Adriamycin-loaded albumin-heparin conjugate microspheres for intraperitoneal chemotherapy

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Abstract

Adriamycin-loaded albumin-heparin conjugate microspheres (ADR-AHCMS) were evaluated as possible intraperitoneal (i.p.) delivery systems for site-specific cytotoxic action. The biocompatibility of the microspheres after intraperitoneal injection was tested first. 1 day after i.p. administration of empty as well as drug-loaded AHCMS to male Balb/c mice, only a moderate increase in i.p. neutrophils was measured. 3 days after injection neutrophil levels were comparable with the controls. No significant increases in the numbers of other cell types were observed, indicating an acute inflammatory response which can be considered to be mild. Antitumour efficacy was tested in an L1210 tumour-bearing mouse model and in a CC531 tumour-bearing rat model. The use of ADR-AHCMS leads to longer survival times of mice and improved tumour growth delay in rats, as compared with untreated controls and free drug treated animals. In both animal models higher adriamycin doses were initially tolerated if the drug was formulated in microspheres, although long-term adriamycin toxicity effects were evident in all treated groups. Doses and dosage schedules may be optimized to further reduce the toxic effects of the drug.

Key words: Adriamycin; Microsphere; Biocompatibility; Intraperitoneal chemotherapy; Controlled release; Drug targeting

1. Introduction

In certain applications regional chemotherapy is established for elevating the therapeutic in-

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dices of conventional anticancer drugs. In the case of intracavitary malignancies, like brain tumours and regionalized abdominal pelvic or mediastinal cancer, intracavitary administration of therapeutic agents allows higher drug levels at the site of the tumour than could be achieved with systemic chemotherapy (Knapp et al., 1983).

In the treatment of brain tumours the blood brain barrier is by-passed if the drugs are administered intracranially. Intracranial administration of drugs by means of refillable silicon devices (Ueno et al., 1982) or by using chronically implanted catheters (Bouvier et al., 1987) to treat brain tumours has been reported. Biodegradable polyanhydride discs loaded with bis(2-chloroethyl)-1-nitrosurea (BCNU), implanted intracranially after surgical resection of the primary tumour, appear to be more effective than standard intravenous treatments (Brem, 1990; Langer, 1991). Another example of intracavitary chemotherapy is the intrapleural administration of cytarabine in the treatment of malignant pleural effusions (Markman and Howell, 1987).

Intraperitoneal (i.p.) chemotherapy for the treatment of malignant diseases that remain largely confined there (e.g., ovarian carcinoma) has also been widely investigated. In the clinical situation prolonged infusions of drugs have been used. Using a dialysis catheter, high intracavitary concentrations of drug can be achieved and large volumes of fluid can be instilled, permitting treatment of all peritoneal surfaces (Knapp et al., 1983; Regelson, 1986). The results have been promising though not spectacular, since most drugs are transferred rapidly out of the peritoneal cavity (Wahl et al., 1988). Upon entering the systemic circulation via either the portal route or the lymphatic route, systemic toxicity is inevitable. Markman and Howell (1987), however, mention that i.p. cytarabine, which mainly exits the peritoneal cavity via the portal circulation, is inactivated in the liver before entering the systemic circulation. Another approach is the co-administration of neutralizing agents via the intravenous route to neutralize the drug transferred from the peritoneal cavity into the bloodstream. Systemic toxicity of i.p. cisplatin, for instance, can be neutralized by systemic thiosulphate (Howell et al., 1982).

An alternative approach in i.p. chemotherapy has been the use of implantable drug delivery systems, designed for retention within the peritoneal cavity following administration there. Upon intracavitary implantation the drug can be released over an appropriate period. Heller et al.

(1991) developed bioerodible, 5-FU-loaded poly (ortho ester) devices which, after i.p. implantation, prolonged survival times of DBA_2 mice inoculated i.p. with L1210 cells. Ulbrich et al. (1992) studied the in vivo release of targeted and non-targeted adriamycin conjugates from intraperitoneally implanted, biodegradable hydrogels. A disadvantage which these systems have in common is that laparotomy is needed to implant the device.

Injectable formulations based on soluble polymers, synthesized for selective retention within the compartment, can be administered without surgery. Their application relies on physical size to regulate their drainage into the systemic circulation. Physical size (i.e., molecular mass) of soluble materials, however, is unable to mediate selective retention, materials up to 778 kDa showing uninhibited lymphatic transfer into the bloodstream (Seymour et al., 1987).

One possible solution is the use of injectable formulations of particles, large enough to be selectively retained in the compartment but small enough to be injected without laparotomy. Albumin-heparin conjugate microspheres (AHCMS) loaded with adriamycin (ADR) appear to be ideal for this purpose because they can be synthesized in any appropriate size (Cremers et al., 1990, 1993a; Kwon et al., 1991) and they are known to release the drug in vitro by an ion exchange mechanism within a few hours after injection. The literature suggests that particles up to a diameter of 5 μm are transferred relatively easily out of the compartment, with reports of some transfer of materials up to 22.5 μm (Allen, 1956). Previously, Miyazaki et al. (1986) reported reduced toxicity and increased antitumour activity of ADR formulated in relatively small fibrinogen microspheres (1.7 $\pm$ 0.7 μm) in an Ehrlich ascites carcinoma-bearing mouse model. In this paper the evaluation of ADR-AHCMS with a mean particle size of 23 μm as i.p. targeting devices against i.p. localized tumours is described.

The i.p. biocompatibility of empty and drug-loaded particles was tested in a slightly modified version of the intraperitoneal biocompatibility test described by Pizzoferrato et al. (1987). Antitumour efficacy was determined in two different

animal models. The first tumour model system used, intraperitoneal L1210 leukaemia, is long established and routinely employed for the screening of novel anticancer agents (Duncan et al., 1988) and polymeric drug delivery implants (Heller et al., 1991). In the second study, a single intraperitoneal tumour model in the rat was used (Los et al., 1990). The tumour used, CC531, is a carcinoma originating in the colon of a rat exposed to methylazoxymethanol (Zedeck and Sternberg, 1974).

2. Materials and methods

2.1. Materials

Albumin-heparin conjugate (AHC) containing 10.9% w/w of heparin was synthesized and purified according to procedures developed by Hennink et al. (1983). Highly refined olive oil was obtained from Sigma Chemical Co., St Louis, U.S.A. Glutaraldehyde (25% aqueous solution), obtained from Merck, Darmstadt, Germany, was purified by distillation and adjusted to 2.5% w/v aqueous solutions prior to use (Gillett and Gull, 1972). Adriamycin was a kind gift from Farmitalia Carlo Erba, Milan, Italy. All other reagents were obtained, in the highest possible grade, from Merck, Darmstadt, Germany.

2.2. Microsphere preparation and drug loading

AHCMS were prepared as described before (Cremers et al., 1990, 1993a). Briefly, to 125 ml of prestirred (850 rpm) olive oil, 0.6 ml of a conjugate solution in phosphate-buffered saline (100 mg/0.6 ml PBS) was added. After emulsification for 15 min, 0.15 ml of glutaraldehyde solution was added. The emulsion was stirred at 850 rpm for 1 h. 50 ml of acetone was then added and the emulsion was stirred for a further 10 min. The microspheres were isolated by centrifugation for 15 min at 2700 rpm, followed by decanting of the supernatant. The microspheres were then washed several times with acetone. After collection, the microspheres were washed with distilled water and phosphate-buffered saline to remove un-

bound material. Subsequently the AHCMS were dehydrated with ethanol and sieved, giving a dry size of 10–25 μm.

Adriamycin loaded AHCMS (ADR-AHCMS) were prepared by 24 h incubation of 10 mg of AHCMS in 1 ml of adriamycin solution in distilled water (5 mg/ml) and subsequent washing with water and acetone. Payloads were calculated from the ADR depletion in the loading solution and the ADR recovery in the washing steps (Cremers et al., 1993b).

2.3. Biocompatibility test

ADR-AHCMS (44.93 mg) were resuspended in 1 ml of ethanol by sonication. To the suspension, 51.6 ml of 5% w/v glucose solution was added. After 2 h of rotation at 4°C in the dark, the microspheres were centrifuged and the supernatant was decanted. Fresh glucose solution was added. Three suspensions were prepared: 1.5, 0.75 and 0.375 mg/ml. Empty AHCMS were resuspended in 5% glucose solution and diluted to concentrations of 1.0, 0.5 and 0.25 mg/ml. The suspensions were stored overnight at 4°C, until use.

Male Balb/c mice (body weight approx. 40 g) were obtained from the Central Animal Laboratory, University of Groningen, The Netherlands. Seven groups of mice were injected with approx. 1 ml of a microsphere suspension as depicted in Table 1. One more group of mice served as a control and received only the 5% w/v glucose

Table 1
Experimental set-up of the intraperitoneal biocompatibility test

Code	No. of mice	Material	Dose (mg)	Sampling time (day)
C	15	control	–	1–2–3–5–7
M1	9	AHCMS	0.25	1–3–7
M2	9	AHCMS	0.5 [a]	1–3–7
M3	15	AHCMS	1.0	1–2–3–5–7
AM1	15	ADR-AHCMS	0.375	1–2–3–5–7
AM2	15	ADR-AHCMS	0.75 [a]	1–2–3–5–7
AM3	15	ADR-AHCMS	1.5	1–2–3–5–7

[a] This dose was calculated based on an adriamycin dose of 5 mg/kg, which is the therapeutic dose used in the tumour model studies described below.

solution. At different time intervals (Table 1) three mice of each group were killed and a peritoneal wash was carried out. The particles and cells in the peritoneal exudate were counted using a Coulter counter. Then the exudate of the three mice was pooled and the particles and cells were identified using light microscopy on sections cut after embedding in Technovit 7100® (Kulzer, Wehrheim, Germany). Five different groups were distinguished at identification: neutrophils, macrophages, lymphocytes, basophils/eosinophils and a group containing cells that could not be identified, microspheres, debris and dead cells which was categorized as 'other'. Their percentage in the LM sections was determined after classification of 200 cells per section.

2.4. In vivo anti-tumour efficacy, L1210 mouse model

Male DBA$_2$ mice (7–8 weeks old, 23–26 g each) were purchased from Bantin & Kingman Ltd, Hull, U.K. They were administered L1210 cells (10^5) intraperitoneally, under light inhalational anaesthetic (Fluothane) on day 0.

The mice were treated i.p. on days 1, 2 and 3, also under Fluothane anaesthetic, between 14:00 and 15:00 to minimize circadian variations. Control animals received 0.1 ml injections of 5% w/v glucose solution. Animals receiving free ADR were administered either 5 or 10 mg/kg daily in glucose solution (0.1–0.2 ml). ADR-AHCMS was formulated as described above and administered i.p. at a dose of either 5 or 10 mg/kg ADR on each day.

Mice were monitored closely and weighed daily. Any animal losing 20% of body weight was killed immediately. Similarly, animals showing either obvious discomfort or extensive tumour burden (usually reflected as a weight greater than 34 g) were also killed at once. Survival was monitored up to 100 days postadministration.

2.5. In vivo anti-tumour efficacy, CC531 rat model

Male Wag/Rij rats, 8–12 weeks old and weighing 220–260 g at the time of the experiments, were bred at the animal department of The Netherlands Cancer Institute (Amsterdam, The Netherlands) under specific pathogen-free (SPF) conditions. The animals were kept on a 12 h light/12 h dark cycle and fed standard rat chow and plain tap water ad libitum. Rats were anaesthetized with ether. After preparation for operation, a 1 cm incision was made into the median ventral abdominal wall, at about the transition of the upper and lower quadrants. A small part of the small intestines was exposed and a tumour disc, with a diameter of 3 mm and a thickness of 1.5 mm, was implanted by a haemoclip fixation on the distal part of the mesentery. The tumour disc was obtained from the viable parts of subcutaneous CC531 tumours. The bowel was repositioned and the abdomen closed by three to four interrupted sutures. For assessing i.p. tumour growth a new laparotomy was performed at each measurement made at 2 weekly intervals. The size of the tumour was assessed by measuring three diameters of the tumour using Vernier calipers. The geometric mean of the three values was then calculated and used as the size estimate, since this gives a better estimate of volume for non-spheroid tumours. The overall mean and SD were then calculated from the geometric means of tumours in each group at set time points.

The tumour was implanted at day −11. Animals were treated in three groups of four rats each and one group of five animals (control) on day 0. All animals were administered 20 ml of solution or suspension. The control animals received 5% w/v glucose solution. The first treatment group was administered free ADR at a dose of 5 mg/kg. The second and third group were treated with ADR-AHCMS at doses of 5 and 10 mg/kg of ADR, respectively. Tumour growth was determined at days −5, 7, 21, 35 and 49.

3. Results and discussion

AHCMS and ADR-AHCMS were prepared according to standard procedures as previously described by Cremers et al. (1993a,b). ADR release from ADR-AHCMS upon suspension of the microspheres in aqueous medium was as described previously. The amount of drug released
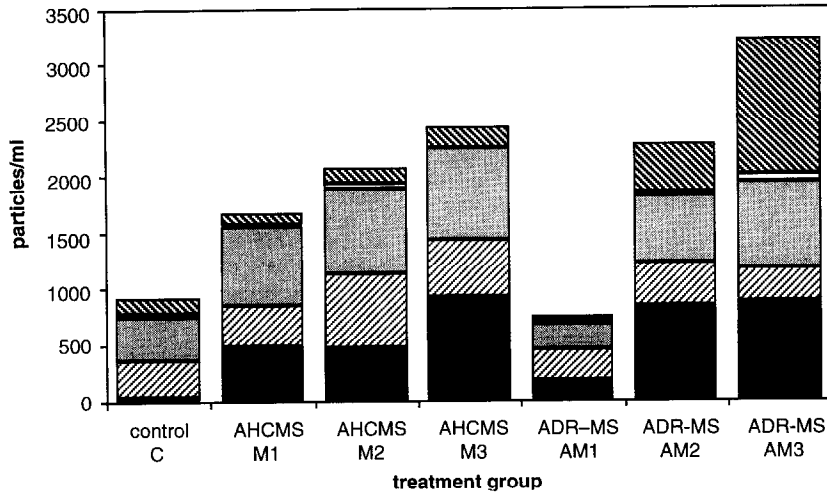
Fig. 1. Number of particles measured in peritoneal exudate, 1 day after i.p. administration of microspheres in Balb/c mice. (■) Neutrophils; (▨) lymphocytes; (▦) macrophages; (□) basophils/eosinophils; (▨) other (codes explained in Table 1).

varied between 2 and 30% w/w of the incorporated drug depending on the batch of ADR-AHCMS used. This was taken into account in the preparation of the injections.

3.1. Intraperitoneal biocompatibility test

The microsphere concentration of the suspensions used in the i.p. biocompatibility experiments is based on a therapeutically applied ADR dose of 5 mg/kg. The mice seemed to be unaffected by the i.p. administration of the microsphere suspensions, except for those receiving the highest ADR-AHCMS dose (10 mg/kg, AM3). These mice seemed to be less lively on the first day after administration. The peritoneum of these mice showed some milky spots, indicating necrosis at sites where the microspheres adhered to the peritoneum. It was also noticed that up to day 3 after administration of ADR-AHCMS, the supernatant of the peritoneal exudate was coloured red. This indicates the presence of free adriamycin or metabolites. At day 7 only the pellet obtained after centrifugation of the peritoneal exudate was coloured.

Fig. 1 shows the number and identification of particles and cells measured in the i.p. exudates on day 1. From Fig. 1 it can be seen that there is

a dose-dependent inflammatory response in the case of empty AHCMS as well as ADR-AHCMS: In both cases the infiltration of cells increases with increasing dose. This can also be observed from Fig. 2 which shows the neutrophils measured after administration of particles, standardized with respect to control animals. It can be seen that the dose dependence of the response to the ADR-AHCMS lasts for a few days.
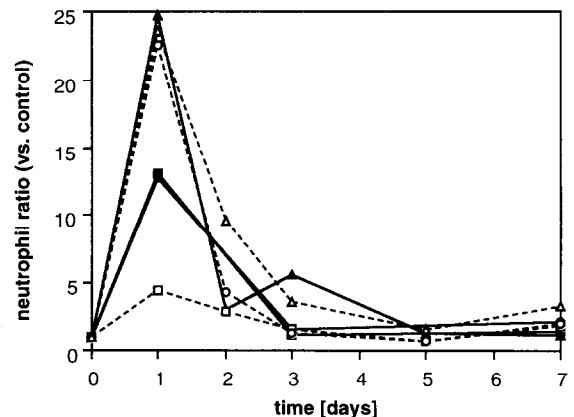

Fig. 2. Number of neutrophils measured after i.p. administration of microspheres in Balb/c mice, relative to controls. (■) M1; (●) M2; (▲) M3; (□) AM1; (○) AM2; (△) AM3 (codes explained in Table 1).
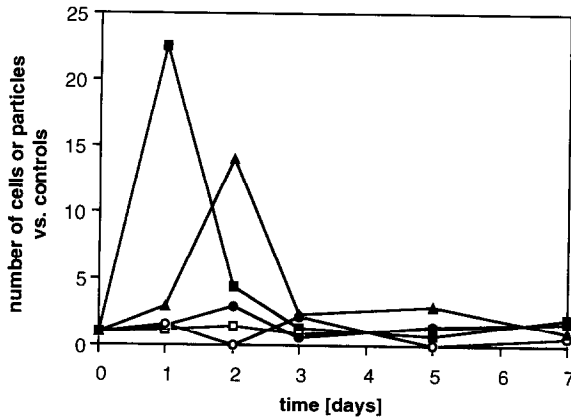
Fig. 3. Number of cells and particles after i.p. administration of 0.75 mg ADR-MS (AM2) in male Balb/c mice, relative to controls. (■) Neutrophils; (□) lymphocytes; (●) macrophages; (○) basophils/eosinophils; (▲) other.

An acute inflammatory response is characterized by the infiltration of primarily neutrophils and to a lesser extent, of macrophages. The increase in cells and particles as shown in Fig. 3, with a predominant increase in the number of neutrophils, is typical of an acute inflammatory response. Fig. 3 shows the amounts of cells and particles after i.p. administration of ADR-AHCMS at the 'normal dose', relative to the controls. At day 1 the neutrophil level is increased significantly. The number of lymphocytes and basophils/eosinophils, which is an indication for immunological and allergic reactions, is barely increased.

Fig. 3 also shows a significant increase in unidentifiable cells and debris (categorized as 'other'), mainly at day 2. This phenomenon only occurred with ADR-AHCMS at normal and at double dose (AM2 and AM3, see also Fig. 1). This increase is most likely due to the cytotoxicity of the ADR, causing more cell death at higher doses. At day 2, the peritoneal infiltration was still characterized by an increased number of neutrophils, although the response was already strongly reduced. At day 3 the response was further decreased and could only be observed with the highest doses of microspheres and at days 5 and 7 no significant responses were observed (data not shown). Viewed overall, these

Table 2
Survival of mice inoculated with L1210 cells, after treatment with ADR or ADR-AHCMS

Treatment [a]	Median T/C [b] (%)	Alive at day 100	Significance [c]
Free ADR, 5 mg/kg	155	1/5 (20%)	–
Free ADR, 10 mg/kg	45	0/5 (0%)	+
ADR-AHCMS, 5 mg/kg	236	2/5 (40%)	+
ADR-AHCMS, 10 mg/kg	209	0/5 (0%)	+

[a] Single administration dose; animals were adminstered three doses.
[b] T/C values calculated with control median = 11 days; mean = 13 days.
[c] Differences, as compared with controls, are significant at $p < 0.05$ levels as determined by log-rank test.

acute inflammatory responses are quantitatively very slight and they suggest that the AHCMS and ADR-AHCMS are well tolerated after i.p. administration to mice.

3.2. L1210 tumour-bearing mouse model

The results of the anti-tumour efficacy investigation of ADR-AHCMS in L1210 i.p. inoculated mice are listed in Table 2 and are shown in Fig. 4. Median survival times are shown in Table 2, whereas the graph shows the survival of the mice. The controls (sham-treated animals) showed rapid
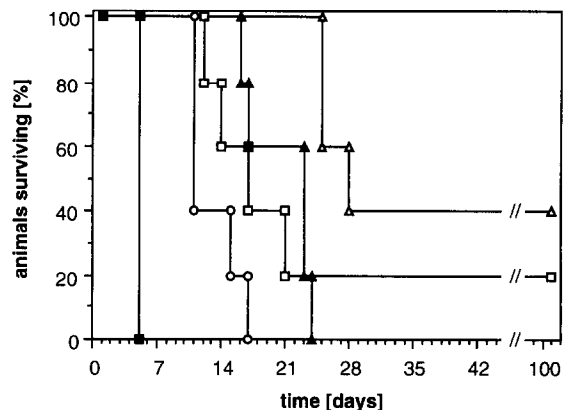

Fig. 4. Survival time of DBA2 mice inoculated i.p. with L1210 tumour. (○) Control; (□) ADR, 3×5 mg/kg; (■) ADR, 3×10 mg/kg; (△) ADR-AHCMS, 3×5 mg/kg; (▲) ADR-AHCMS, 3×10 mg/kg.

weight gain, beginning on day 8; all animals died from tumour burden. Animals treated with free ADR (3×10 mg/kg) showed weight loss indicative of severe toxicity and were killed on day 5. Those receiving the optimum therapeutic dose of free ADR (3×5 mg/kg) died from a mixture of tumour burden and toxicity with one animal still alive at day 100 (Fig. 4). The increase in survival times with respect to controls was not significant at a $p < 0.05$ level as determined using a log-rank test (Table 2).

Animals receiving ADR-AHCMS were treated with three doses of either 5 or 10 mg/kg. Those receiving 10 mg/kg demonstrated a substantial decrease in toxicity compared with the same dose of free drug. All animals showed some signs of toxicity like peritoneal oedema and weight loss although none met the criteria for early killing. The animals showed some recovery from these effects which were worst on about day 5, although all eventually succumbed to treatment-related deaths. On autopsy there were no signs of any tumour burden in these mice. Animals treated with 3×5 mg/kg of ADR as ADR-AHCMS showed only slight toxicity effects, with just a small initial fall in body weight. They appeared outwardly healthy for the first 20–25 days of the experiment. On about day 18 some animals began to show a regular increase in weight, and tumours were manifest in three animals on day 24. Tumours then grew rapidly. Two animals were free of tumour and still alive at day 100. Treatment with ADR-AHCMS at both doses improved the survival of mice significantly, as compared with controls (Fig. 4 and Table 2). Treatment with empty AHCMS did not significantly increase the survival time of L1210 inoculated mice as compared to untreated controls (data not shown).

3.3. CC531 tumour-bearing rat model

CC531 tumour growth curves are shown in Fig. 5. The plots show that i.p. treatment with 5 mg/kg free ADR reduced the tumour growth significantly. Treatment with 5 mg/kg ADR-AHCMS reduced tumour growth even more. At day 35, two out of three surviving animals were free of tumour. All animals treated with the
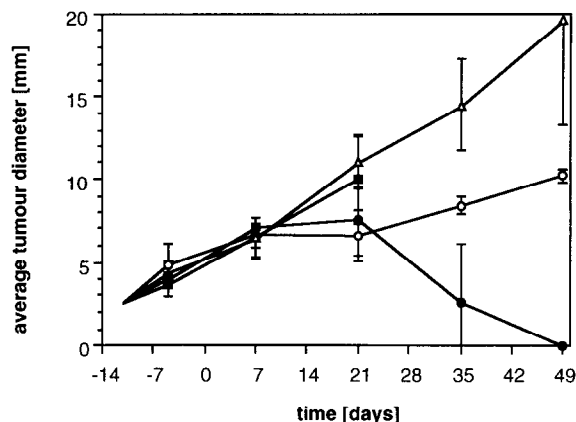

Fig. 5. Tumour growth delay of mesenteric CC531 tumours, implanted at day -11 in Wag/Rij rats, following i.p. treatment at day 0. Treatment: (○) ADR, 5 mg/kg; (●) ADR-AHCMS, 5 mg/kg; (■) ADR-AHCMS, 10 mg/kg; (△) control. Each point represents mean; error bars indicate SD.

highest dose of ADR-AHCMS (10 mg/kg) had died from toxicity, evident as peritoneal oedema, by day 35. In this group tumour growth over the first 20 days was not significantly different from the controls.

At day 21 one animal in the ADR-treated group and one animal in the ADR-AHCMS (10 mg/kg) group had died. Most other animals in these groups showed severe ascites. This was also noticed in the ADR-AHCMS (5 mg/kg) group at later stages. At day 21 only one animal in this group suffered from ascites and at day 35 one animal had died and the remaining animals all showed signs of ascites.

The cause of the ascites is still not completely clear. In a control experiment animals were treated with empty AHCMS. This treatment did not have any effect on the tumour growth. None of the animals showed any signs of ascites. This implies that the ascites is ADR-related. Los et al. (1990) treated i.p. CC531 tumour-bearing Wag/Rij rats intraperitoneally with ADR at a dose of 4 mg/kg. At this dose, tumour growth was initially delayed. At day 21, however, tumours started growing at a rate comparable to that of controls. The authors did not report any adverse effects due to ADR administration. This suggests that the applied dose (4 mg/kg) was safe in terms of

toxicity, but less effective in tumour growth control than the treatment (5 mg/kg) applied here. Ozols et al. (1982) reported that the dose-limiting factor in intraperitoneal ADR in patients was chemical peritonitis. Treatment at concentrations greater than 36 μM induced sterile ascites and peritoneal adhesions. Hence, intraperitoneal ADR toxicity is dose dependent as well as species dependent. In the case of rats, ascites was already evident at the 5 mg/kg dose, in the case of both free drug and ADR-AHCMS. In the L1210 mouse model, acute toxicity was observed with free ADR only at a dose of 3 × 10 mg/kg. Long-term effects indicated by ascites were only observed after 3 × 10 mg/kg ADR-AHCMS treatment, not at the therapeutically applied dose of 3 × 5 mg/kg ADR-AHCMS.

4. Conclusions

The intraperitoneal biocompatibility test in male Balb/c mice showed that empty AHCMS provoked only a mild dose-dependent inflammatory response, indicated by the infiltration of predominantly neutrophils. Incorporation of ADR into the AHCMS did not significantly enhance this response. At a drug dose of 10 mg/kg, however, a greater extent of cell death was initially observed, probably due to the presence of high amounts of ADR.

The L1210 tumour-bearing mouse model and the CC531 tumour-bearing rat model both showed an increase in antitumour efficacy when the drug was formulated in AHCMS. The administration of ADR-AHCMS instead of ADR reduced the acute toxicity in both models. Higher drug doses were initially tolerated. At a dose of 3 × 10 mg/kg, however, mice developed ascites. In rats this was observed at lower doses (5 mg/kg).

It is encouraging that formulation of ADR in AHCMS increases the antitumour activity of ADR against two different types of tumour model. There appears to be a balance between reduction of the acute toxicity and induction of chemical peritonites and ascites as a long-term effect. This calls for more research directed towards the improvement of dose and dosage schedules of mi-

crosphere drug delivery systems in i.p. chemotherapy.

It might be expected that regional therapy with a particulate formulation would show particularly good activity against tumour models which tend to grow in the omentum (eg. L1210), since this is also known to be the main site of accumulation of particles injected intraperitoneally (Seymour et al., 1991). However, the good activity demonstrated against solid CC531 tumours also implicates a component of sustained drug release.

Acknowledgements

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