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Intraperitoneal Tumor Growth and Chemotherapy in a Rat Model*

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Abstract—*Animal models are important to evaluate new treatment modalities. In the present paper a new animal model is described, in which the effects of intraperitoneal (i.p.) administration of cytostatic drugs on cancers restricted to the peritoneal cavity can be studied. The tumor cell line used is a chemically induced carcinoma (CC531), sensitive* in vitro *to cisplatin (cDDP), carboplatin (CBDCA), 5-fluorouracil (5-FU), doxorubicin and mitoxantrone. Three to* 5 *weeks after i.p. inoculation of* 2×10^6 *CC531 cells,* 80% *of Wag/Rij rats develop small tumor nodules on peritoneal surfaces. Both tumor size and localization at this time are comparable to the human situation, especially to cases of minimal residual disease ovarian carcinoma. The model has been used to determine the usefulness of i.p. treatment in comparison to i.v. Changing the route of administration of cDDP from i.v. to i.p. increases tumor platinum concentrations and prolongs survival. The model offers the possibility to study drug pharmacokinetics and tumor drug penetration related to i.p. drug administration.*

INTRODUCTION

OVARIAN CANCER is difficult to diagnose and monitor clinically. Consequently the majority of patients have advanced disease (FIGO stages III or IV) at the time of diagnosis [1]. Since ovarian cancer usually grows intraperitoneally and rarely disseminates to distant sites [2] attempts at salvage therapy, after failure of intravenous chemotherapy, have focused on an intraperitoneal approach. Following the development of the theoretical basis of i.p. drug administration [3, 4], a number of cytostatic drugs have been administered intraperitoneally [5, 6]. Promising results have been reported for i.p. administration of cDDP, 5-FU, mitoxantrone and anthracyclines [7–10]. It seems possible that pharmacological advantages may result in drug concentration ratios between peritoneal fluid and plasma of 1 to 3 logs [3], while i.p. administration of cDDP and mephalan also results in effective plasma levels [11, 12]. An additional advantage of i.p. drug administration would be the direct penetration of these drugs into tumors, a subject about which very little is known. After i.p. administration the presence of doxorubicin can be demonstrated intranuclearly in only four to six cell layers on the outside of a solid ovarian carcinoma [13] and it has been calculated that 5-Fu penetrates into the tumor no more than 60 cell layers [14].

In order to define the relationship between drug penetration and clinical response, a rat model has been developed and is described in the present study. This model resembles ovarian cancer closely and show similarities to other intraperitoneal xenograft tumor models [15–17]. The model has been exploited to study the advantages of i.p. over i.v. chemotherapy.

MATERIALS AND METHODS

Rats

Male Wag/Rij rats, 8–12 weeks old and weighing 220–260 g at the time of the experiments, were obtained from the animal department of The Netherlands Cancer Institute and bred under SPF conditions. The animals were kept on a 12 h light–12 h dark schedule and fed standard rat chow and tap water *ad libitum*.

Tumor

The tumor used (CC531) is a carcinoma originating in the colon of rats exposed to methylazoxymethanol and is well defined [18]. It grows *in vitro*,

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subcutaneously and intraperitoneally, forming small tumor nodules on peritoneal surfaces and in a preterminal phase an exudate. *In vitro* it is replated at a density of 1×10^5 cells in fresh Dulbecco's modified medium (DMEM) with 10% fetal calf serum (FCS, Flow Laboratories). The doubling time of the CC531 tumor cells was 16 h/cell cycle.

Single cell suspensions were prepared by enzymatically detaching the CC531 cells with trypsin. Trypsin was inactivated by added DMEM + 10% FCS. The solution was centrifuged and cell concentrations were prepared in PBS. The viability of the cells was assessed with trypan blue exclusion.

Drugs

5-FU (Fluorouracil Roche, Hoffman-la Roche, Mijdrecht, The Netherlands) was supplied as a solution in vials containing 250 mg (5 ml). The product also contains sodium chloride 0.9% (w/v). Dilutions, with final concentrations of 0.1–10 μM, were made in 0.9% sodium chloride before every experiment. Vials were stored at room temperature.

Doxorubicin (Adriablastina, Farmitalia, Rotterdam, The Netherlands) was supplied as a red lyophilized powder. Before every experiment the product was reconstituted with sterile water (concentrations of 0.1–100 μM). Intact vials were stored at room temperature.

Mitoxantrone (Novantron, Lederle, Etten-Leur, The Netherlands) was supplied as a dark solution in vials containing 20 mg (10 ml). The product also contains sodium chloride 0.8% (w/v), sodium metabisulfite 0.01% (w/v), sodium acetate 0.005% (w/v), and acetic acid 0.046% (w/v) in water for injection. Dilutions, with final concentrations of 0.01–1 μM, were made in 0.9% sodium chloride. Vials were stored at room temperature in the dark.

Cis-diamminedichloroplatinum(II) (cDDP) (Platinol®, Bristol Myers, Weesp, The Netherlands) was supplied as a solution in vials containing 10 mg (20 ml). The product also contains sodium chloride 0.9% (w/v) and hydrochloric acid at pH 2–3. Dilutions, with final concentration of 0.1–500 μM, were made in 0.9% sodium chloride before every experiment. Vials were stored at room temperature.

Cis-diammine (1, 1-cyclobutanedicarboxylato) platinum(II) (CBDCA) (Bristol Myers, Weesp, The Netherlands) was supplied as a white lyophilized powder, composed of 150 mg CBCDA and 150 mg mannitol in a 20 ml amber vial. Before every experiment the product was reconstituted with sterile water (concentrations from 10 μM to 10 mM). Intact vials were stored under refrigeration (2–8°C).

Rat model

Groups of Wag/Rij rats (5–15 rats in each group) were inoculated intraperitoneally (i.p.) with 0.5×10^6, 1×10^6, 2×10^6 and 4×10^6 CC531 tumor cells in 2 ml PBS at day 0. Tumor load and tumor localization were determined by repeated laparotomies (3–4) from days 14 to 35 after inoculation. Definitive tumor take was assessed on day 35. Tumor nodules were situated on the diaphragm, peritoneum and on the intestinal mesentery. No ascites or any distant metastases were detected during the time experiments were performed. However in a late stage of the disease, 9–10 weeks after inoculation of the tumor, some animals produced some ascites. The volume doubling time of the tumor in the peritoneal cavity was 3.9 days, determined by repeated surgery at fortnightly intervals during 60 days in 10 rats. The size of the tumor was assessed by measuring three diameters by digital calipers. The geometric mean of the three values was used. The tumor also grows subcutaneously and in the liver, if injected into the portal vein [19].

Sensitivity of CC531 for cytostatic drugs

Sensitivity of CC531 for different cytostatic drugs was tested by clonogenic assays. Drug sensitivity curves were determined using clonogenicity in 6-well tissue culture plates (Costar, U.K.). CC531 cells were harvested with a trypsin solution (0.05% w/v/EDTA (0.02% w/v) and counted. Cells in single cell suspension were plated in 6-well plates (100 cells/well) in a volume of 2.9 ml medium [Dulbecco's modified medium + 10% fetal calf serum (Flow Laboratories)]. A dilution of each drug (100 μl) was added after 24 h. The ultimate concentration in each well differed in the different experiments for cDDP from 0.01 to 10 μM, for CBDCA; 0.1 to 100 μM, for mitoxantrone from 0.1 to 100 nM, for doxorubicin from 1 nM oto 1 μM and 5-FU from 1 to 100 nM. After 1 h incubation cells were washed three times with PBS and fresh medium was added. Colonies containing more than 50 cells were scored 7 days after plating the cells. Every experiment was performed in triplicate.

Pt *concentrations in intraperitoneal tumors after i.p. and i.v. chemotherapy*

Wag/Rij rats were inoculated with 2×10^6 CC531 cells on day 0. Groups of three to eight rats were treated i.p. or i.v. with 5 mg/kg cDDP on day 28, or three times with 4 mg/kg cDDP on day 28, 35 and 42. Cisplatin was injected i.p. in a volume of 20 ml 0.9% NaCl and i.v. in a volume of 2.5 ml (0.5 mg cDDP/ml). On day 35 or 49 tumor tissue was collected and total platinum concentrations in tumors were determined by flameless atomic absorption spectroscopy (FAAS).

Exposure of the tumor to the cytostatic drug

Pharmacokinetic studies were performed after cannulation of the jugular vein and the carotid

artery in groups of three rats each. Cisplatin (5 mg/kg) was administered i.v. in a volume of 2.5 ml or i.p. in a volume of 20 ml. Blood samples were taken at different time points (0, 3, 10, 20, 30, 45, 60, 120, 180, 240, 360, 1440 min). Platinum (Pt) concentrations in plasma, total and ultrafiltered, were determined by FAAS. Pt concentrations in the peritoneal cavity were studied after cannulation of the peritoneal cavity. Samples (100 µl) were taken from the injected cDDP containing peritoneal fluid via a peritoneal cannula at the same time points as blood as no ascites was present. The areas under the concentration–time curves (AUC), from 0 to 1440 min, were determined from plasma as well as from the installed peritoneal fluid for total platinum.

Survival

Wag/Rij rats were inoculated with 2×10^6 CC531 cells on day 0. On day 14 rats were treated i.p. or i.v. with 4 m/kg cDDP. For i.p. treatment cDDP was dissolved in 20 ml 0.9% NaCl solution, for the i.v. treatment cDDP was injected into the tail vein in a volume of 2.5 ml. Each group (i.v.-treated, i.p.-treated and control group) consisted of 10 tumor bearing animals.

Flameless atomic absorption spectroscopy (FAAS)

A model AA40 atomic absorption spectrometer with a GTA 96 graphite tube atomizer (with Zeeman background correction) from Varian (Victoria, Australia) was used for Pt analysis. Platinum concentrations were determined in plasma, peritoneal fluid and in tumor tissue. Samples were diluted (1:4 v/v for plasma and ultrafiltrate) with a solution containing 0.2 M HCl and 0.15 M NaCl. Tumor samples were disrupted by scissors. An aliquot of tumor tissue (about 200 mg) was digested with 2.5 ml 65% HNO_3 at 170°C in a Parr Teflon-lined acid digestion (PTAD) bomb for 2 h. After cooling the liquid was evaporated under a stream of air after addition of 5 mg NaCl. The residue was dissolved in 0.2 ml 0.2 M HCl and 0.15 M NaCl. If necessary the tubes were placed in an ultrasonic bath for 10 min. All standards were treated in the same way as the samples, diluted with, or digested in, the appropriate matrix. A 4-stage heating program was used consisting of drying at 110°C for 65 s, ashing at 1400°C for 75 s, atomizing at 2650°C for 3 s using maximum power, and conditioning at 2550°C for 5 s. The inert gas was nitrogen.

Statistics

The Wilcoxon test or Student's *t* test, as indicated in the legend, were used to determine statistical significance, with P values > 0.05 considered to be not significant.

RESULTS

Tumor 'take'

Different numbers of CC531 cells (0.5×10^6, 1×10^6, 2×10^6, 4×10^6) were inoculated into the peritoneal cavity of male Wag/Rij rats. Figure 1 demonstrates that the highest tumor take, determined after 5 weeks, was 80% and occurred in the group which received 2 and 4×10^6 CC531 cells at time of inoculation. Inoculations with more than 2×10^6 were not therefore considered to be of added use.

Tumor load and localization

The tumor load in the peritoneal cavity was determined at several time points after inoculation of 2×10^6 CC531 cells in the peritoneal cavity. After 2 weeks no tumor nodules could be detected, after 3 weeks 50% of the animals had more than 10 small tumor nodules, with a size between 1.0 and 4.0 mm and after 5 weeks nodules were detectable in 80% of the animals (Fig. 2). No increase in tumor take was detected after 5 weeks. This indicates that in the period between 1 and 3 weeks after inoculation microscopic metastases are present in eight out of 10 animals, thereby making this model a suitable one to study microscopic disease. The fact that 20% of the rats will not develop tumors means that for adjuvant studies, the number of rats in each experiment have to be increased, to increase the power of the statistical analysis. Tumor localization is demonstrated in Fig. 3. Tumor nodules with different size (diameter: 2–5 mm) can be detected on all peritoneal surfaces, e.g. mesentery, diaphragm, and abdominal wall, resembling the anatomical pattern of transperitoneal metastasis of ovarian, gastric and colon carcinomas and mesothelioma.

Figure 4 shows the histological features of the tumor. No necrotic areas could be detected, indicating a well-vascularized tumor. This was confirmed by the distribution of i.v. Hoechst 3342 fluorescent dye (data not published). This dye can be used to visualize blood vessels in frozen sections of biopsies taken after i.v. injection.

Sensitivity of CC531 tumor cells to different cytostatic drugs

Table 1 demonstrates maximal drug concentrations (C_{max}) in plasma and peritoneal cavity of rats after i.p. administration of a non-toxic dose of doxorubicin (4 mg/kg), mitoxantrone (2.5 mg/kg), cisplatin (4 mg/kg) and carboplatin (20 mg/kg) and 5-fluorouracil (30 mg/kg) all used in human i.p. chemotherapy trials. It indicates that after i.p. administration of these drugs the maximal concentration (C_{max}) achieved in plasma lies between 2 and 90 µM and in the peritoneal cavity between 40 and 3000 µM. Further, the sensitivity of CC531

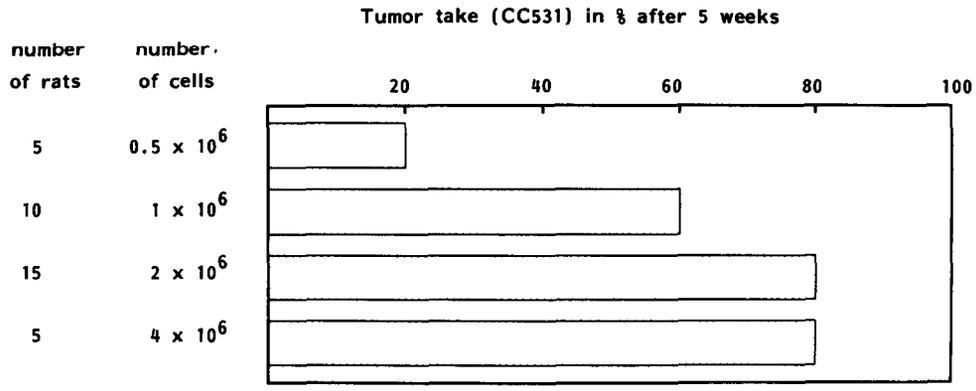

Fig. 1. Tumor take of CC531 cells in Wag/Rij rats after intraperitoneal inoculation of different numbers of cells.

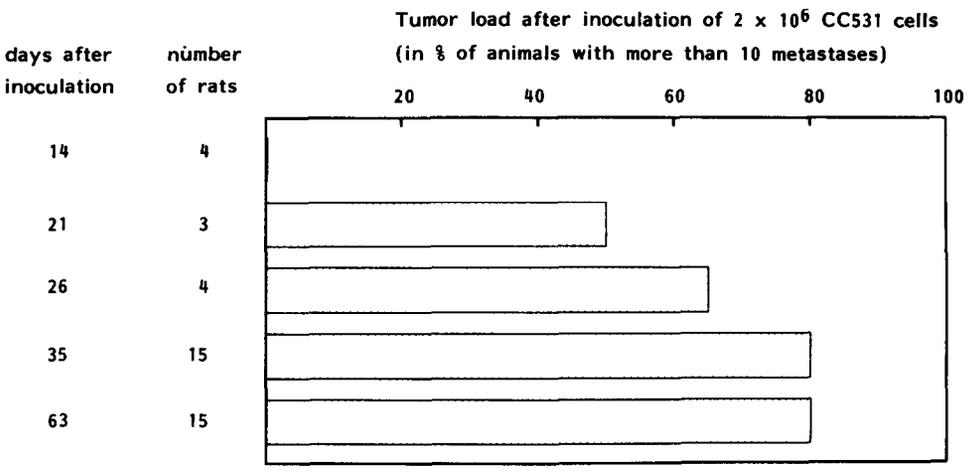

Fig. 2. Determination of the tumor load in the peritoneal cavity of Wag/Rij rats after inoculation of 2 × 10^6 CC531 cells after 2-7 weeks.

tumor cells for 5-fluorouracil, doxorubicin, mitoxantrone, cisplatin and carboplatin was tested in clonogenic assays, performed to determine the LD_{50} after 1 h and continuous incubation for these drugs (Table 2). The LD_{50} of these drugs lay between 1 nM and 1.5 μM after continuous incubation and between 0.03 to 45 μM after 1 h incubation. Comparing the LD_{50}s in clonogenic assays *in vitro* after 1 h incubation with the C_{max} achieved in plasma after i.p. administration *in vivo*, it is demonstrated that the drug concentration needed to kill 50% of the CC531 cells is much lower than that obtained in plasma after i.p. administration. The difference in LD_{50}s and C_{max} lies between a factor 1 to 2 for carboplatin to a factor 133 to 200 for 5-fluorouracil. From these data, presented in Table 1 and 2, it can be expected that CC531 tumor cells will be sensitive to 5-FU, mitoxantrone and doxorubicin and moderately sensitive to cDDp and CBDCA, when administered *in vivo*, in tumor bearing rats.

Platinum concentrations in intraperitoneal tumors

Drug concentrations in intraperitoneal tumors were measured, to study the accessibility of intraperitoneal tumors to cytostatic drugs after i.p. administration. By measuring the drug concentration after both i.v. and i.p. administration, data can be obtained about possible advantages of one route over the other. The cisplatin concentration in tumors is significantly higher after intraperitoneal administration, both after single administration ($P = 0.05$) and after repeated injections ($P = 0.01$) (Table 3).

Pharmacokinetics of intraperitoneal cisplatin

Values of the areas under the concentration–time curves (AUC) in plasma and peritoneal fluid have been constructed to provide an indication for the exposure of the tumor to the cytostatic drug. As demonstrated in Table 4, no difference between the i.p. and i.v. administration of 5 mg/kg cDDP could

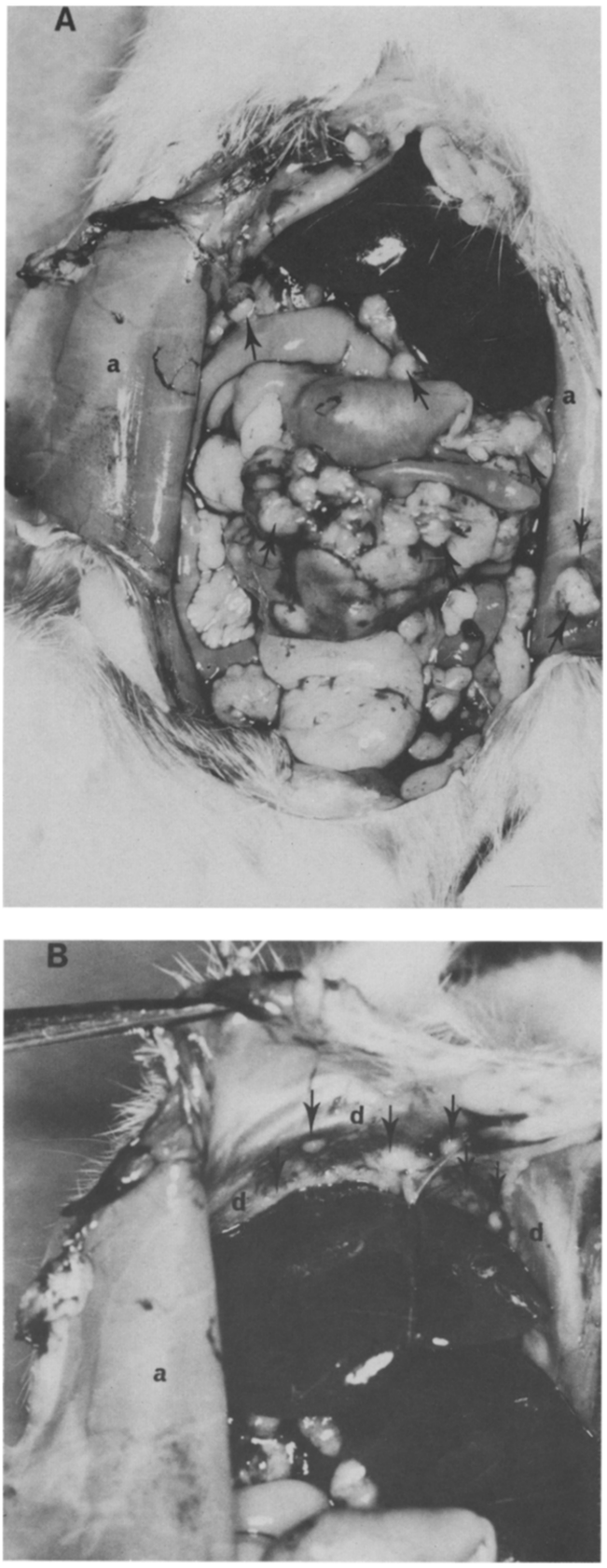

Fig. 3. Localization of CC531 tumor nodules 5 weeks after inoculation of 2 × 10⁶ CC531 tumor cells. A: Tumors (arrows) on the abdominal wall and all over the mesentery between the intestines. B: Localization of tumors (arrows) on the diaphragm. C: Magnification of tumors on the mesentery, size of tumor nodules between 2 and 5 mm (scale 0–70 mm). a = abdominal wall, d = diaphragm.

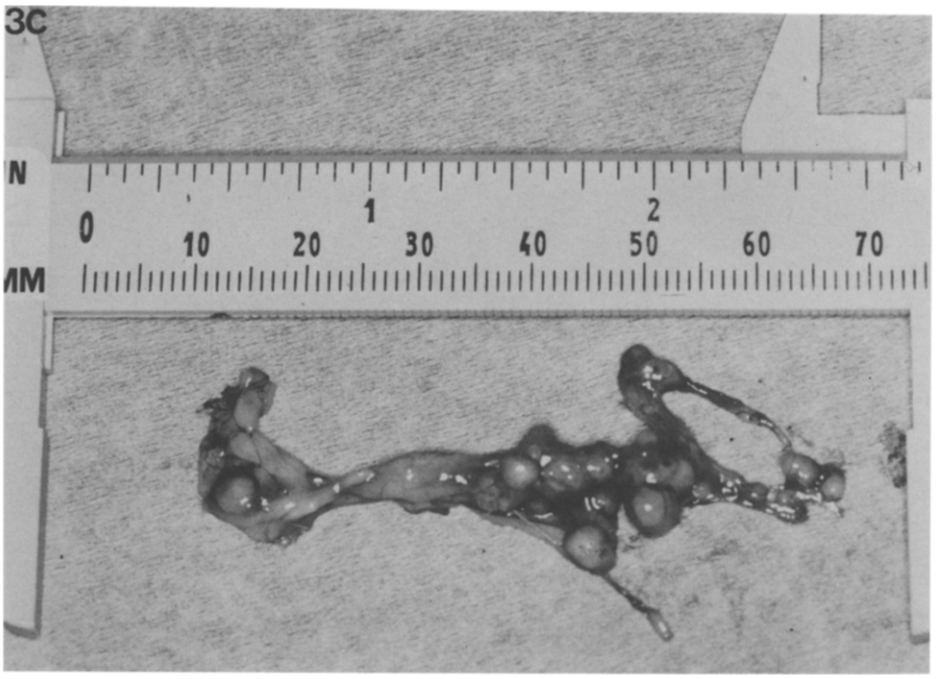

Fig. 3. continued.

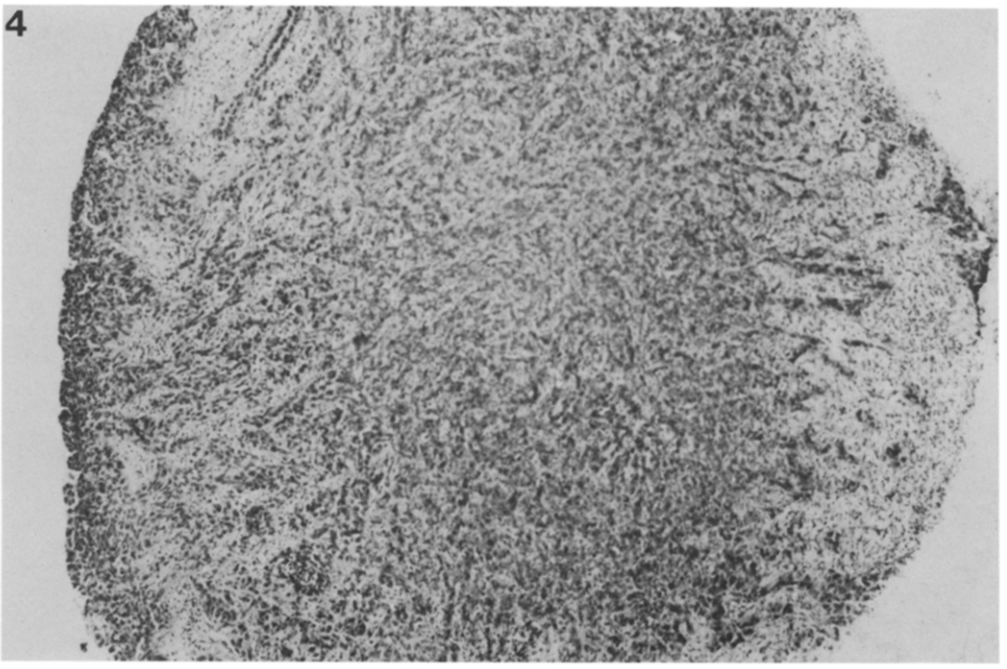

Fig. 4. Histology of an intraperitoneal CC531 tumor nodule, 5 weeks after inoculation. 36 × *Hematoxylin and eosin.*

Table 1. Drug concentration (C_{max}) in plasma and peritoneal cavity after i.p. administration of a non-toxic dose

Drug	Dose (mg/kg)	C_{max} in peritoneal cavity (μM)	C_{max} in plasma
5-FU	30	2000–3000	4–6
Doxorubicin	4	40–60	2–5
Mitoxantrone	2.5	80–120	2–4
cDDP	4	200–250	10–15
CBDCA	20	800–1200	50–90

*Table 2. In vitro sensitivity of CC531 cells for cytostatic drugs**

Drug	LD_{50}† continuous incubation	LD_{50}† 1 h incubation (μM)
5-FU	nd‡	0.03
Doxorubicin	18 nM	0.48
Mitoxantrone	1.1 nM	nd
cDDP	0.16 μM	4.0
CBDCA	1.5 μM	45.8

*Data obtained from at least two experiments, performed in triplicate.
†LD_{50} was determined by clonogenic assays.
‡nd: not determined.

be detected in the AUC measured in plasma. The advantage of i.p. treatment therefore lies in the peritoneal cavity, an AUC of 9561 (μM.m) after i.p. administration in comparison to 1497 (μM.m) after i.v. This indicates that the higher drug concentration, measured in tumors after i.p. administration, is probably caused by direct diffusion of the drug from the peritoneal cavity into the tumor.

The advantages of i.p. over i.v. administration expressed in survival time

A survival experiment has been performed to test whether the increased drug concentration in the tumor obtained after i.p. administration of cDDP, also leads to a better antitumor response. Figure 5 shows that survival of i.p. treated rats is longer than for i.v. ($P = 0.025$) which in turn is longer than untreated controls ($P = 0.025$). This would seem to indicate a correlation between Pt tissue concentration and the antitumor response.

*Table 4. Areas under the curve (AUC × S.D. μM.m) in plasma and peritoneal fluid after i.p. and i.v. administration**

Pt detection	AUC
In plasma after i.p. administration	3915 ± 305†
In plasma after i.v. administration	3466 ± 365
In peritoneal fluid after i.p. administration	9561 ± 325
In peritoneal fluid after i.v administration	1497 ± 81

*Number of rats: $n = 3$.
†Not significantly different ($P > 0.05$).

DISCUSSION

There is growing evidence that small tumor nodules in the abdominal cavity, occurring for instance in patients with ovarian cancer, may be particularly difficult to eradicate [2]. About 40% of the patients with ovarian cancer (stages III and IV) obtain surgically proven complete responses after systemic chemotherapy [20]. Patients who fail to achieve a complete response often have minimal residual disease [5, 6], defined as nodules on peritoneal surfaces smaller than 1 or 2 cm in diameter. One explanation why about 60% of the patients do not achieve complete responses is probably the fact that insufficient drug levels are reached in tumor nodules. One way to achieve higher drug concentrations in tumors located in the peritoneal cavity is to change the route of administration from i.v. to i.p. We have developed a well-defined intraperitoneal tumor model in the rat to study the basic mechanisms involved in the effects of this change in route of administration.

Intraperitoneal tumors were obtained in 80% of the animals after i.p. injection of 2×10^6 CC531 adenocarcinoma tumor cells. Injections with more than 2×10^6 cells did not lead to a better tumor take. These tumors grew on the peritoneal surfaces and formed small nodules but did not metastasize outside the peritoneal cavity, thereby mimicking the anatomical features of ovarian cancer closely.

Table 3. Platinum concentration in intraperitoneal tumors

Dose	i.p. administration	i.v. administration	*P* value
1 × 5 mg/kg	1.9 ± 0.12†	1.5 ± 0.2‡	0.05
3 × 4 mg/kg	10.5 ± 1.6§	5.4 ± 0.75‖	0.01

*Significance, measured with the Wilcoxon test.
†Number of rats: $n = 5$.
‡Number of rats: $n = 8$.
§Number of rats: $n = 3$.
‖Number of rats: $n = 4$.

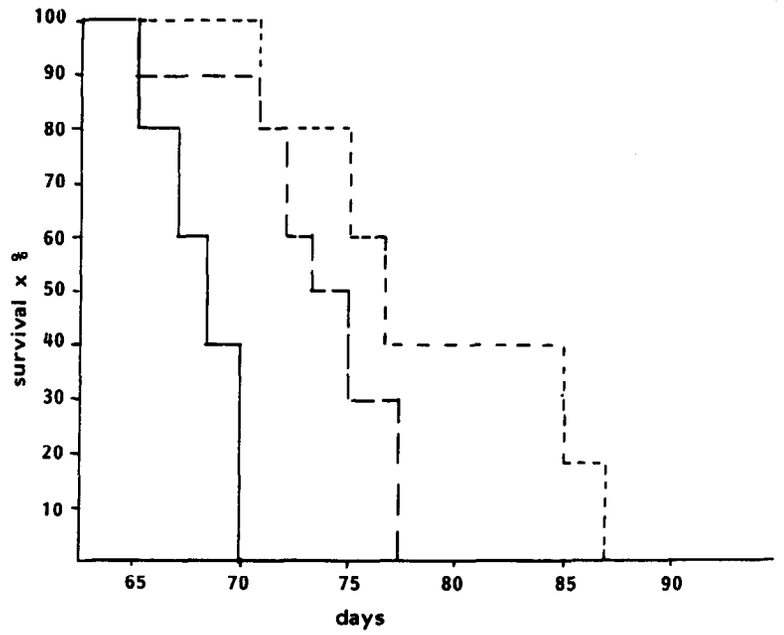

Fig. 5. Survival of Wag/Rij rats after inoculation of 2 × 10^6 *CC531 tumor cells and treated with 5 mg/kg CDDP i.v. or i.p.* ——— = *control,* – – – *i.v. and* ..... *i.p. Significant differences were present between the control group and both the i.v.* (P = 0.025) *and i.p.* (P = 0.005) *groups and between the i.v. and i.p. treated groups* (P = 0.025). *Significance was measured with Student's t test.*

The time to the appearance of detectable tumor nodules (diameter > 2 mm) after the inoculation was about 20 days. An important consequence is that in the period between days 1 and 20 microscopic metastases are present in 80% of the animals, which closely resembles the human situation [5, 6]. Furthermore, the tumor is sensitive to different drugs including mitoxantrone, cisplatin and carboplatin, drugs used in the clinical setting [8, 10, 21]. The dose-limiting toxicity for i.p. mitoxantrone and doxorubicin in patients is chemical peritonitis [10, 22], which also occurred in this tumor model (data not shown).

This is one of several intraperitoneal models, the others being mainly xenografts of human ovarian cancers in nude mice [15–17, 23–26]. It is true that xenografts maintain close morphological similarity to the parent primary tumor [15, 17, 23–26], nevertheless variation in tumor take (4 weeks to 4 months), in the antitumor effect of cytostatic drugs, in the expression of hormone receptors in human ovarian cancer cell lines [23] and in receptor expression between the original tumor and the xenograft [28] are disadvantages of the xenograft models.

Another obvious difference between the xenograft models and that presented here is the immunological status of the nude mouse in comparison to the syngeneic situation. It seems that metastases at a distance from implanted xenografts do not generally occur [29], which is related to the function of circulating natural killer cells [30]. This would imply that processes, involved in achieving optimal clinical responses after i.p. chemotherapy, such as pharmacokinetics, tumor drug distribution and antitumor response, might be better studied in our kind of model. The morphological similarity of the human tumor, as demonstrated in human xenograft models, is of less importance in investigating the above parameters.

The present study demonstrates that the syngeneic model can be used to provide supplementary information about intratumor drug concentrations, drug pharmacokinetics and antitumor responses. The highest Pt concentrations in tumors were always reached after i.p. administration of cDDP (Table 1). An explanation for this phenomenon could be drawn from the AUC in plasma and peritoneal fluid. Further the survival experiment has demonstrated that the survival increases after i.p. administration in comparison with i.v. indicating that the drug concentration achieved in the tumor has positive effects on the ultimate goal. This is also the case in the human situation [8, 31]. The change in the route of administration of cisplatin from i.v. to i.p. in patients who had failed to respond completely to initial cDDP treatment (i.v.) led to complete remissions in 30%.

In conclusion, we have developed an intraperitoneal tumor model which demonstrates not only similarity with other *in vivo* models [15, 16, 27] but also with the anatomy of the human cancers restricted to the peritoneal cavity. We suggest that this model can be used to study in more detail processes which play an important role in the uptake of drugs into tumors.

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