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Myocardial toxicity of high-dose cyclophosphamide in rabbits treated with daunorubicin*

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Summary. The present study was performed to evaluate experimentally the possible cardiotoxicity of high doses of cyclophosphamide after pretreatment with anthracyclines, a regimen used prior to bone marrow transplantation. A total of 27 rabbits received daunorubicin at a dose of 2.25 mg/kg per week for 10 weeks. At 1 week after the last daunorubicin dose, 13 of these rabbits received cyclophosphamide at 100 mg/kg per day ×2 (total dose, 200 mg/kg). All animals were killed after 1 additional week. Seven rabbits received cyclophosphamide at 100 mg/kg per day ×2 and two animals were given 50 mg/kg per day ×2 without additional treatment. In all, 18 untreated rabbits served as controls. At 3 h before the animals were killed, they received [^{99m}Tc]-pyrophosphate i.v. Myocardial isotope activity was determined using a detector, and cardiac specimens were examined with a gamma-camera. Cardiotoxic effects were evaluated by myocardial isotope accumulation and pathologic changes were determined by morphology and by light and electron microscopy. The pathologic evaluation showed more frequent and widespread acute myocyte necrosis in daunorubicin/cyclophosphamide-treated rabbits as compared with those treated with daunorubicin or cyclophosphamide only. Myocardial isotope accumulation in rabbits treated with daunorubicin/cyclophosphamide was significantly higher then that in animals treated with either drug alone ($2\alpha \leq 0.001$). Rabbits receiving cyclophosphamide as a single agent showed minor myocyte lesions but did not differ from controls in terms of isotope accumulation. We conclude that high-dose cyclophosphamide treatment on a dose schedule similar to that used prior to bone marrow transplantation and given soon after long-term daunorubicin therapy is considerably cardiotoxic.

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Introduction

In acute leukaemia the anthracyclines doxorubicin or daunorubicin are used in most treatment regimes. The most important side effect of these agents is chronic cumulative toxic cardiomyopathy [5, 13, 14, 17, 19]. Experimentally, the myocardial toxicity of high-dose cyclophosphamide has been reported in dogs [4], monkeys [24] and rats [15]. Concurrent cyclophosphamide therapy has been suggested to potentiate doxorubicin-induced cardiotoxicity [6, 12, 19]. Most patients with acute leukaemia who undergo bone marrow transplantation (BMT) have previously received significant doses of anthracyclines. The cytoreduction therapy given before BMT includes high-dose cyclophosphamide and total body irradiation so as to eliminate the leukaemic cells and induce immunosuppression [25].

A number of case reports have described cardiotoxic effects occurring after BMT [2, 3, 18]. Mild to moderate cardiac effects associated with echocardiographic changes have been reported in children [23]. Although the incidence of acute lethal cardiotoxic complication is probably low, the improved long-term survival of patients after BMT makes it important that the risk be evaluated for organ damage that can subsequently become manifest. The aim of the present investigation was to study experimentally the acute cardiotoxic effect of high-dose cyclophosphamide therapy given after long-term treatment with daunorubicin.

Materials and methods

A total of 54 New Zealand White rabbits of both sexes weighing 2.1–4.3 kg were used in the present study. In all, 27 animals received 2.25 mg/kg i.v. daunorubicin once weekly for 10 weeks, and 13 of these rabbits received 100 mg/kg i.v. cyclophosphamide daily (total dose, 200 mg/kg) on days 6 and 7 after the last dose of daunorubicin. The other 14 daunorubicin-treated rabbits received no additional treatment. Seven animals were given 100 mg/kg i.v. cyclophosphamide daily ×2 (total dose, 200 mg/kg) and two rabbits received 50 mg/kg daily ×2 (total dose 100 mg/kg) without additional treatment. Another 18 untreated rabbits served as controls (Table 1).

Table 1. Drug schedules and number of rabbits treated with daunorubicin and cyclophosphamide (*D+Cy*), daunorubicin (*D*) and cyclophosphamide (*Cy*)

Treatment	Total number	Dose	Duration	Total dose
D+Cy	13	*D:* 2.25 mg/kg/week	10 weeks	22.5 mg/kg
		Cy: 100 mg/kg/day	2 days	200 mg/kg
D	14	2.25 mg/kg/week	10 weeks	22.5 mg/kg
Cy	7	100 mg/kg/day	2 days	200 mg/kg
	2	50 mg/kg/day	2 days	100 mg/kg
Controls	18			

Six rabbits died spontaneously at different points during the treatment period and were not examined using the isotope technique: three had been treated with daunorubicin and cyclophosphamide, one died during daunorubicin therapy, one had received cyclophosphamide and one was a control animal. At 14 days after the completion of daunorubicin treatment and/or 7 days after the last cyclophosphamide injection, the rabbits were anaesthetized (0.8 ml Hypnorm i.m.) and then rapidly killed by i.v. injection of Mebumal-natrium; 3 h prior to anaesthesia and euthanasia, 1 ml [^{99m}Tc]-pyrophosphate (100–130 MBq) was injected i.v.

A complete necropsy was performed immediately after euthanasia. Blood samples (2 ml) for isotope activity determinations were initially collected from the inferior vena cava and then the hearts were removed, the ventricular walls were incised along the anterior septum and the parietal pericardium and the blood remaining in the cavities were removed. Myocardial specimens were taken from the lateral left ventricular wall for electron microscopy. The rest of the hearts were weighed and saved for isotope evaluation.

Isotope activity evaluation. The isotope activity (technetium Tc 99m) in the entire hearts (obtained after the necropsy procedure) and the blood samples (2 ml) were measured in a 51-mm diameter NaI (Tl) detector (Canberra 1790C). The blood samples and the hearts were kept in small plastic cases. For correction of the activities due to blood in the coronary vascular bed, myocardial isotope accumulation was calculated as the ratio between the isotope activity in the myocardium (counts/g) and that in the blood (counts/ml).

The material was collected in 10 series of experiments. Each series involved 2–10 rabbits that were handled concurrently, including 1 or 2 control animals. The rabbits within each series were injected with equal doses of [^{99m}Tc]-pyrophosphate from the same preparation; however, the injected activity differed between experimental series (range, 100–130 MBq). Labelling efficiency, i.e. the fraction of [^{99m}Tc]-pyrophosphate complex in the prepared solutions in relation to the remaining free [^{99m}Tc]-O_4 and hydrolysed-reduced ^{99m}Tc, was not evaluated. For comparison of different series of experiments according to isotope accumulation in treated rabbits, the activity ratios in controls were used as references. Thus, the above-defined ratios calculated for treated rabbits within a given series were normalized to the mean ratio of non-treated controls in the same series (reference level of activity). Normalized activity ratios were calculated for all series of experiments.

Gamma-camera examination. The hearts from rabbits that had been euthanized on the same occasion were simultaneously examined with a gamma-camera in the left anterior oblique view. In gamma-camera images, a visual gradation of isotope uptake and distribution was done (Table 2). Data acquisition and computer-aided quantitative analysis were not performed. Gamma-camera examination was not carried out in heart preparations from four rabbits that had received cyclophosphamide alone.

Pathologic evaluation. At necropsy, representative tissue samples of all major organs were fixed in buffered neutral formalin for histopathologic examination. After gamma-camera examination, the hearts were cut into longitudinal slices of ventricular walls and septum. The slices were made parallel to the heart axis and perpendicular to the walls. Myocardial slices fixed in buffered neutral formalin were embedded in paraffin, sectioned and stained with Masson's trichrome or haematoxylin and eosin.

Table 2. Visual gradation of myocardial isotope uptake in gamma-camera images of cardiac specimens from rabbits injected with [^{99m}Tc]-pyrophosphate

Grade	isotope uptake
0	Normal
+	Slightly increased
++	Moderately increased and/or highly increased in focal parts
+++	Highly increased

Table 3. Qualitative and quantitative scoring at histopathologic evaluation

Qualitative score

- 0 – Normal
- 1 – Degeneration only
- 2 – Myocytic necrosis accompanied with a sparse inflammatory reaction
- 3 – Atrophy and degeneration of myofibers and development of connective tissue

Quantitative score

- 0 – Normal
- 1 – Involvement of single scattered myocytes at one or more levels
- 2 – Involvement of focal groups of myocytes at one level
- 3 – Involvement of focal groups of myocytes at 2 or more levels
- 4 – Confluent areas of involvement at one or more levels

Total score = qualitative score + quantitative score (maximum 7)

Qualitative and quantitative scoring of the myocardial lesions was conducted on sections of the longitudinally sliced ventricular myocardium using the criteria of Jaenke [16], with modifications (Table 3). For electron microscopy, small pieces of myocardium from each chamber were collected immediately and then fixed overnight in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer. After postfixation in phosphate-buffered 1% OsO_4 at 4°C for 1 h, the tissues were dehydrated in ethanol, passed through propylene oxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate followed by lead citrate [22] and then examined in a Philips 201 electron microscope. The results of pathologic evaluation of cardiac preparations from animals that died spontaneously are not presented. For

Table 4. Results of histopathologic scoring, myocardial isotope accumulation and gamma-camera imaging in rabbits treated with daunorubicin and cyclophosphamide (*D+Cy*), daunorubicin (*D*), and cyclophosphamide (*Cy*) and in control rabbits (*Cont*). Normalized activity ratios are used for treated animals and the reference level of activity for controls

Treatment	Number	Histopathology Total score			Isotope accumulation Normalized ratio			Gammacamera imaging Number			
		Mean	Median	Range	Mean	Median	Range	+++	++	+	0
D+Cy	10	6.2	6	5–7	3.1	3.4	1.4–5.2	2	5	3	–
D	13	4.5	5	2–6	1.3	1.3	0.9–1.7	–	–	8	5
Cy	8	2.9	3	2–3	1.1	1.1	0.7–1.3	–	–	1	3*
Cont	17	0	0	0	1.0	Rev. level 1.0	1.0	–	–	–	17

* 4 animals not gamma-camera examines

the statistical analysis of isotope accumulation and histopathologic scores, the two-tailed, two-sample Wilcoxon test was used.

Results

Isotope activity evaluation

In 17 control, the mean activity ratio was 0.2 (range, 0.12–0.29; Fig. 1). In 7/10 different series of experiments there were 2 controls in each series. The activity ratios of the controls within each series were close, the mean difference being 0.017 (median, 0.017; range, 0.004–0.04).

Rabbits treated with daunorubicin and cyclophosphamide showed myocardial isotope accumulation expressed as normalized ratios that was significantly higher than that detected in animals treated with either of the separate drugs alone ($2\alpha \leq 0.001$ and $2\alpha \leq 0.001$, respectively; Table 4, Figs. 1, 2). In the daunorubicin/cyclo-

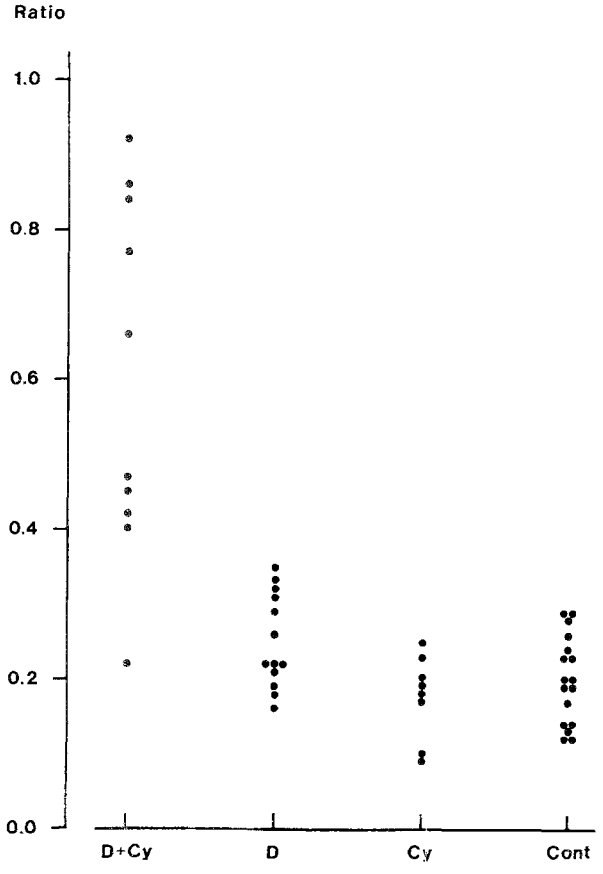


Fig. 1. Myocardial isotope accumulation from 10 series of experiments in rabbits treated with daunorubicin and cyclophosphamide (*D+Cy*), daunorubicin (*D*) or cyclophosphamide (*Cy*) or handled as controls (*Cont*). The isotope accumulation is expressed as the ratio between the isotope activity in myocardium (counts/g) and that in blood (counts/ml)

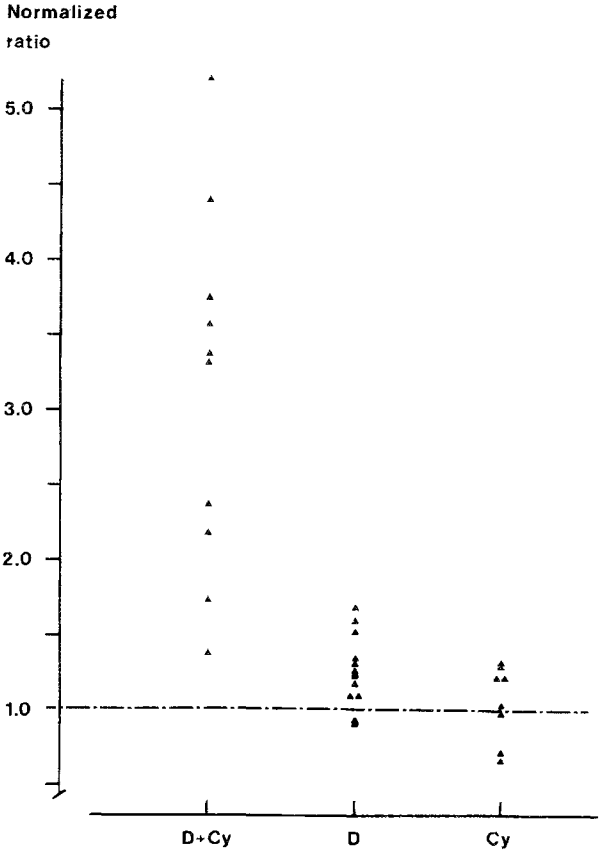


Fig. 2. Myocardial isotope accumulation from 10 series of experiments in rabbits treated with daunorubicin and cyclophosphamide (*D+Cy*), daunorubicin (*D*) or cyclophosphamide (*Cy*). The isotope accumulation is expressed as the ratio between the isotope activity in myocardium and blood, normalized to the mean ratio of controls in the same series of experiments. The *broken line* represents the reference level of activity (level in controls)

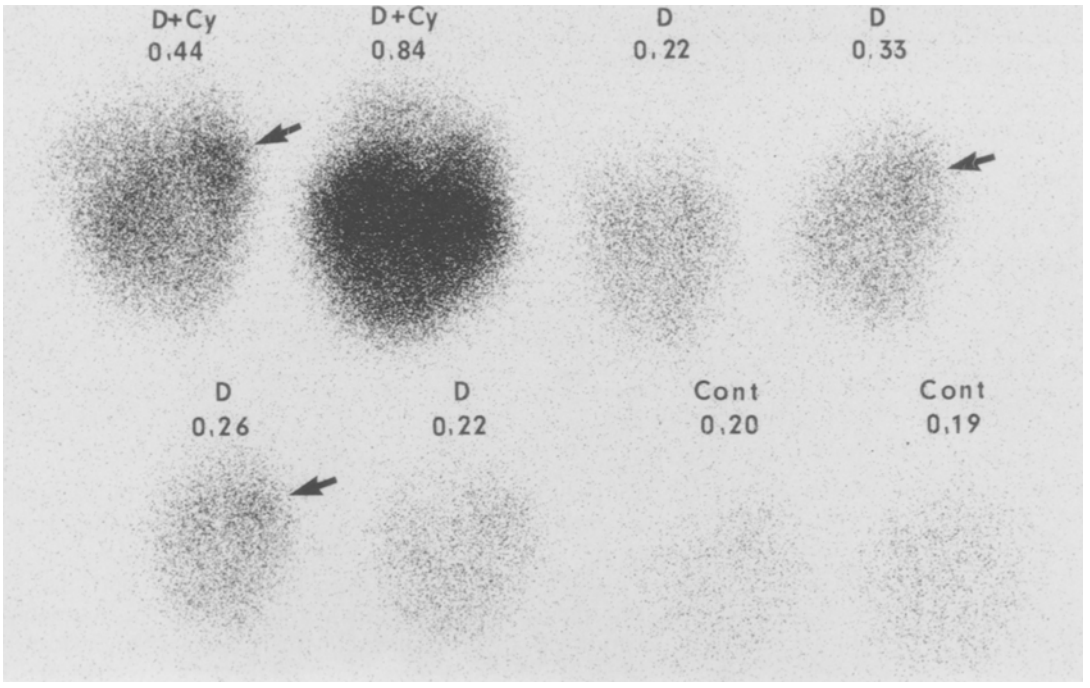


Fig. 3. Gamma-camera image in a left anterior oblique view of cardiac specimens from one of the series of experiments. Corresponding activity ratios are presented. The rabbits were treated with daunorubicin and cyclophosphamide (*D+Cy*) or daunorubicin (*D*) or were handled as controls (*Cont*). Focally increased uptake is indicated in the base of the left ventricle (*arrows*)

phosphamide-treated group the normalized activity ratios varied considerably (Fig. 2). However, in the separate series of experiments, without exception the daunorubicin/cyclophosphamide-treated rabbits had higher activity ratios than did animals in the same series that received daunorubicin only. Most of the daunorubicin-treated rabbits showed slightly elevated myocardial activity ratios as compared with controls ($2\alpha \leq 0.1$). Cardiac specimens from animals receiving cyclophosphamide alone showed activity ratios similar to those in controls; the difference was not significant (NS, $2\alpha \leq 0.1$).

Gamma-camera examination

None of the heart specimens from control animals showed increased isotope uptake. The visual gradation of the gamma-camera images corresponded to the observed differences in normalized activity ratios (Tables 4, 5; Fig. 3). All daunorubicin/cyclophosphamide-treated rabbits exhibited increased myocardial isotope uptake, whereas in 5/13 of the daunorubicin-treated animals the gamma-camera images showed the same isotope intensity and distribution displayed by controls (Table 5). One of the rabbits receiving only cyclophosphamide exhibited slightly increased isotope uptake; the others examined showed uptake and distribution equal to that in controls. In 10/17 hearts from all treatment groups displaying slightly or moderately increased isotope activity, the accumulation showed irregular distribution, with more increased uptake occurring in focal parts, mainly in the base of the left ventricle (Table 5, Fig. 3).

Table 5. Isotope uptake and distribution in gamma-camera images and range of normalized activity ratios in cardiac specimens from rabbits treated with daunorubicin and cyclophosphamide (*D+Cy*), daunorubicin (*D*) or cyclophosphamide (*Cy*). For control animals (*Cont*), the reference level of activity is presented

Treatment	Isotope uptake	*n*	Isotope distribution		Normalized ratio
			Homogeneous	Irregular	Range
D+Cy	+++	2	2		3.6–5.2
	++	5	1	4	1.7–4.4
	+	3	2	1	1.4–2.4
D	+	8	3	5	0.9–1.7
	0	5	5		0.9–1.3
Cy	+	1	1		1.3
	0	3	3		1.0–1.2
Cont	0	17	17		1.0

Pathologic evaluation

Macroscopic changes. The control rabbits showed no cardiac changes. In 7/10 euthanized rabbits treated with daunorubicin and cyclophosphamide, the myocardium appeared pale at necropsy and slight cardiac dilatation was observed in 2 hearts. A hydrothorax with a clear, reddish appearance that contained small fibrin clots was seen in 5/10 animals in this experimental group. Macroscopic changes including pale foci of the ventricular myocardium associated with watery fluid in the thorax were seen in only 2/13 hearts from euthanized rabbits in the daunorubicin-treated group. Subendocardial haemorrhages were seen in only one daunorubicin-treated animal and were otherwise absent. No macroscopic changes were observed in rabbits treated with cyclophosphamide only.

Histopathology. The control rabbits showed no alterations. The histopathologic changes in the hearts from the daunorubicin- and the daunorubicin/cyclophosphamide-treated groups were to a great extent qualitatively the same. The cardiac changes consisted of degeneration and atrophy of cardiac muscle cells, interstitial oedema and fibrosis (Fig. 4). There were two forms of degeneration in both groups: the first type was characterized by myofibrillar loss with pale-staining but non-vacuolated cells; the second manifested as extensive vacuolization (Fig. 4).

Quantitatively, most of the rabbits treated with daunorubicin and cyclophosphamide showed extensive, acute myocyte necrosis in the septum and the left ventricular free wall (Fig. 5). In some cases, subendocardial interstitial oedema was seen. The injuries caused by daunorubicin were most severe and frequent in the base of the lateral left ventricular wall and septum. Changes were particularly predominant in the muscle cells close to large and medium-sized intramural arteries (Fig. 6). No vascular changes were observed. There were only mild, infrequent lesions in the right ventricular wall; even in rabbits with diffuse, very severe transmural damage to most of the left ventricular free wall and septum, the right ventricular wall was only rarely affected. Animals that received only cyclophosphamide generally had very small and infrequent foci of necrosis. Intramyocardial haemorrhages were not observed in any treatment group. Myocardial necrosis was more severe and extensive in rabbits given daunorubicin and cyclophosphamide than in those receiving either daunorubicin or cyclophosphamide alone.

The qualitative and quantitative scores of the treatment groups are presented in Fig. 7. In all, 6/10 rabbits receiving daunorubicin and cyclophosphamide had extensive, acute myocyte necrosis and degenerative lesions, resulting in top qualitative and quantitative scores; 2/10 had the same lesions but these were observed only as single foci at one level, whereas in 2/10 rabbits the lesions were less severe, albeit extensive. Overall, 7/13 rabbits that received daunorubicin alone were given the highest qualitative scores, mainly because of focal preexisting fibrosis; these animals also had degenerative lesions and atrophy, which were extensive in some cases. In animals lacking fibrosis the score was based either on degenerative alterations (4/13), which were infrequent in 3 of these rabbits, or on slight to moderate focal necrosis associated with a cellular reaction (2/13). The scores given to animals treated with cyclophosphamide alone were based on infrequent, very small areas of cellular necrosis (7/8) or degeneration (1/8). The total pathologic scores (qualitative + quantitative scores, Fig. 3) were significantly higher for rabbits treated with both drugs as compared with those treated with either drug alone ($2\alpha \leq 0.005$ and $2\alpha \leq 0.001$, respectively; Table 4).

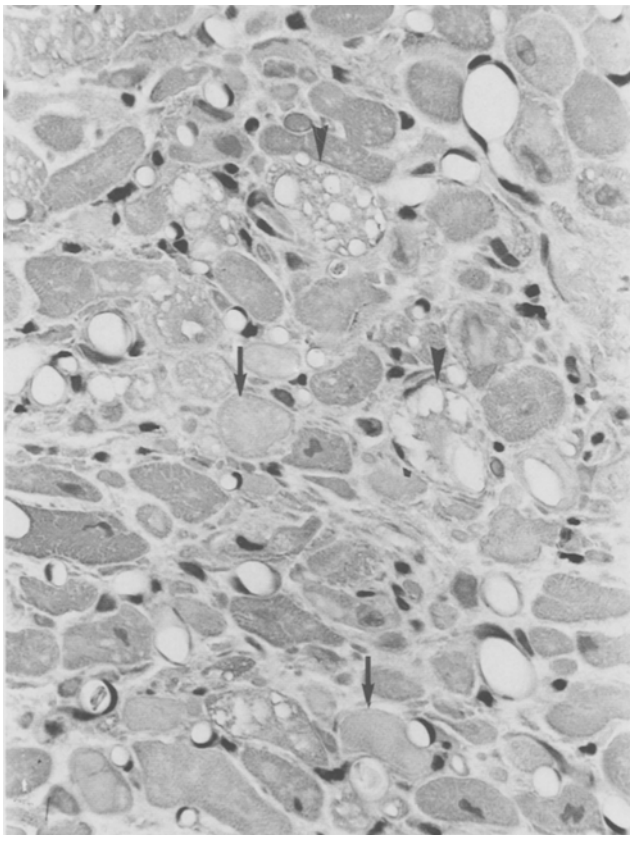


Fig. 4. Severely damaged myocardium from a rabbit treated with daunorubicin and cyclosphosphamide. Pale nonvacuolated cells (*arrows*) and cells with cytoplasmatic vacuolization (*arrowheads*) are visible. Atrophic fibers and interstitial fibrosis can also be seen. Masson's trichrome, ×450.

Ultrastructural changes. The ultrastructural studies showed that the degeneration of cardiac cells in the daunorubicin-treated group and in the daunorubicin/cyclophosphamide-treated group was complex, involving several organelles. The degenerated myocardial cells were characterized by cytoplasmic vacuolization, lysis of myofibrils and mitochondrial changes. Cytoplasmic vacuolization was prominent and resulted from hydropic distension of the sarcoplasmic reticulum and the T-tubules (Fig. 8). The vacuoles were variably sized and diffusely scattered in the fibers and often contained a faintly granular material. To a lesser degree, accumulation of lipids contributed to the vacuolated appearance of the cytoplasm.

The degenerated fibers showed atrophy, which was at least partly caused by lysis of the myofibrils. Short fragments of both thick and thin filaments were scattered in the granular cytoplasmic material. In some cases, fibers exhibited prominent lysis of myofibrils accompanied by sar-

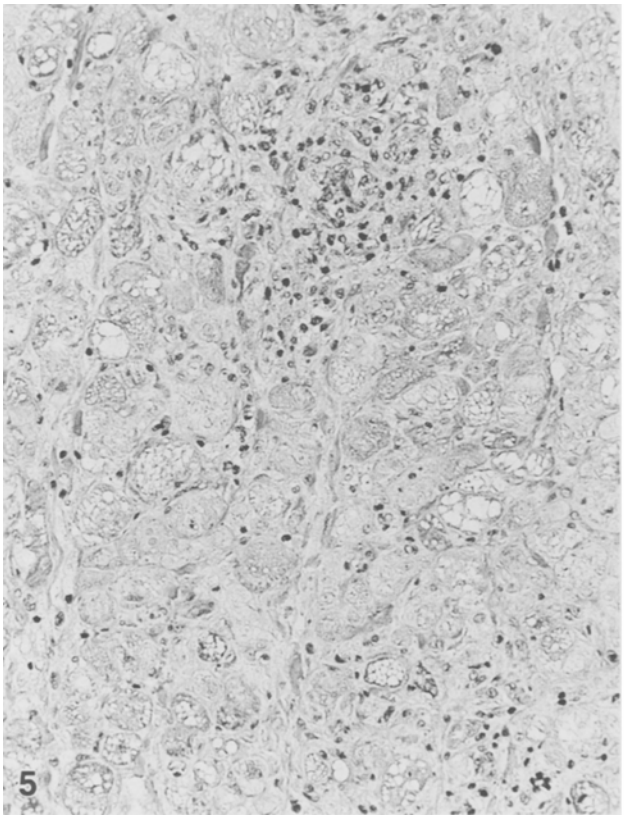


Fig. 5. A daunorubicin/cyclophosphamide-treated animal with a necrotic area infiltrated by neutrophils and macrophages. The surrounding myocytes reveal varying severity of vacuolar degeneration and interstitial fibrosis is prominent. Masson's trichrome, ×280

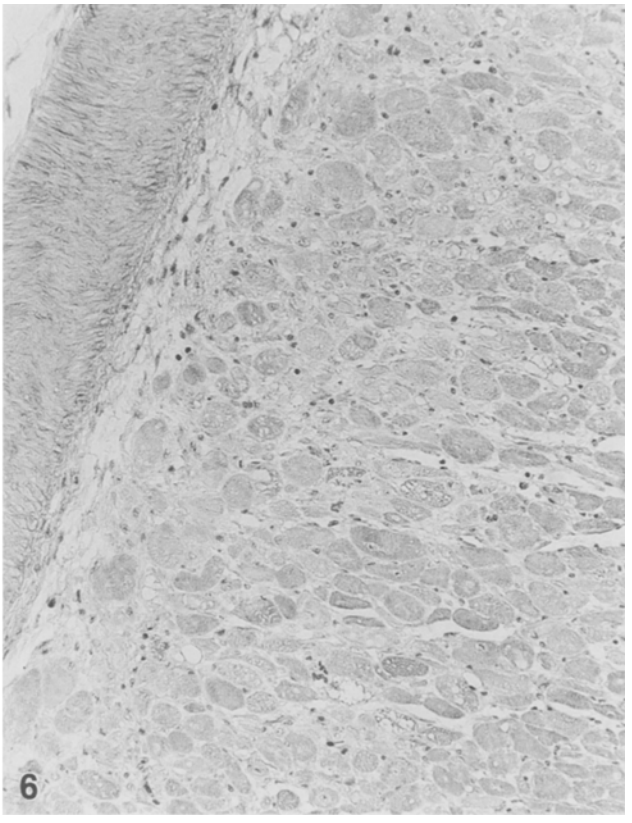


Fig. 6. A daunorubicin-treated rabbit with severely damaged myocardium close to an intramural artery. Masson's trichrome, ×180

coplasmic vacuolization. The appearance of the mitochondria was pleomorphic, showing alterations in the matrix density. Swollen and disrupted mitochondria as well as electron-dense myelin figures were frequently seen (Fig. 9). Generally, mitochondrial changes were more prominent in acute than in chronic lesions, whereas alterations in sarcoplasmic reticulum were mainly seen in chronic lesions but were minimal in acute lesions.

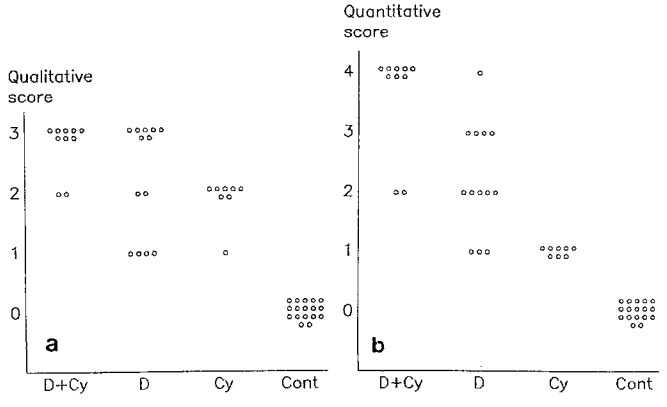


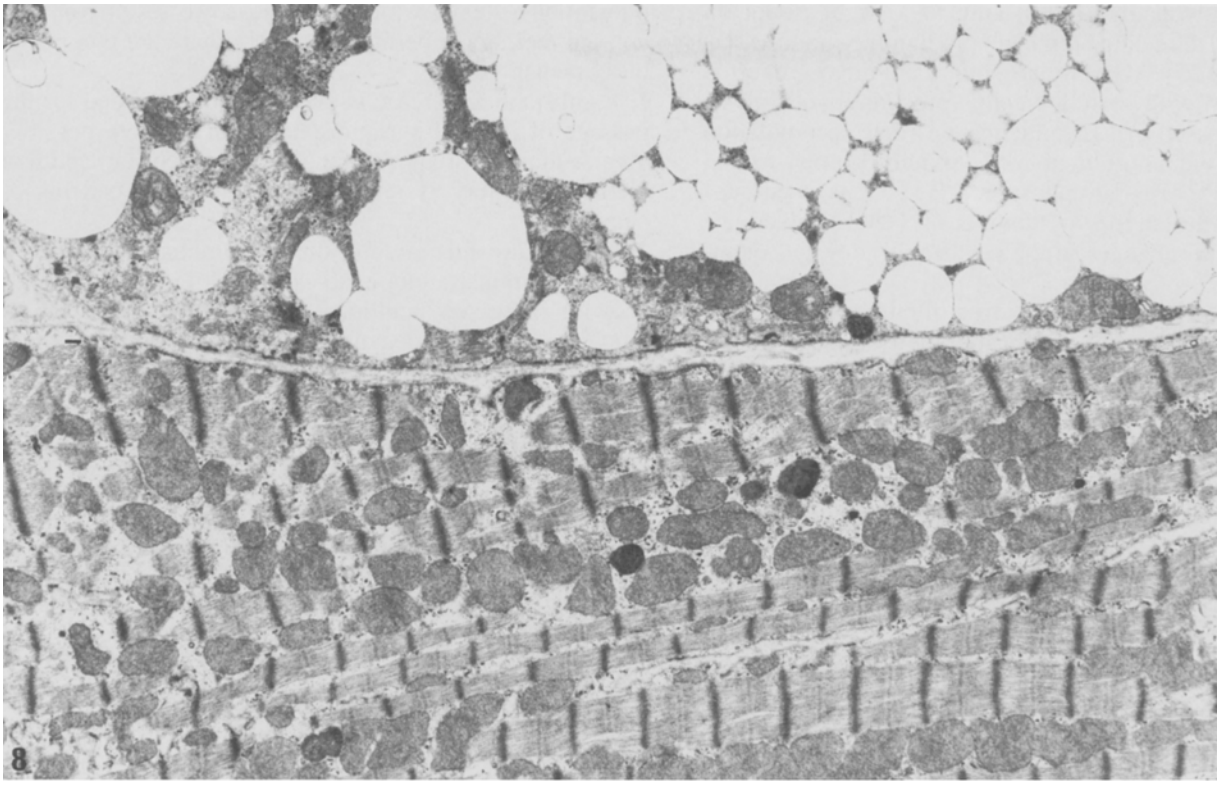
Fig. 7. a Qualitative score and **b** quantitative score reflecting histopathologic changes in rabbits treated with daunorubicin and cyclophosphamide (*D+Cy*), daunorubicin (*D*) or cyclophosphamide (*Cy*) or handled as controls (*Cont*)

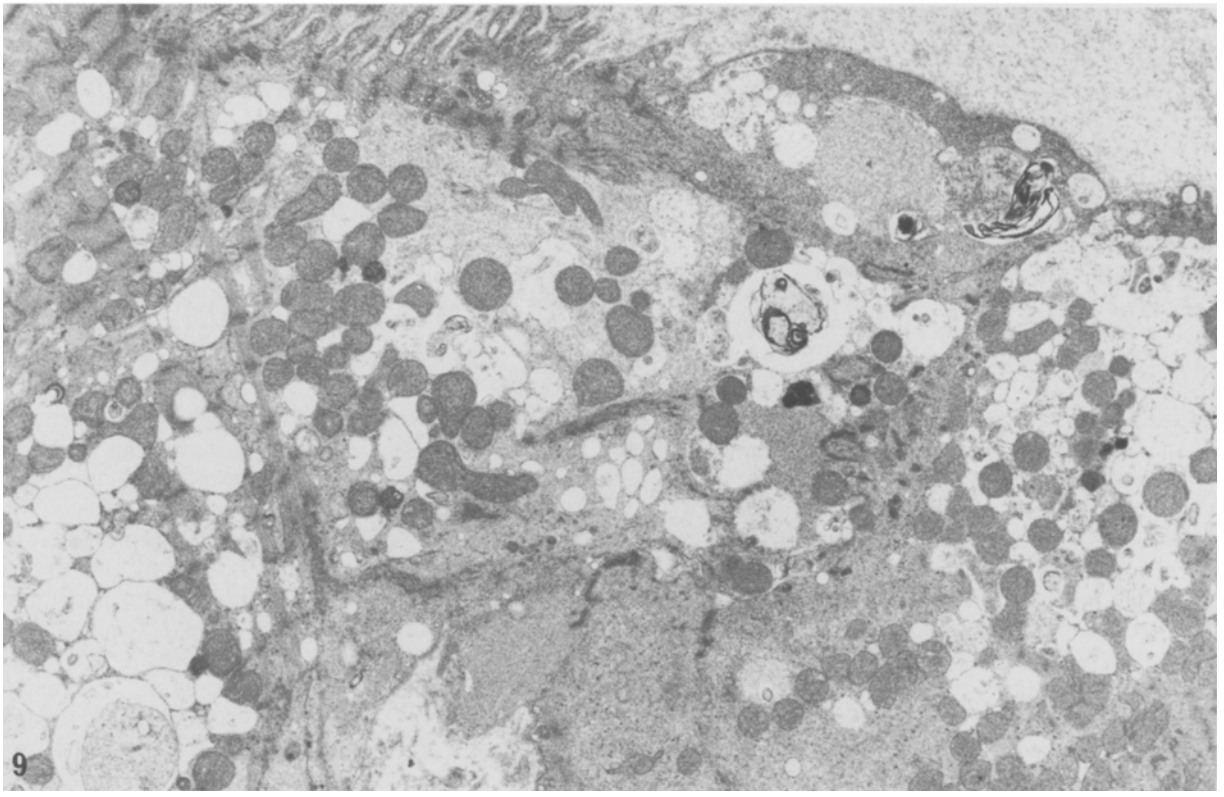
Several alterations, including oedema, fibroblasts and collagen fibrils, were observed in the interstitial tissue that surrounded degenerate fibers. Inflammatory reactions were limited to the presence of scattered macrophages, if seen at all. Ultrastructural differences between the daunorubicin and the daunorubicin/cyclophosphamide treatment groups were not seen. Rabbits treated with cyclophosphamide alone were not evaluated ultrastructurally.

Discussion

In the present study, rabbits received treatment with cardiotoxic cytostatics similar to that given most patients with acute leukaemia prior to allogeneic or autologous BMT. This implied the administration of high doses of cyclophosphamide to animals that had been pretreated with daunorubicin. In contrast to the clinical situation, radiation therapy was not included in the present experiment. In a previous study we showed that a cumulative dose of 22.5 mg/kg daunorubicin caused moderate to severe cardiac lesions, resulting in a low incidence of fatal congestive heart failure in rabbits [1]. This dose probably corresponds to the maximal recommended cumulative dose for humans [13, 14].

Increased [^{99m}Tc]-pyrophosphate uptake occurs in acute myocardial infarction [21], experimental anthracy-





Figs. 8, 9. Electron micrographs of the left ventricle from a rabbit given daunorubicin and cyclophosphamide. **Fig. 8.** The myocyte in the upper part of the figure is severely damaged, with extensive sacroplasmic vacuolization arising from the hydropic distension of elements of sacroplasmic reticulum. The adjacent cardiac muscle cell with slight myofibrillar lysis shows scattered mitochondria with an electron-dense matrix. ×7,500. **Fig. 9.** A cardiac muscle cell with advanced and extensive myofibrillar lysis. The sarcoplasm contains vacuolated sarcoplasmic reticulum and several myelin-like profiles of membranous debris. ×4,500

cline cardiomyopathy [1, 10] and, to a slight extent, in experimental adrenaline- and theophyllamine-induced cardiomyopathy [7]. Myocardial isotope binding is reported to correlate with histologically proven myocytolysis [7, 21]. Increased [^{99m}Tc]-gluconate cardiac accumulation occurs in late daunorubicin cardiotoxicity, similar to that seen using [^{99m}Tc]-pyrophosphate [1]. It has been suggested that the myocyte binding of [^{99m}Tc]-gluconate correlates with changes in the permeability of cell membranes [9].

In the present study, the majority of rabbits treated with daunorubicin showed cardiac alterations typical of anthracycline cardiotoxicity and generally similar to those previously reported by other investigations [16, 20, 27]. The cardiac lesions were most severe in the left ventricular wall and the ventricular septum but occurred only occasionally in the right ventricular wall. There was a tendency for myocardial alterations to be most severe and frequent towards the base of the heart. The lesions were predominantly located around the intramyocardial arteries. Van Vleet et al. [26] suggested that differences in structure, metabolism and work load could be involved in this selective destruction of cardiac muscle fibers in various parts of the myocardium.

In agreement with previous studies, degenerated fibers developed marked atrophy following the lysis of numerous myofibrils. The interstitial tissues were distended by oedema, scattered fibroblasts and macrophages and as increased number of collagen fibrils. Ferrans [8] suggested that oedema might be the result of increased membrane permeability with subsequent calcium overload and increases in the tissue content of sodium and water.

Despite pronounced pathologic changes represented by frequent vacuolization and focal necrosis fibrosis, most of the rabbits from the daunorubicin group showed only slightly elevated myocardial isotope accumulation. This indicates that vacuolized myocytes or focal fibrotic lesions reflecting post-necrotic scars in areas of prior damage are responsible to only a small extent for isotope accumulation. These results contrast with our previous finding in which rabbits receiving daunorubcin on a similar schedule showed clearly increased myocardial isotope accumulation [1]. However, in that study, the rabbits were killed within 3 days following the last injection of daunorubicin, whereas in the present study the animals were kept alive for 2 weeks after the daunorubicin treatment had been terminated. In line with our results are the findings by Gorton et al. that [^{99m}Tc]-pyrophosphate accumulates in hearts from rabbits treated weekly with doxorubicin but that positive myocardial scan images return to normal levels at 2–6 weeks after the discontinuation of doxorubicin [10].

A correlation has been reported between myocyte accumulation of [^{99m}Tc]-gluconate and extracellular leakage of intracellular lactate dehydrogenase, reflecting changes in the permeability of cell membranes [9]. A similar correlation is probable for [^{99m}Tc]-pyrophosphate [21]. It is therefore likely that at 14 days following the last daunorubicin dose, the majority of the remaining myocytes showing daunorubicin-induced degenerative alterations have not incurred severe cell membrane damage. However, this assumption seems to contradict the histologic observation of interstitial oedema, which is proposed to result from increased membrane permeability [8]. There are two possible explanations:

1. A substantial increase in permeability is needed for the passage of [^{99m}Tc]-pyrophosphate into the cell, possibly greater than that required for [^{99m}Tc]-gluconate, and less membrane injury is required for interstitial oedema to occur.
2. The 14-day interval after the discontinuation of daunorubicin administration enables some injured myocytes showing increased membrane permeability to recover, although progressive degenerative changes occur in other myocytes and some seriously damaged myocytes undergo lysis. This lysis leads to the formation of additional interstitial oedema. However, after 14 days the extent of progressive myocytolysis is greatly reduced, leaving myocytes (normal and degenerate) and fibrosis accompanied by low isotope binding together with preexisting interstitial oedema. Furthermore, there is a possibility that interstitial oedema created at an earlier stage remains because of a low disappearance rate.

Animals receiving only high-dose cyclophosphamide showed no increase in myocardial isotope accumulation. This observation is in accordance with the histopathologic analysis, which showed mild myocyte alterations associated with infrequent small areas of acute necrosis. The cardiotoxic pathogenesis of high-dose cyclophosphamide treatment in patients probably results from endothelial toxicity due to transsudation of the drug, with subsequent damage occurring in the surrounding tissues [3, 12]. In agreement with our experiments, Hopkins et al. [15] did not find evidence of haemorrhages or inflammatory reaction after high-dose cyclophosphamide treatment in rats. High-dose cyclophosphamide given to rabbits that had been pretreated with daunorubicin resulted in acute myocardial necrosis and, in some cases, interstitial oedema. The increased myocardial isotope accumulation observed in daunorubicin/cyclophosphamide-treated rabbits appeared to be related to the acute myocardial necrosis found in these animals.

The evaluation of myocardial [^{99m}Tc]-pyrophosphate accumulation carried out in this and in a previous study [1] included heart scintigraphy and measurements of the activity in the hearts and blood using a NaI (Tl) detector. The accumulation of isotope in the normal heart is partly due to uptake in the myocardium (interstitial fluid and tissue, myocytes, endocardium and pericardium) and partly depends on blood activity within the coronary vascular bed. Although coronary blood activity represents a significant portion of the normal cardiac isotope activity, the actual relationship between coronary blood and the myocardial contribution to the total heart activity is not known. However, assuming an interindividually equal relationship between coronary vascular volume and heart weight, the activity ratio defined in this study corrects for varying blood activity and should therefore provide a better estimate of myocardial isotope accumulation than does the activity concentration (total heart activity/heart weight) itself.

Our scintigrams showed regional variations of isotope distribution in many hearts showing toxic damage. This

observation indicates a potential error in the detector's evaluation; the measured counts would vary, depending on how the hearts were placed in the plastic cases during counting. In analysis of the counting geometry, the error was estimated to be ±6%. When two control rabbits were used in the experimental series, the activity ratios of these two controls were almost the same; however, there were larger variations between experimental series. These results are in line with our assumption that the systematic errors of isotope labelling and counting between series were greater between than those within experimental series. The observation that none of the controls showed gross or histopathologic cardiac alterations indicated that the evaluations of isotope activity in these animals could be used as references. Normalizing the activity ratios of treated animals to that or those obtained for controls in the same experimental series would accordingly result in more comparable data between series of experiments than would the use of non-normalized data.

The question as to the prognostic value of [^{99m}Tc]-pyrophosphate scanning for early detection of drug-induced myocardial damage cannot be answered on the basis of the present results. The experimental design, with simultaneous in vitro examination of heart specimens from treated rabbits and controls, reveals even a slight increase in myocardial isotope accumulation. The significance of these findings for the clinical situation is uncertain. It is obvious that the experimental evaluation of cardiac specimens with the gamma-camera enables more sensitive detection than does imaging of living subjects, in which heart movements and varying isotope activity in the blood and the lung influence the sensitivity, whereas other possible etiologies of increased cardiac isotope accumulation (for instance, pericarditis) influence the specificity.

One interesting finding was that gamma-camera imaging made it possible to detect foci showing increased isotope uptake. The detection of such foci seems to be more indicative of pathology. A majority of the daunorubicin-treated rabbits exhibiting slightly or moderately increased myocardial isotope uptake on gamma-camera images showed focal increments, mainly in the base of the left ventricle, whereas isotope distribution in all control hearts was homogeneous. This focal isotope distribution was in accordance with the results of our pathologic evaluation, which often revealed more intensive alterations in the base of the left ventricular wall.

In the present study we confirmed previous findings that long-term treatment with anthracyclines induces chronic degenerative cardiac damage. We also showed that the addition of high-dose cyclophosphamide caused significant acute cardiac toxicity when late anthracycline-induced cardiac alterations had developed. The experimental design did not enable a long-term follow-up of this toxicity, since the cyclophosphamide dose was lethal in the absence of bone marrow transplantation (BMT).

Furthermore, the importance of the interval between the last injection of daunorubicin and the onset of cyclophosphamide administration has not been evaluated. A period longer than the 6–7 days used in the present study might lead to some recovery from cell injury and to less toxic effects of high-dose cyclophosphamide.

With regard to late anthracycline-induced cardiotoxicity, it has been suggested that cardiac dysfunction resulting from doxorubicin toxicity is manifested several years after the discontinuation of therapy [11]. It has also been reported that congestive heart failure has developed at >6 months after the last dose of doxorubicin [13]. A recent experimental study in rats receiving single-dose doxorubicin and epirubicin showed a rapid decline of cardiac output in the first 12 weeks and a persistent depression of cardiac function after 12 weeks following drug injection [28]. High-dose cyclophosphamide given as a part of cytoreduction therapy prior to BMT has been reported to cause lethal haemorrhagic cardiac necrosis [2, 3, 18]. A high incidence of severe cardiotoxicity has also been shown in monkeys receiving 240 mg/kg cyclophosphamide [24]. Moreover, cyclophosphamide-induced cardiotoxicity in rats has resulted in chronic myocardial degeneration [15]. Thus, it is conceivable that patients who are treated with anthracyclines and subsequently receive high-dose cyclophosphamide prior to BMT are at greater risk of developing manifest cardiac damage.

The use of allogeneic as well as autologous BMT is increasing and the indications are continuously reconsidered. It is reasonable that attention be paid to important risk factors such as myocardial injuries that may subsequently become clinically overt. Thus, the importance of further attempts to reduce the cardiotoxicity associated with cytostatic therapy must be emphazised. Variation of treatment schedules and, perhaps, modification of BMT conditioning therapy are means of accomplishing this goal.

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