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Dose response and toxicity of doxorubicin microspheres in a rat tumor model

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The therapeutic response and toxic effects of chemotherapy using several doses of doxorubicin in conventional solution form or bound to an ion-exchange resin were compared in a rat tumor model, to assess the relationship of drug dose to therapeutic efficacy and associated toxicity. Single bolus injections of 3.0, 4.5, 6.0, 7.5 and 9.0 mg/kg were administered via the abdominal aorta to rats bearing hindlimb tumors. Tumor size was measured serially and the growth rates of treated groups were compared with a control growth curve. In addition, the effect of empty microspheres on tumor growth rate was assessed. The levels of circulating white blood cells were measured and compared to control levels to provide an indication of the severity of bone marrow toxicity experienced by each form of treatment. Finally, any difference in the distribution of doxorubicin to tumor, hindlimb and cardiac tissue following administration of doxorubicin as free drug or on microspheres was ascertained. Empty ion-exchange resin exerted a small although significant detrimental effect on tumor growth which may be explained by the embolization of microspheres in the precapillary blood vessels of the tumor resulting in a transient delay in tumor growth rate. The lowest dose of doxorubicin produced a significantly better therapeutic response when administered in the free drug form, but higher doses elicited an equivalent delay in tumor growth for both drug microsphere and free drug groups in a dose-dependent manner, with the maximum anti-tumor response occurring at the highest dose. Treatment with free doxorubicin at high doses resulted in significant reductions of circulating white blood cells suggesting the occurrence of bone marrow toxicity. However, addition of ion-exchange microspheres evinced no significant change in white cell count. Consistently higher levels of doxorubicin were present in the normal hindlimb and tumor tissue of microsphere treated animals over a period of 96 h, indicating prolonged release of drug from the microsphere matrix. Conversely, there was a reduction in the amount of doxorubicin present in cardiac tissue of drug microsphere treated animals compared to free drug treated animals shortly after treatment. In summary, the administration of doxorubicin on ion-exchange microspheres reduced bone marrow toxicity without altering cytotoxic function and demonstrated the potential of microspheres in the prevention of long-term cardiac toxicity.

Key words: Doxorubicin, microspheres, toxicity.

Introduction

The major limiting factor in the efficacy of chemotherapy for most solid tumors is the local and systemic toxicity associated with high drug doses. However, therapeutic response is directly related to high drug doses. A number of mechanisms have been proposed to overcome the problems of drug toxicity, such as regional or arterial drug infusions and extended low dose administration. Another alternative has been the utility of drug carriers such as microspheres,[1] liposomes[2] or macromolecular conjugates.[3] These drug carriers are designed to deliver large doses of drug directly to the site of the tumor resulting in reduced levels of circulating drug and hence reduced toxicity.

Microspheres have been formulated from a variety of natural and synthetic materials. The choice of material will determine such factors as drug loading and rate of drug release. Formulations of biodegradable protein microspheres, e.g. albumin, have been unable to provide sufficient drug levels required for optimal therapeutic responses in a clinical situation.[4] Non-biodegradable microspheres produced from ion-exchange resins have been developed for the transport of a variety of different cytotoxic compounds.[6]

Ion-exchange microspheres for arterial administration have been previously described by Jones *et al.*[6] with loading levels of between 40 and 50% w w. These levels of drug combined with the physiological constraints of introducing limited numbers of particulate microspheres have resulted

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in a formulation ensuring therapeutic doses of drug can be targeted to the tumor site. Microspheres are sized to become entrapped in the pre-capillary arterioles of the tumor vasculature where drug release is sustained by ionic exchange with counter ions in the tumor environment.

We have previously described the therapeutic responses of implanted tumors in rats to doxorubicin loaded ion-exchange microspheres compared to free doxorubicin in solution.[5] However, there has been only limited examination of the therapeutic effects associated with toxicity of different doses of doxorubicin microspheres in relation to free drug administration. The present study was designed to assess tumor response to escalating drug doses and the relationship of drug dose to bone marrow toxicity. The propensity for systemic toxicity was also addressed in an analysis of the distribution of doxorubicin to cardiac, tumor and normal hindlimb tissue following bolus administration of free or microsphere bound drug.

Materials and methods

Doxorubicin microsphere preparation

The preparation of doxorubicin loaded microspheres has previously been described.[6] In brief, 40 mg of doxorubicin (Farmitalia, Sydney) was dissolved in milliQ water (4 ml) and slurried with 40 mg of ion-exchange resin (Aminex 50WX4, BioRad) at 4°C for 24 h. The resulting drug–resin combination was washed several times to remove excess doxorubicin and finally resuspended in milliQ water and stored at −20°C until required. Drug loading on the resin was determined using UV spectrophotometry at 495 nm with doxorubicin composition and concentration validated by HPLC.[8]

Ion-exchange resin microspheres with a mean diameter of 32 ± 2 μm were used in this study and were administered to animals in a dose-dependent manner with total microsphere numbers not exceeding 0.4×10^6 per animal. Microsphere numbers were calculated from suspensions in water with a hemocytometer and light microscopy.

Animals

The study was carried out using DA rats of both sexes with mean body weight 170 ± 19 g. Pieces of tumor derived from a salivary adenocarcinoma (1 mm^3) were implanted s.c. into both left and right hindlimbs. Tumor growth was assessed by the product of the minimal and maximal dimensions of the tumor measured with calibrated callipers. This was repeated every 2–3 days up to 15 days following treatment. Mean tumor size at the time of treatment was 64 ± 9 mm^2.

Therapeutic protocol

To evaluate the anti-tumor efficacy of doxorubicin on microspheres, tumor response to microspheres containing 3.0, 4.5, 6.0, 7.5 and 9.0 mg/kg body weight of doxorubicin was compared to equivalent doses of free doxorubicin. Animals were anesthetized by i.p. injection of Nembutal at 60 mg/kg, and treated by injection into the blood flow of the descending aorta below the junction with the renal arteries and above the iliac artery bifurcation. In this way microspheres were distributed proportionally to both hindlimbs. Blood samples were taken by heart puncture from all rats at 13–14 days after treatment (at time of sacrifice) for determination of white cell counts. Samples of tumor tissue and normal muscle surrounding the tumor tissue were also removed at this time for histological examination.

Four treatment schedules were used:

(i) Control: injection of 0.5 ml of 0.9% saline.
(ii) Free doxorubicin: 3.0, 4.5, 6.0, 7.5 or 9.0 mg/kg body weight was injected as a single bolus into the abdominal aorta over a 30 s period.
(iii) Doxorubicin microspheres: 3.0, 4.5, 6.0, 7.5 and 9.0 mg/kg of drug attached to ion-exchange microspheres was injected in a similar fashion.
(iv) Sham microspheres: 0.3×10^6 and 0.4×10^6 empty microspheres, the latter corresponding to a dose of 9 mg/kg, were injected in the same manner to assess any effects microspheres carrying no drug may have had on tumor growth.

Doxorubicin distribution

The levels of doxorubicin in tumor and normal tissues were investigated in a separate experiment. Two animal groups, each involving nine rats, were treated with either microspheres or free drug at 6 mg/kg. Three of the animals in each group were sacrificed at each of 3, 48 and

96 h, and plasma samples, heart, tumor and hindlimb tissue were removed. Doxorubicin was extracted from these tissues by homogenizing in phosphate buffered saline and subsequent extraction with chloroform–isopropanol.[8] The concentration of doxorubicin and its relatively inactive metabolite doxorubicinone were then determined by HPLC.

Statistics

A two-tailed Student's *t*-test was used to compare white blood cell counts between treated groups and the control group. The tumor growth for control animals was statistically compared to all treated groups by comparison of regression lines by analysis of covariance.[9]

Results

All animals in the study tolerated the surgical procedures without event. However, animals treated with free doxorubicin at a dose of 9 mg/kg were required to be sacrificed earlier than the corresponding microsphere treated animals due to extreme weight loss, lethargy and general cachexia. Histological examination of sections of tumor and normal hindlimb tissue taken at the time of sacrifice revealed no differences in cell autolysis, necrosis or fibrosis between sham, free drug or microsphere groups at any of the drug doses given.

Dose response

Control animals undergoing laparotomy and saline injection experienced a small transient decrease in body weight for 3–5 days followed by complete recovery by 10 days. Animals treated with sham microspheres or injected with low doxorubicin doses displayed a similar degree of weight loss. There was a dose-dependent decrease in body weight experienced by all animals following treatment with doxorubicin. Doses under 6 mg kg produced a drop in weight of less than 5% up to 5–6 days post-treatment after which the weights recovered to control levels. Doses above 6 mg kg decreased body weight from 10 to 20% for the 9 mg kg group before recovery after 5–8 days. There was no significant difference between the animals in the free drug or microsphere groups in the degree of weight loss except at 9 mg/kg where the loss was not reversed for the free drug group before their poor condition required euthanasia.

Injection of 0.4×10^6 sham microspheres produced a small but statistically significant ($p < 0.01$) delay in tumor growth relative to the control growth curve (Figure 1a) with the number of microspheres injected being equivalent to a doxorubicin microsphere dose of 9 mg/kg. Administration of a smaller dose of 0.3×10^6 microspheres resulted in a curve which was situated between the control curve and the high dose sham curve and was only different to the control curve at a $p < 0.2$ significance level. This suggests that a dose-response relationship may exist between the number of microspheres injected and the anti-tumor response evoked.

Treatment with doxorubicin produced a delay in tumor growth irrespective of the form of administration either as free drug or loaded onto microspheres (Figure 1). At a dose of 3 mg/kg, injection of both free drug and microspheres produced a significant ($p < 0.01$) decrease in tumor growth rate compared to the control growth curve but the response to free doxorubicin was significantly superior to that of the drug microsphere group. At all doses above 3 mg/kg there was no significant difference ($p < 0.05$) between the microsphere and free drug groups. The highest doses examined (7.5 and 9 mg/kg) demonstrated the most prolonged responses but in all cases the tumors eventually recurred. Treatment with doxorubicin microspheres at 9 mg/kg resulted in complete regression of tumor growth (tumors could not be palpated or measured) for 2–3 days in six of the 11 tumors in that group. Tumors in the free drug group decreased in size but were still present throughout the treatment period.

White blood cell counts of animals treated with sham microspheres did not significantly differ from those of the control group. No change in white cell count was seen on administration of 3.0 or 4.5 mg kg of doxorubicin in free drug or microsphere form but doses of 6.0 and 7.5 mg kg induced a significant decrease (46 and 35%, respectively, with $p < 0.05$) in circulating white blood cells in the free drug group, with the maximum response occurring at 9 mg kg ($p < 0.01$; Figure 2). In this case white cell count decreased by 54%. Doxorubicin microspheres produced no significant change in white cell numbers at any dose.

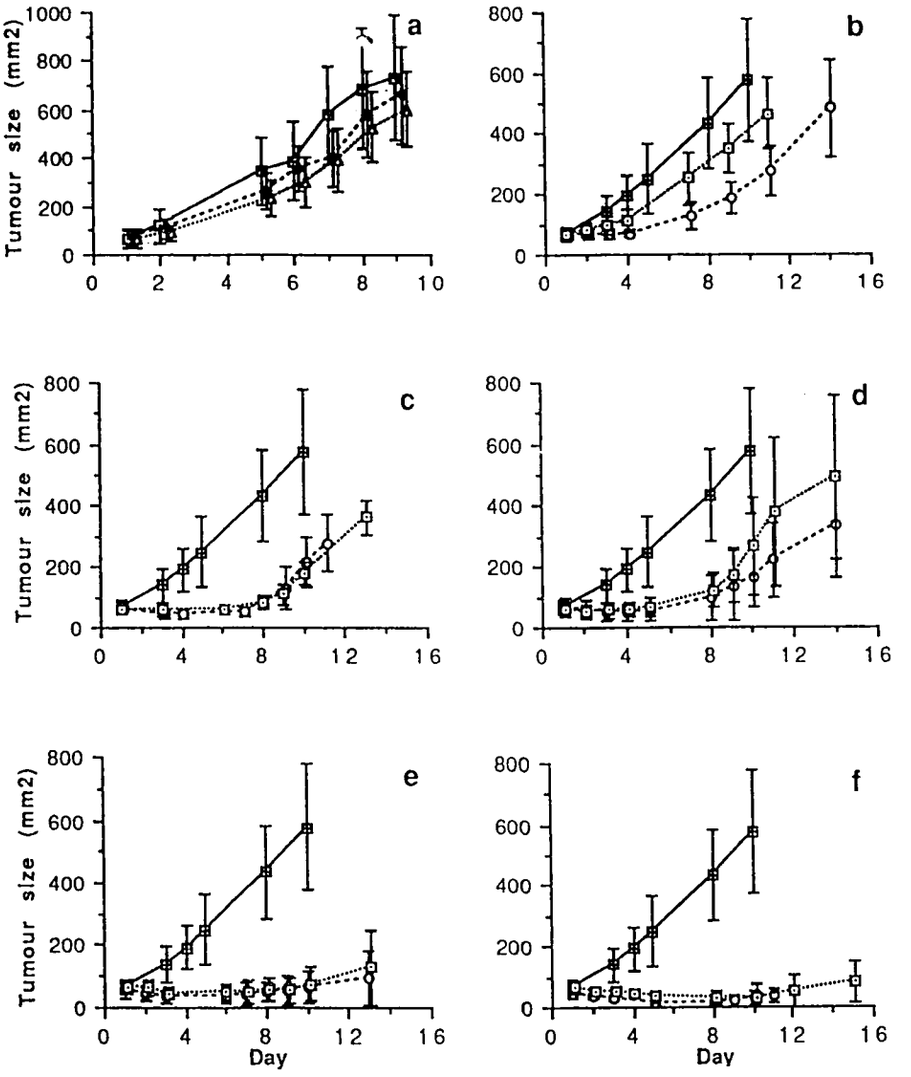

Figure 1. The effect of sham microspheres and five doses of doxorubicin injected intra-arterially on hindlimb tumor growth rate compared to controls. (a) Sham microspheres, 0.3 × 10^6 (--●--) and 0.4 × 10^6 (··△··); (b) 3.0 mg/kg; (c) 4.5 mg/kg; (d) 6.0 mg/kg; (e) 7.5 mg/kg; (f) 9.0 mg/kg. Control (-⊞-), doxorubicin incorporated in microspheres (··⊡··) and delivered as free drug (--⊙--).

Doxorubicin distribution

Similar levels of doxorubicin (0–0.2 μg/ml) were found in the plasma samples from free drug and microsphere treated animals at 3 and 48 h, with only trace amounts remaining in the blood by 96 h. There were consistently higher levels of drug measured in the normal hindlimb tissue of the microsphere treated group at 3, 48 and 96 (5.2, 2.75 and 3.2 μg/g) h compared to animals given free drug (3.87, 2.47 and 0.82 μg/g, respectively). Four times as much doxorubicin (3.2 μg/g) was still present in the drug microsphere group compared to free drug at 96 h (0.82 μg/g), indicating the sustained availability of doxorubicin.

Prolonged presence of the drug in the microsphere group was also demonstrated in tumor tissue. At 3 h, there was slightly more drug in the free drug group but this effect was reversed in the latter samples. Drug concentration in tumor tissue in the microsphere treated groups were 2 times that of the free drug group at 48 h (7.5 and 3.6 μg/g) and 30 times by 96 h (5.0 and 0.17 μg/g). Furthermore, in the microsphere groups there were high levels of drug in the normal tissue at 3 h, but at 48 and 96 h the levels were higher in the tumor

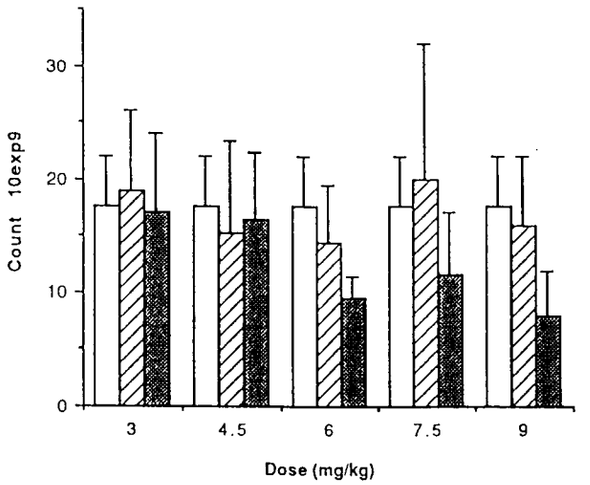

Figure 2. White cell count 13 or 14 days after treatment with five doses of doxorubicin injected as free drug (▦) or incorporated on microspheres (▨) compared with control value (□). Values presented are means with standard deviation bars.

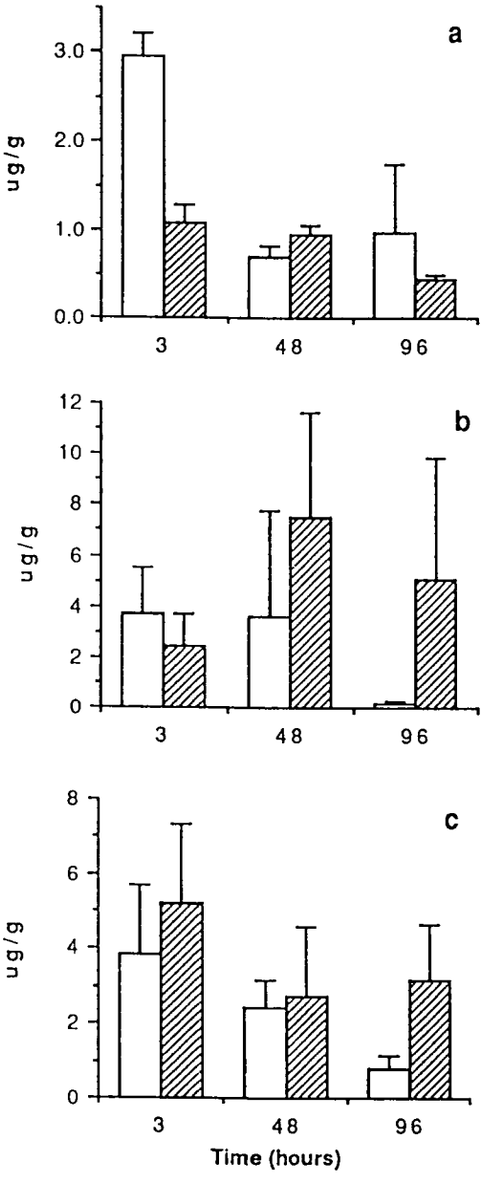

Figure 3. Distribution of doxorubicin to (a) heart, (b) tumor and (c) hindlimb tissue at 3, 48 and 96 h following administration of doxorubicin on microspheres (▨) or as free drug (□). Values presented are means with standard deviation bars.

than in normal tissue. This may indicate movement of the microspheres[10] from the normal tissue into the tumor after the 3 h time sample. The ratio of doxorubicin in tumor tissue compared with that of the surrounding normal hindlimb tissue was higher in microsphere treated animals compared to the free drug group at 48 h (2.74 and 1.46 μg/g, respectively) and at 96 h (1.58 and 0.21 μg/g, respectively).

Cardiac levels of doxorubicin (mean 1.08 μg/g) were 36% lower in the microsphere treated animals compared with the free drug group at the 3 h time sample. After 48 h the doxorubicin levels were similar for both treatment groups (Figure 3) but at 96 h there was again higher levels measured in the free drug group.

Doxorubicinone was found at low levels in tumor and hindlimb tissue in both groups, but could not be detected in plasma. At 3 h, however, 6 times as much of the metabolite was found in cardiac tissue taken from the free drug group (4.44 μg/g) compared with that present in the microsphere treated group (0.7 μg/g). As was seen with doxorubicin in the heart, doxorubicinone levels were similar for both groups at 48 h and higher again in the free drug group at 96 h (0.73 and 0.27 μg g for the microsphere group).

Discussion

Doxorubicin, an anthracycline antibiotic, exhibits a broad spectrum of anti-tumor activity against solid tumors, unfortunately its effectiveness is often limited by hematological and cardiac toxicity related to high doses. Microspheres have been proposed as a means of delivering high doses of drug to a tumor site

whilst hindering the distribution of drug into the systemic circulation in order to limit exposure of normal tissues to cytotoxic effects.[4]

Albumin microspheres have been suggested as a possible mechanism of sustained release, as incorporated drugs are slowly released by a combination of diffusion and matrix degradation[11] and also due to the non-toxic nature of the matrix when used *in vivo*. However, their therapeutic effectiveness in a clinical situation is minimal due to the low levels of drug loading which can be achieved. Ion-exchange microspheres, however, can contain up to 60% (w/w) doxorubicin in their matrix. The ability to target[12] these microspheres to tumor tissue via the arterial system provides a potential mechanism for preferential tumor destruction. They have also been shown to be non-toxic and without any significant immunological impact on human tissues.[13]

Previous studies using doxorubicin on multilamella non-ionic surfactant vesicles, labelled niosomes,[14] have demonstrated a prolonged circulation time of doxorubicin in plasma compared to free drug. Administration of niosomes, as opposed to free drug, resulted in higher levels of doxorubicin in tumor tissue which was reflected by an enhanced anti-tumor response. Liposome encapsulation of doxorubicin has also been shown to greatly reduce the drug's cardiac and acute toxicities without altering its anti-tumor efficacy.[2]

The present study demonstrated the anti-tumor activity of doxorubicin to be consistent whether administered as a free solution or loaded onto ion-exchange microspheres. A dose-dependent decrease in growth rate was demonstrated for both free drug and drug microspheres (Figure 1). However, at the lowest dose examined, delay in tumor growth rate was significantly greater in the free drug group than in the microsphere group (Figure 1b). This is probably due to slow and sustained release of drug from the microspheres. The low dose may have provided an insufficient concentration of drug to the tumor tissue at any one time to induce a maximal response. Administering the same dose as free drug provided a transient but higher concentration in the tumor thereby exerting more influence on its growth pattern.

Doses above 3 mg/kg produced similar anti-tumor responses for both treatments and doses of 7.5 and 9.0 mg/kg elicited the maximum response which was extended for the longest time period.

Microspheres containing no doxorubicin also demonstrated a small, transient influence on the tumor growth curve (Figure 1a). However, even when the number of microspheres administered corresponded to the highest drug dose, the magnitude of the response did not account for the magnitude of the changes in growth rate of the drug microsphere group. The tumor response of the sham microspheres commenced 3 days after treatment followed by rapid recovery of tumor growth by 7 days. This would have resulted from embolization of microspheres in the pre-capillary blood vessels of the tumor with subsequent compensatory development of collateral arterial blood flow.[10]

Despite the response being similar for both treatments there was a significant relationship between the presence of general drug toxicity and free drug administration which was absent in the microsphere treated animals as well as in the sham microsphere animals.

Decreases in white blood cell count associated with systemic exposure to doxorubicin were used as a measure of the relative toxicity. At lower drug doses (less than 6 mg/kg) there was no significant difference between the mean white cell counts of the treated animals and the untreated animals. However, at 6 mg/kg and above there was a significant reduction in white cell counts for the free drug groups alone. This indicates that, in relation to toxicity, there is only non-toxic levels of doxorubicin circulating to bone marrow when the drug is confined to the target by delivery on microspheres. Microspheres become entrapped in the capillaries of the tumor and subsequent sustained release of drug from the microsphere matrix results in more of the drug being taken up by tumor cells before it can enter the general circulation. This implies that higher doses of doxorubicin could be administered to the animal in the form of microspheres thereby enhancing relative anti-tumor response without a corresponding increase in bone marrow toxicity. This latter point was exemplified by the need to sacrifice the animals in the high free drug dose group.

Cardiac toxicity is the major limiting factor in the use of doxorubicin chemotherapy, so any means of reducing the level of drug available to the heart would be a valuable adjunct in the treatment of solid tumors. Microsphere delivery of doxorubicin demonstrated this potential. The level of doxorubicin found in cardiac tissues of animals treated with microspheres was 3 times lower than that seen in animals in the free drug group shortly after treatment.

This indicates that less drug is available to the cardiac tissue of microsphere treated animals, due to reduced systemic circulation of doxorubicin. Additionally, the 3 h time period for this sample would have post-dated the initial maximum drug concentration peak following free drug administration and thus understates the significance of the lower levels measured in the microsphere group. This is further supported by the high levels of the doxorubicin metabolite doxorubicinone, found at 3 h in the hearts of animals given free drug, implicating a prior difference in distribution of doxorubicin favouring the microsphere treated group.

We have previously published results on the therapeutic responses of doxorubicin microspheres in a rat model indicating significantly enhanced tumor growth retardation for microsphere treated animals in comparison to free drug animals.[5,12] In the present study this result has not been repeated. The discrepancy may be explained by the mode of measurement of doxorubicin concentration. The original studies utilized solely UV spectrophotometry for the assay of doxorubicin concentrations prior to injection. Such a method cannot differentiate between doxorubicin and its metabolites. It is conceivable that the microspheres and free drug solutions in the previous study contained some levels of the less active metabolite doxorubicinone which may have resulted in lower and unequal drug concentrations. It is not possible to estimate the degree of drug degradation in the previous study as HPLC data is unavailable. The present study has validated all concentrations from both free and microsphere treatments as consisting solely of natural doxorubicin by the use of the HPLC method. The problems related to drug degradation during the production of ion-exchange microspheres has recently been described.[4]

Conclusion

The administration of doxorubicin on ion-exchange resin in a rat tumor model resulted in an equivalent tumor growth response to doxorubicin in solution, but was less toxic than free drug in that it caused a significant reduction in bone marrow toxicity after treatment and may prevent long-term cardiac toxicity by confining the drug to a localized tumor area and inhibiting its systemic circulation.

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