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Increased Bone Marrow Toxicity of Doxorubicin Bound to Nanoparticles

S. Gibaud, J. P. Andreux, C. Weingarten, M. Renard and P. Couvreur

The *in vivo* myelosuppressive effects of free and polyalkylcyanoacrylate-bound doxorubicin were compared in a mouse model. After intravenous administration of 11 mg/kg body weight of doxorubicin either free or bound to polyisobutyl (doxo-PIBCA) or polyisohexylcyanoacrylate (doxo-PIHCA) nanoparticles, we studied the total and differential counts of blood, bone marrow and spleen cells; the number of granulocyte progenitors (CFU-GM) was determined by culture. Doxorubicin concentrations were measured with an HPLC method in the bone marrow and the spleen. Doxo-PIHCA nanoparticles showed the highest and longest myelosuppressive effects which correlated well with a high concentration of the drug in the bone marrow and the spleen. Moreover, it was found that PIHCA nanoparticles induced the release of colony stimulating factors, which might account for the observed increase of toxic effects of doxorubicin on bone marrow progenitors. These data also indicate that a more precise evaluation of the myelosuppressive effects of targeted formulations of anticancer drugs is needed, which may be attained by studies on bone marrow progenitors.

Key words: doxorubicin, drug carriers, cyanoacrylates, bone marrow, stem cells
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INTRODUCTION

A MAJOR CONCERN in cancer chemotherapy has been to develop drugs with greater tumour specificity: more effective against the tumour and less toxic towards normal tissues. This may be attained by using new drugs or by targeting known ones. Recently, the potential usefulness of polyalkylcyanoacrylate nanoparticles as anticancer drug carriers has been well demonstrated. These biodegradable colloidal systems have several advantages, especially modifying tissue distribution of bound drugs [1,2]. Indeed, a higher accumulation of drugs in the liver, spleen and lungs has been reported after intravenous administration of anticancer compounds loaded on to these

colloidal particles [2,3]. Binding of doxorubicin to nanoparticles has been found to increase the efficacy against experimental tumours, and especially hepatic murine metastasis, while cardiac toxicity has largely been decreased [4,5].

Thus far, the bulk of the research concerning the toxicity of these nanoparticles loaded with doxorubicin has tended to focus on specific organ toxicity (i.e. cardiac toxicity). However myelosuppression is often the main limiting factor of such anticancer drugs [6,7] and it is obviously dangerous not to consider that this secondary effect may be increased by targeting. Surprisingly, this problem was never investigated with nanoparticles. Therefore, the aim of the present study was to evaluate any modifications of doxorubicin haematopoietic toxicity when associated with poyalkylcyanoacrylate nanoparticles.

Special emphasis has been given to bone marrow granulocyte progenitors, as neutropenia remains the most important aspect of myelosuppression in terms of morbidity and mortality [6]. In rodents the spleen plays an important role in haematopoiesis and storage of blood cells and hence special attention has also been given to that organ.

MATERIALS AND METHODS

Chemicals

The two monomers used isobutylcyanoacrylate (IBCA) and isohexylcyanoacrylate (IHCA) were obtained from Sopar (Belgium) and doxorubicin was a gift from Farmitalia Carlo Erba. All other chemicals were analytical grade and pyrogen free.

Preparation of doxorubicin nanoparticles

Polyisobutyl- and polyisohexylcyanoacrylate nanoparticles were prepared by anionic polymerisation as described previously [8]. Briefly, 66.5 mg of the monomer were added with mechanical stirring to 6.5 ml of a sterile aqueous solution containing glucose 5%, dextran-70 1%, citric acid 0.5% and doxorubicin (0.77 mg/ml). The agitation was maintained for 5 h in the case of IBCA and for 20 h for IHCA. The polymerisation occurred at room temperature. The suspension of the nanoparticles obtained was dispensed into vials of 1.3 ml for lyophilisation in an Alpha 1-5 system (Ehviss), then stored at 4 °C. Resuspension of solid nanoparticles before injection was performed by addition of 1 ml of 0.1 M phosphate buffer pH 6.8 to each vial, containing 13.3 mg of polymer and 1 mg of doxorubicin. These loaded particles were named doxo-polyisobutyl (doxo-PIBCA) and doxo-polyisohexylcyanoacrylate (doxo-PIHCA). Drug unloaded nanoparticles were prepared in the same way and used as controls; they were named PIHCA and PIBCA.

Determination of nanoparticles size were carried out using a laser light-scattering method (N4MD, Coulter, U.S.A.). Examination of doxorubicin-loaded particles in serum under a fluorescence microscope did not show agglutinates.

The amount of doxorubicin associated with nanoparticles was estimated by HPLC, after ultra-centrifugation of the samples (30 000 rpm for 1 h). Analytical determinations were performed both in the supernatant (free drug) and in the sediment after dissolution in acetonitrile (bound drug). The quantity of bound drug was expressed as a percentage of the amount of drug initially dissolved in the polymerisation medium.

Analytical assays for doxorubicin determination and characterisation

The HPLC apparatus for doxorubicin assays consisted of a Waters 600 E multisolvent delivery system, with a WISP Waters 712 injector. The column used (1.6 × 30 cm) was packed with nucleosil C18 Bondasorb (SFCC-Shandon). The detector was a spectrofluorimeter (Waters M-470, 470–550 nm). The mobile phase was a mixture of methanol, sodium acetate 0.01 M and acetic acid (65/35/1.3, v/v), used at a flow rate of 1 ml/min. In these conditions, the retention time of doxorubicin was about 9 min.

Animal assays

Groups of five CD1 male mice weighing 25–30 g (Charles Rivers, France) were used. Doxorubicin was given as a single bolus injection into the caudal vein at a dose of 11 mg/kg (0.4 ml) either free or in nanoparticles (doxo-PIBCA or doxo-PIHCA). This dose was chosen because preliminary assays had shown that it was the highest dose which, in a single intravenous (i.v.) injection, induced clear effects on the bone marrow without being lethal. Nevertheless, injections were made slowly (taking about 2 min). During the whole experimental procedure, the animals remained in good physical condition without any weight loss. Control groups received either no treatment, unloaded nanoparticles or solvent alone (0.1 M phosphate buffer pH 6.8). Cytological studies of blood, bone marrow and spleen were performed 1, 2, 4, 7 and 11 days after injection. In another assay, cultures of granulo-monocytic progenitors (granulocyte–macrophage colony forming units, CFU-GM) were made at the same time intervals.

For analyses, the assumption was made that blood volume in ml was 7% of body weight in g, and that the content of a femur represented 6% of the total bone marrow.

Cytological studies

Heparinised blood samples were obtained from ether anaesthetised mice via cardiac puncture. Bone marrow was flushed from femur shafts with Iscove modified Dulbecco medium (IMDM Gibco) supplemented with 20% fetal calf serum. Suspensions of spleen cells were obtained by passage through a stainless steel mesh. All cell suspensions were homogenised by repeated aspiration through a needle. Nucleated cells of blood, bone marrow and spleen were counted in a haemocytometer; slides were prepared by cytocentrifugation, and differential counts performed on blood smears after staining with May Grunewald Giemsa. The morphology of at least 200 cells was analysed for each slide.

Red blood cells and platelets were counted in a haemocytometer.

CFU-GM assay

The methylcellulose culture technique of Iscove [9] was used with slight modifications. The final culture mixture contained 100 000 bone marrow nucleated cells /ml, 0.8% methylcellulose, 20% fetal calf serum (Gibco), 10^{-4}M alpha thioglycerol (Sigma), 100 U penicillin/ml and 100 μg streptomycin/ml in IMDM. The colony stimulating factor was serum of mice injected with lipopolysaccharide (LPS, *E. Coli* 055B5) and was added at optimal concentration (determined by preliminary assays) [10].

The culture mixture was incubated at 37°C in a humidified

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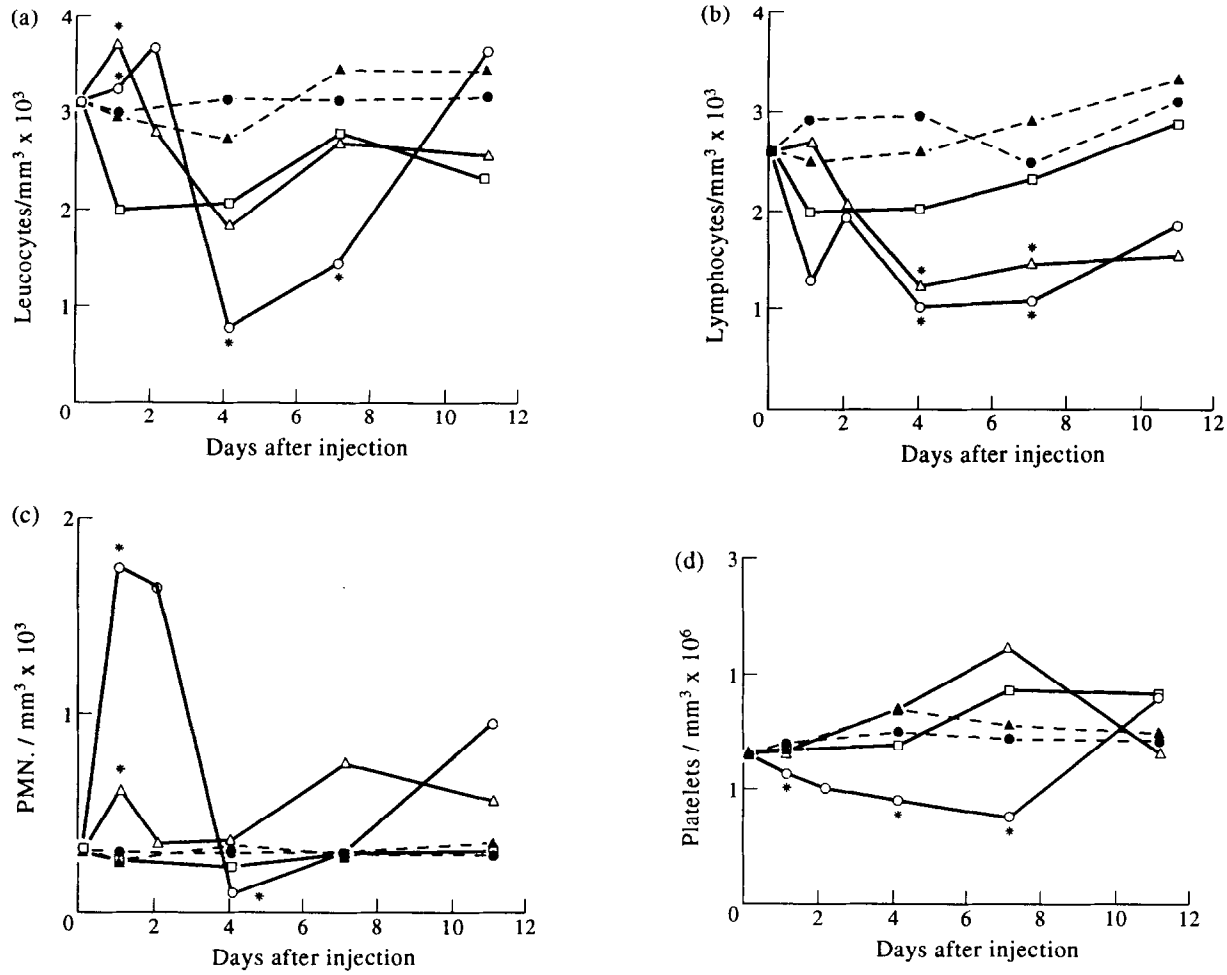

Figure 1. Changes of peripheral blood cells as a function of time following i.v. administration of 11 mg/kg free doxorubicin (—□—), doxorubicin-PIBCA (—△—) or doxorubicin-PIHCA (—○—). Control groups received unloaded nanoparticles at a polymer concentration of 146 mg/kg PIBCA (--▲--) or PIHCA (--●--). Each data point represents the average value obtained with blood samples from five mice. Each experiment was duplicated. (a) Total leucocytes per mm³, (b) lymphocytes per mm³, (c) polymorphonuclear cells (PMN) per mm³, (d) platelets per mm³. * Different from free doxorubicin-treated mice ($P < 0.05$).

5% CO_2 atmosphere. On day 8, CFU-GM-derived colonies containing at least 50 cells, were counted using an inverted microscope.

Determination of serum colony stimulating activity (CSA)

The widely used CFU-GM assay was modified to determine the CSA in serum of treated mice. Groups of five CD1 mice received a single i.v. injection either of free doxorubicin (11 mg/kg), doxo-PIHCA or unloaded PIHCA nanoparticles of isotonic buffer. Blood was collected 4 h after this injection (time corresponding to the peak of circulating CSA, as determined by preliminary assays). After coagulation, the sera of each group of five mice were pooled. This pool of serum was used at a final concentration of 2% as the stimulating factor in the CFU-GM assay. The CSA was expressed as the number of colonies obtained from 100 000 bone marrow cells.

Determinations of doxorubicin in the bone marrow and spleen

Six groups of three mice were injected i.v. with bound (doxo-PIBCA, doxo-PIHCA) or free doxorubicin, at a dose of 11 mg/kg, in a 0.4 ml suspension. At various time intervals (30 min, 4, 8, 24 h, 4 and 11 days), the bone marrow and spleens of the three animals were collected. Each time, six femurs were flushed with 1 ml of a mixture of acetonitrile/water (50/50 v/v), which dissolved the nanoparticles, allowing the complete release of doxorubicin from the polymer. The spleens were homogenised with a Potter homogeniser, and the doxorubicin extracted with 5 ml of the acetonitrile/water mixture. The extracts were immediately centrifuged at 1500 rpm for 5 min, and the doxorubicin content determined by HPLC as described previously.

Results were expressed as weight of total doxorubicin per femur or per spleen.

RESULTS

All experiments were performed at least twice. The Mann–Whitney test was used, at the level of $P < 0.05$, to compare assay groups. In fact, the number of animals per group was small and the results did not show a normal distribution, so using a non-parametric test was appropriate.

Characterisation of nanoparticles suspensions

The diameter of doxorubicin nanoparticles was 235 ± 36 nm for PIBCA and 240 ± 26 nm for PIHCA. The size of unloaded nanoparticles was not significantly different. These particles did not show any agglutination in plasma.

Of the doxorubicin initially dissolved in the polymerisation medium, 77% was firmly bound to PIBCA nanoparticles; with PIHCA, this percentage was 98%.

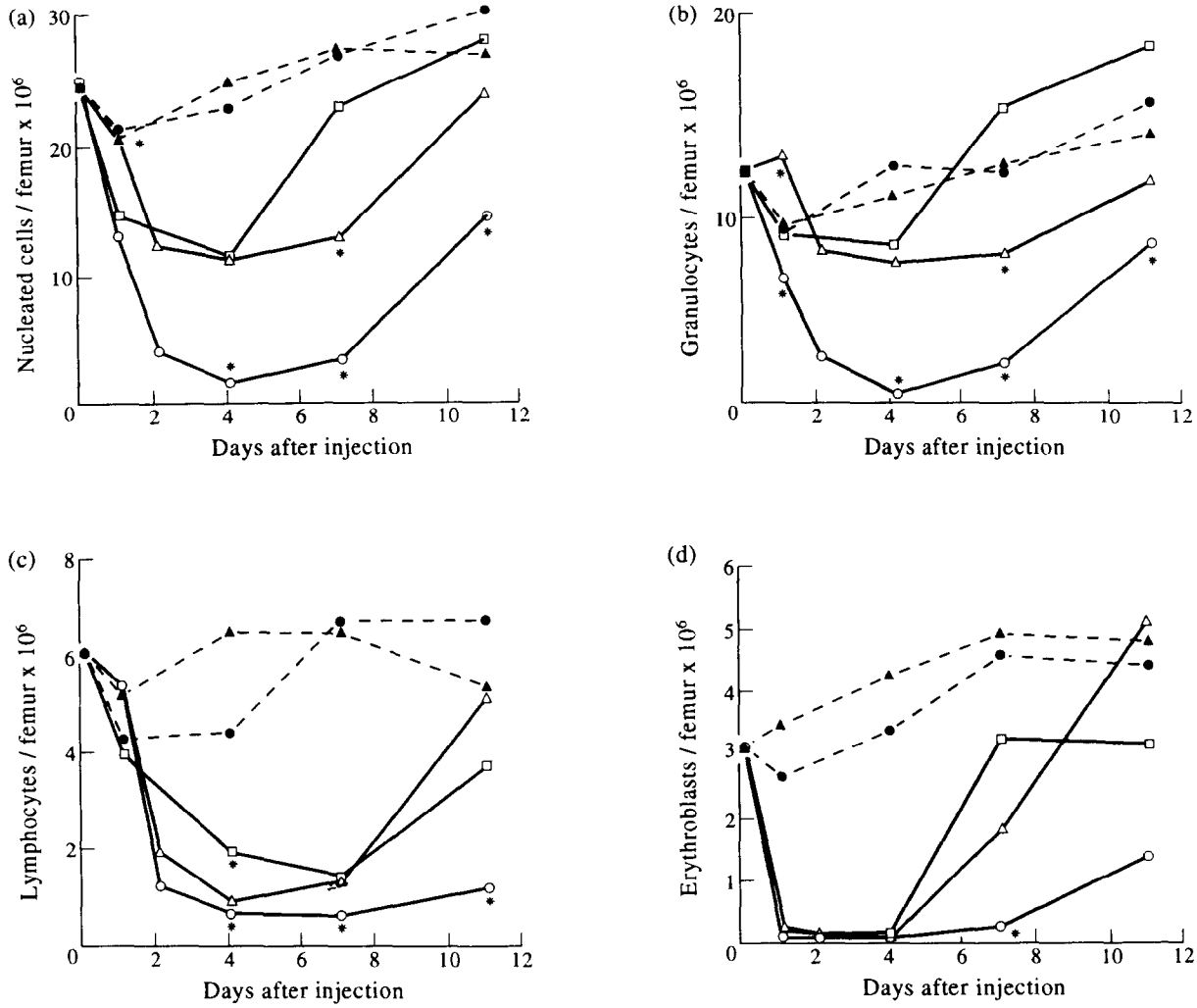

Figure 2. Changes in bone marrow cellularity as a function of time following i.v. administration of 11 mg/kg free doxorubicin (—□—), doxorubicin-PIBCA (—△—) or doxorubicin-PIHCA (—○—). Control groups received unloaded nanoparticles at a polymer concentration of 146 mg/kg PIBCA (--▲--) or PIHCA (--●--). Each data point represents the average value obtained with bone marrow flushed from femur shafts, from five mice. Each experiment was duplicated. (a) Total number of nucleated cells per femur, (b) granulocytes per femur, (c) lymphocytes per femur, (d) erythroblasts per femur. *Different from free doxorubicin-treated mice ($P < 0.05$).

Effect on peripheral blood cells

Comparing the effects of free and nanoparticle-bound doxorubicin, there was no significant variation in red blood cells (data not shown). Leucocyte numbers essentially comprised lymphocytes and polymorphonuclear cell (PMN) counts (Figure 1a). The lymphocytes, which account for more than 80% of mouse white blood cells, began to decrease 1 or 2 days after injection. This decrease was greater and more persistent for nanoparticle-bound doxorubicin than for the free drug (Figure 1b). For PMN an unexpected peak appeared at day 1 and 2 after injection with doxo-PIHCA which was also noted with doxo-PIBCA but to a lesser extent (Figure 1c). A decrease was seen only at day 4 with doxo-PIHCA. Platelets decreased only on days 1, 4 and 7 after doxo-PIHCA injection and on day 11 with doxo-PIBCA (Figure 1d).

Effect on femur bone marrow cells

In all doxorubicin-treated mice, femoral cellularity decreased between 1 and 2 days after injection (Figure 2a). The extent of the reduction was similar with both the free and the PIBCA-bound drug, but the return towards starting values was slower for doxo-PIBCA in comparison to the free drug (day 11 instead of day 7). Doxo-PIHCA induced a greater cytopenia, with values remaining lower than the starting values even at day 11. The granulocytes (60% of nucleated bone marrow cells in mice) showed the same variations (Figure 2b). For lymphocytes, doxorubicin-loaded nanoparticles (doxo-PIBCA and doxo-PIHCA) were more toxic than the free drug (Figure 2c). Erythroblasts showed a high sensitivity to all doxorubicin treatments, but a slower recovery was noticed for doxo-PIHCA, for which low values were still observed at day 11 (Figure 2d).

Effect on spleen cells

Doxorubicin generally reduced the number of spleen cells (Figure 3a). Lymphocytes (more than 80% of spleen cells) showed a steep and quick decrease, which was more pronounced for doxo-PIHCA (Figure 3b). With free doxorubicin, granulocytes decreased slightly at day 1 but increased strongly at day 11 (Figure 3c). With bound drug, especially with doxo-PIHCA, a peak appeared at day 1, preceding a dramatic decrease of the granulocyte counts (Figure 3c), although recovery occurred by day11. As in bone marrow, erythroblasts fell quickly to very low levels and recovered later with free doxorubicin and doxo-

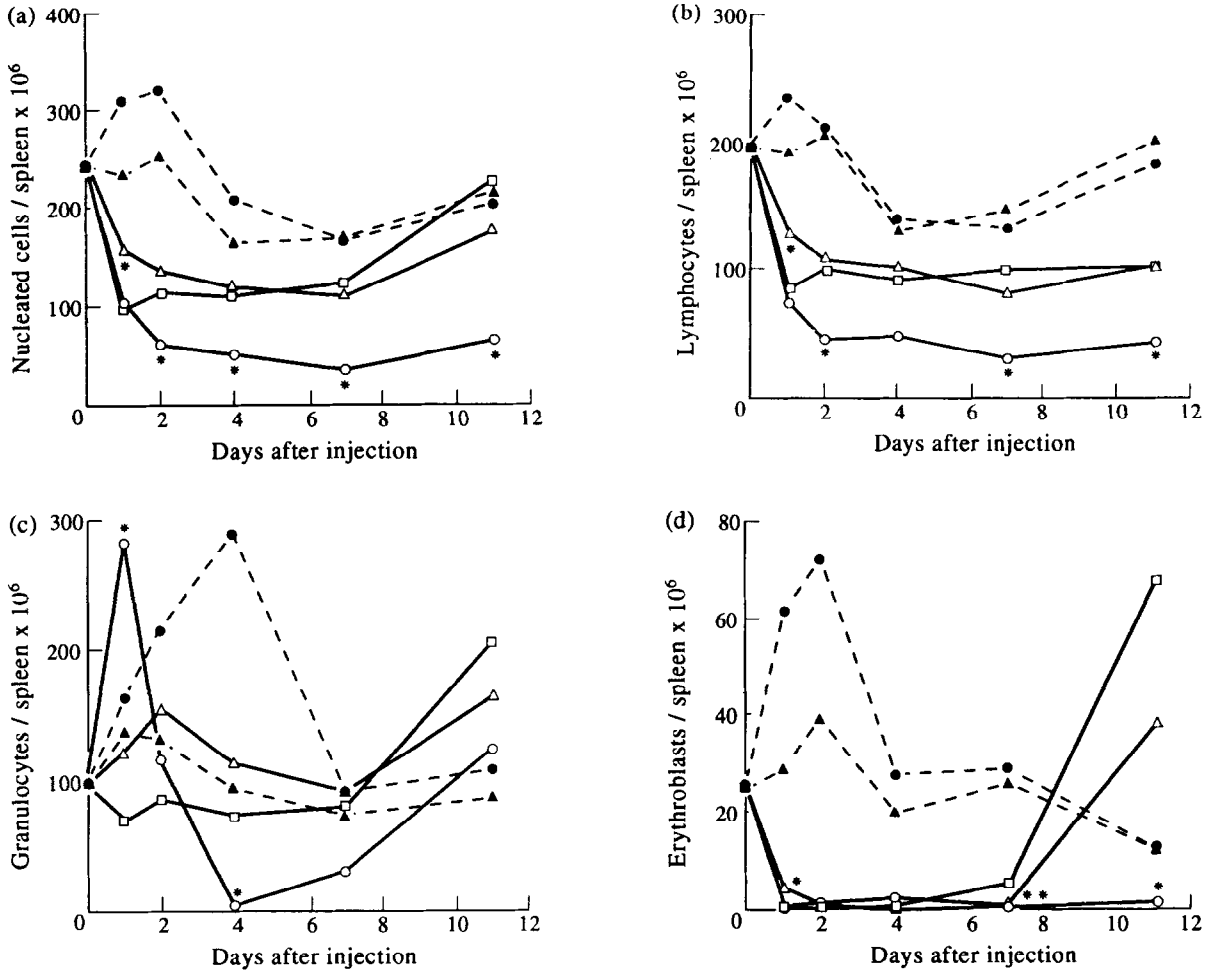

Figure 3. Changes in spleen cells as a function of time following i.v. administration of 11 mg/kg free doxorubicin (—□—), doxorubicin-PIBCA (—△—) or doxorubicin-PIHCA (—○—). Control groups received unloaded nanoparticles at a polymer concentration of 146 mg/kg PIBCA (--▲--) or PIHCA (--●--). Each data point represents the average value obtained with spleen from five mice. Each experiment was duplicated. (a) Total number of nucleated cells per spleen, (b) lymphocytes per spleen, (c) granulocytes per spleen, (d) erythroblasts per spleen. * Different from free doxorubicin-treated mice ($P < 0.05$).

PIBCA but not at all with doxo-PIHCA during the assay (Figure 3d).

It should be noted that unloaded nanoparticles, especially the PIHCA, induced an increase in granulocytes and erythroblasts, while for the lymphocytes, the short increase observed was followed by a significant decrease between days 4 and 7.

Effect on bone marrow granulocytes progenitors (CFU-GM)

CFU-GM decreased sharply as early as day 1 after administration of doxorubicin but recovery occurred at day 4 for free doxorubicin and doxo-PIBCA (Figure 4). Doxo-PIHCA induced a more severe decrease lasting 6 days.

CSA released in the circulating blood

Four hours after injection of doxo-PIHCA nanoparticles, a high CSA was observed in the serum of treated animals, corresponding to 99 ± 12 colonies. Neither free doxorubicin (4 ± 5 colonies) nor unloaded PIHCA nanoparticles (9 ± 3 colonies) produced such CSA, giving a number of colonies not significantly different from that which was produced after isotonic buffer injections (7 ± 3 colonies) (figure 5).

Bone marrow and spleen pharmacokinetics

The bone marrow concentrations of doxorubicin were higher for nanoparticles than for the free drug as early as 1 day after

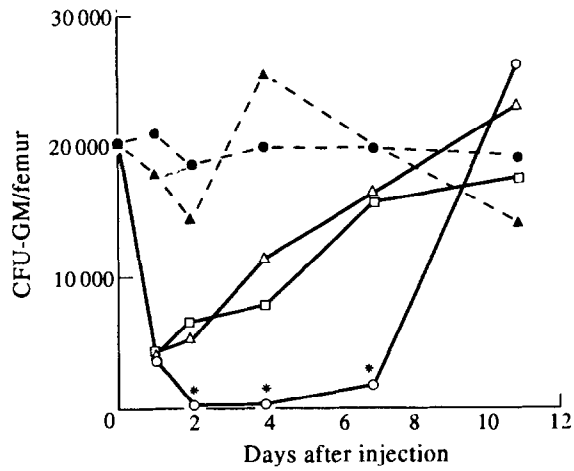

Figure 4. Changes in bone marrow granulo-monocytic progenitors as a function of time following i.v. administration of 11 mg/kg free doxorubicin (—□—), doxorubicin-PIBCA (—△—) or doxorubicin-PIHCA (—○—). Control groups received unloaded nanoparticles at a polymer concentration of 146 mg/kg PIBCA (--▲--) or PIHCA (--●--). Each data point represents the average CFU-GM value obtained with cultures of bone marrow cells of five mice. * Different from free doxorubicin-treated mice ($P < 0.05$).

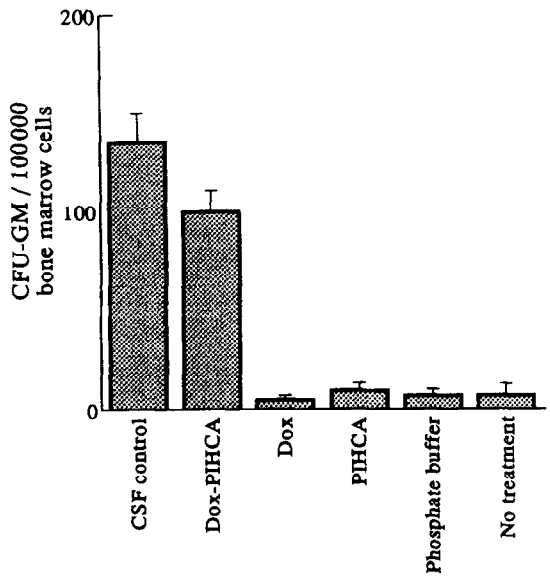

Figure 5. Serum colony-stimulating activity 4 h after i.v. administration of 11 mg/kg free doxorubicin, doxorubicin-PIHCA, PIHCA or phosphate buffer. Each data represents the number of CFU-GM obtained with 2% of the pooled plasma of five mice.

injection (Figure 6a); these concentrations were twice as high for doxo-PIBCA and 10-fold higher for doxo-PIHCA. Moreover, in this later case, the concentration remained high until day 11.

Spleen concentrations showed similar variations, with a very high and early peak for doxo-PIHCA, approximately 50-fold higher than the concentration with free doxorubicin 8 h after the injection (Figure 6b).

DISCUSSION

This study clearly shows that doxorubicin bound to polyalkylcyanoacrylate nanoparticles had higher myelosuppressive effects than the free drug. This was more evident with doxo-PIHCA nanoparticles, which gave the highest doxorubicin bone marrow concentrations, suggesting a direct targeting to that organ, as was previously shown by Grislain and colleagues [11], with ^{14}C radiolabelled nanoparticles. Nevertheless, the reason for the huge differences in bone marrow concentration and persistence of doxorubicin with doxo-PIHCA compared with doxo-PIBCA is unknown but different hypotheses can be put forward.

It could be due to the polymer biodegradation velocity which depends on the alkyl chain length of the polymer, as proposed by other investigators [12]: the longer the alkyl chain, the slower the biodegradation process. A quicker bioerosion of PIBCA nanoparticles in plasma may explain a lower bone marrow uptake than for PIHCA nanoparticles, and after absorption into the bone marrow, a slower release of the drug from PIHCA nanoparticles could induce a more prolonged effect.

Alternatively, the difference in retention of doxorubicin, in the spleen and the bone marrow, between PIBCA and PIHCA may be due to specific opsonins interacting differently [13,14]. Moreover, the presence of "dysopsonins", having a higher affinity for PIHCA nanoparticles, could also decrease their uptake by Kupffer cells, thus increasing their direct capture by bone marrow. Such "dysopsonins" were indeed described as being serum components which discourage the uptake of particulate carriers by the reticuloendothelial system [7,14,15]. As proposed by Moghimi and Patel, the process behind the selective opsonins' recognition depends on the surface characteristics of the carrier [14]. In our study, the nature of the polymer used (PIBCA or PIHCA) could, thus, influence the opsonisation process. Nevertheless, Muller and colleagues [15], comparing isobutyl and isohexylcyanoacrylate nanoparticles, found no significant differences in charge, zeta potential, interaction with charged serum components or surface hydrophobicity.

Another original point of the present study was related to the size of the carriers used. As previously shown by Illum and Davis (with polystyrene particles coated with poloxamer 338 or 407) [16], the size of the particles greatly influenced their uptake across the marrow sinusoids: only the smallest particles (less than 100 nm) were able to concentrate in the bone marow [7]. Surprisingly, we observed that the large PIHCA nanoparticles (240 nm) significantly increased the medullar concentration of doxorubicin. Decreasing the size of these particles could further improve their uptake, if the level of drug binding in such small sized carriers remained sufficient.

In addition to the results on doxorubicin concentration in the spleen and bone marrow, this study also showed that bone

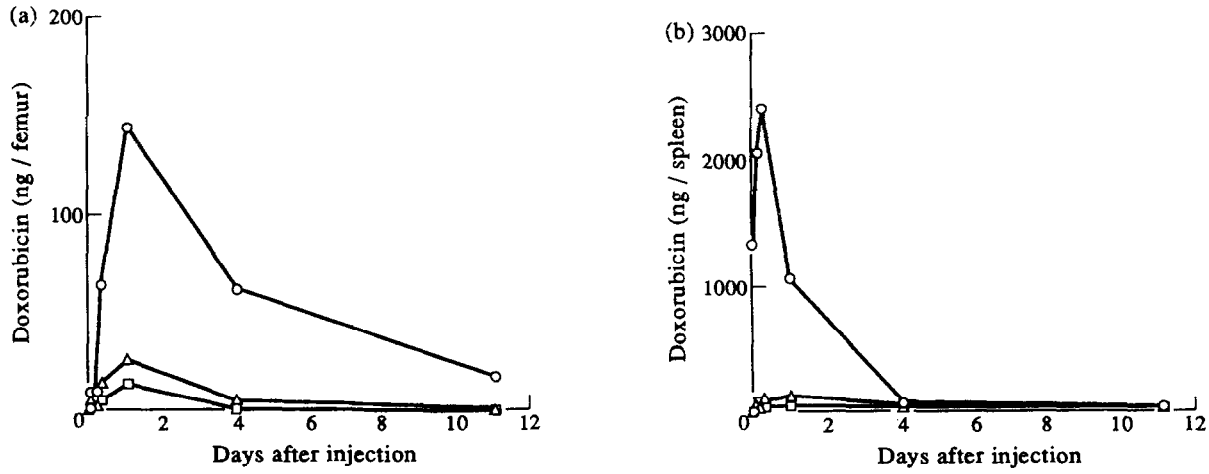

Figure 6. Changes of concentration of doxorubicin as a function of time following i.v. administration of 11 mg/kg free doxorubicin (—□—), doxorubicin-PIBCA (—△—) or doxorubicin-PIHCA (—○—). Each data point represents the average of concentrations values of total doxorubicin extracted from the organs of three mice. (a) Bone marrow, (b) spleen.

marrow granulocytes decreased following doxorubicin injection, but peripheral blood polymorphonuclear cells did not. In fact, doxo-PIHCA gave a clear increase in blood polymorphonuclear cell counts 1 or 2 days after injection. This may be discussed in the light of the fact that these doxo-PIHCA particles gave high and prolonged concentrations of doxorubicin in bone marrow and spleen, probably because of a high trapping rate in the macrophages of those tissues. Therefore, it could be postulated that macrophages and lymphocytes of these tissues released certain stimulating factors able to increase the bone marrow output of granulocytes. Using carboplatin liposomes, Fichtner and colleagues also observed such a blood increase and proposed a similar explanation [17]. In fact, we tested the sera of treated mice for the ability to stimulate cultures of granulocyte progenitors (CSA), and showed a high activity for doxo-PIHCA treated mice. The CSA release observed might increase the myelotoxicity of doxo-PIHCA, as it is known that colony stimulating factors induce divisions of bone marrow progenitors exposing these cells to the toxic effects of doxorubicin. Neither the free drug nor unloaded nanoparticles were able to induce such a release in these conditions.

Other surprising effects were noted in the spleen after injection of unloaded nanoparticles, especially PIHCA ones: a brief increase of spleen lymphocytes was quickly followed by a decrease and an increase of granulocytes and erythroblasts. Stimulated or injured lymphocytes might have released cytokines able to induce locally the observed stimulations of spleen granulopoiesis and erythropoiesis. Such effects, never previously described with unloaded nanoparticles, are now under study. Alternatively, part of the lymphocyte decrease observed between days 4 and 7 after injection of doxorubicin loaded nanoparticles may also be related to an effect of the polymer or its degradation products.

From the methods used in this study to assess myelosuppressive effects of new forms of anticancer drugs, it is obvious that bone marrow studies are more sensitive than single blood cell counts [18]. This is due to the fact that anticancer pharmaceuticals kill bone marrow dividing cells and have less effects on the non-dividing blood cells. Bone marrow cells, which produce blood cells, in constant renewal, while circulating red blood cells, for example, have long life spans and do not divide. This may explain why a steep but relatively short decrease of bone marrow erythroblasts produced no significant variation in red blood cells. Cultures of bone marrow granulocyte progenitors (CFU-GM, Figure 4) enabled us to show quick and clear decreases which correlated well with doxorubicin bone marrow concentrations. Moreover, the cells studied were the stem cells of bone marrow and blood granulocytes. Therefore, this new and more accurate method could give more meaningful information than that available from rather crude estimates based on peripheral white blood cell counts and differentials.

In conclusion, this study showed that, depending on the nature of the polymer used, nanoparticles may modify the profile of doxorubicin haematopoietic toxicity. This was clearly correlated with an increased concentration of doxorubicin in the bone marrow when associated with PIHCA nanoparticles. In the future, the affinity of these particles for haematopoietic organs could be valuable for the targeting of certain stimulating factors to those tissues.

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