.001$).

DISCUSSION

The use of human xenografts in athymic mice provides an *in vivo* test model that closely resembles the human situation with respect to morphology, tumour products, kinetics, drug activation and detoxification mechanisms. Furthermore, the small size of the transplants may perhaps mimic the residual tumour mass or metastases remaining after surgery [12]. The alternating schedule was adopted to avoid pharmacokinetic interactions between levamisole and 5-FU. The data obtained with levamisole and 5-FU clearly demonstrate the beneficial effect of combining both drugs ($P < 0.001$), while either monotherapy was ineffective.

Surgical–pathological staging is useful in predicting the prognosis of cancer patients providing important information about invasion and metastasis, but bears less relevance to our experimental conditions where fragments of the primary tumour were transplanted. Furthermore invasion and metastasis of human tumour transplants are very rare phenomena in nude mice. To correlate our observations to the clinical situation, the differentiation grade of the tumours should be considered as an important parameter. All resections transplanted in our experiments were grade I or II. Only 31% of grade I or II tumours were associated with positive lymph nodes (stage Dukes' C), whereas invaded lymph nodes were found in 81% of

grade III or IV tumours [13]. It may be suggestive from this finding that patients classified as stage A or stage B may also benefit from the adjuvant setting with levamisole and 5-FU.

Another intriguing observation is the activity of levamisole/5-FU combination on rectal and sigmoidal adenocarcinoma grafts. Although these tumours have a different behaviour compared with colon carcinoma [14], regression of implants was also demonstrated. Therefore further preclinical and/or clinical evaluation of levamisole in other oncologic settings is warranted to elucidate fully its potential use for the treatment of neoplasms.

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The Influence of Hyperthermia on the Uptake of Cisplatin in the Rat Cervical Spinal Cord

Peter Sminia, Gerrit Los, Jos J.G.W. Hendriks, Marianne J.H. van Vugt, Jaap Haveman and Dionisio González González

The influence of local hyperthermia on the uptake of cisplatin in the rat cervical spinal cord was investigated. After single intraperitoneal or intravenous injection of cisplatin (5 mg/kg body weight), the spinal cord region cervical 5-thoracic 2 was heated for 60 min at mean (S.D.) 41.2 (0.4) °C or 40 min 42.4 (0.3) °C using a 434 MHz microwave heating device. One day after treatment with either hyperthermia alone, cisplatin alone or the combination, none of the animals expressed neurological symptoms. The spinal cord was dissected and platinum levels were measured by flameless atomic absorption spectroscopy. No difference was found in uptake of platinum in the spinal cord between control- and heat treated animals. In a second series of experiments, the spinal cord was heated for 30–60 min. during a 2 h infusion of cisplatin. One day after treatment at 42.3°C for 60 min, neither motor nor sensory functions were affected and platinum levels did not differ significantly between control and treated animals. Also, platinum levels measured in the spinal cord immediately after cisplatin infusion were not influenced by heat treatment at 42.1 or 43.0°C for 30 min. However, after a heat dose of 60 min 43°C, cisplatin uptake was significantly increased ($P < 0.001$) by a factor of 2.8 (1.3). The data demonstrate that mild hyperthermia has no effect on the uptake of cisplatin in the spinal cord, while an injurious heat dose leads to a significant increase in cisplatin uptake. The present findings indicate that, in case of treatment of tumours of the central nervous system with hyperthermia and cisplatin, a treatment which might be toxic for the tumour is well tolerated by the normal nervous tissue.
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INTRODUCTION

THE MAJOR side-effects of the widely used anticancer drug cisplatin are acute and chronic nephrotoxicity and neuropathy [1–4]. The cisplatin induced nephrotoxicity has however become managable using a regimen of hydration and forced diuresis [5] or by chemoprotection [6]. Neurotoxicity is therefore now considered to be the dose limiting factor in platinum based chemotherapy [7]. The most common neurological complication is peripheral sensory neuropathy, which has been described in detail by a number of clinicians [8–10]. The frequency of neuropathies increased with increasing cumulative doses of cisplatin. Discontinuation of the therapy is generally followed