Failure of the Antioxidant Vitamin E to Protect against Adriamycin-induced Cardiotoxicity in the Rabbit

Johan G. S. Breed, Ariaen N. E. Zimmerman, Jan A. M. A. Dormans, and Herbert M. Pinedo

Department of Oncology, Free University Hospital and Netherlands Cancer Institute, de Bollelaan, Amsterdam [J. G. S. B., H. M. P.]; Department of Cardiology, University Hospital, Utrecht [A. N. E. Z.]; and Laboratory of Pathology, National Institute of Public Health, Bilthoven [J. A. M. A. D.], The Netherlands

ABSTRACT

Recently, vitamin E has been proposed to protect against Adriamycin-induced cardiotoxicity. We studied contractile decline and ultramicroscopic alterations of the heart of rabbits chronically treated with Adriamycin up to a cumulative dose of 400 mg/sq m. High doses of vitamin E did not protect against the Adriamycin-induced development of severe contractile decline as evaluated by means of measurement of the interval-force relationship curve. Light and electron microscopic analysis did not show any signs of protection against Adriamycin-induced morphological alterations. Biochemical and hematological alterations caused by the antineoplastic agent were similar in both Adriamycin-treated animal groups. Coadministration of vitamin E did result in an increased life span. This study indicates that vitamin E does not protect against the development of cardiomyopathy and contractile decline after chronic exposure to Adriamycin.

INTRODUCTION

The anthracycline antibiotic ADM is highly active against a variety of solid tumors and malignant hematological processes. Its most unpredictable toxic manifestation is an insidious, irreversible cardiomyopathy (3). Endocardial biopsies show marked pathological alterations of the heart tissue after administration of a cumulative dose as low as 200 mg/sq m body surface area (4). The incidence of this side effect increases significantly when the cumulative dose exceeds 550 mg/sq m (16). At present, dose limitation is the only way to prevent this myopathy. The pathogenesis of ADM-induced cardiotoxicity is unknown, but several mechanisms have been proposed (1, 7, 24). Recently, Myers, et al. (21) showed in a short-term experiment that ADM-induced cardiotoxicity can be reduced in BALB/c × DBA/2 F1 (hereafter called CD2F1) mice by pretreatment of the animals with α-tocopherol. The purpose of the present study was to find out whether in the rabbit chronic coadministration of vitamin E could prevent the development of changes in contractile behavior of the heart as measured on the basis of the IFR (2).

MATERIALS AND METHODS

Animals. Male New Zealand White rabbits, weighing 2.9 to 3.9 kg, were obtained from ENKI Farms, Someren, The Netherlands, and were kept in a climatized environment, isolated from other animals, in individual cages meeting recommended space requirements. The rabbits were given injections of polyvalent antipasteurella immune globulin. The animals were given water and pelleted food (Hope Farms, Woerden, The Netherlands), containing a-tocopherol acetate (89 mg/kg) in the form of a-tocopherol acetate and selenium (0.41 mg/kg) ad libitum. One week after the predeterined dose of ADM, the 400-mg/sq m cumulative dose level was reached, and the animals were killed. The predeterined dose was calculated according to the formula 56.33 (weight in g) 0.428 = surface in sq m (25).

Drugs. Lyophilized ADM hydrochloride (NSC 123-127) was a gift of Dr. H. Wood (Drug Synthesis and Chemistry Branch, National Cancer Institute, NIH, Bethesda, Md.) and Dr. C. de Sloop (Montedison, Brussels, Belgium). Pure, unesterified α-tocopherol acetate was obtained from Serva Feinbiochemica, Basel, Switzerland. At regular intervals, the vitamin E and α-tocopherol quinone levels were assessed by means of thin-layer chromatography using freshly received vitamin E as reference standard.

Drug Administration. Group 1 consisted of 14 male rabbits receiving ADM hydrochloride (1 mg/kg) as single agent twice a week. The drug was reconstituted at a concentration of 2 mg/ml and administered to the rabbits via the marginal ear vein. This dose is considered to be sublethal to rabbits (10). Ten rabbits received in addition vitamin E (300 mg/kg/day i.p.) 5 times a week (Group 2). In these animals, ADM was given 3 hr after vitamin E administration. The dose of vitamin E was calculated on the basis of studies done by Myers in CD2F1 mice (20). Seven animals received the vitamin alone (Group 3). To assess the uptake of vitamin E after i.p. injection, blood samples were taken from 3 rabbits before and 1, 3, and 72 hr after the administration of the vitamin alone. Six rabbits received 0.9% NaCl solution i.p. 5 times/week and also i.v. 2 times/week (Group 4).

Biochemical Studies. The following biochemical parameters were determined in serum every 4 weeks throughout the treatment period: calcium (direct fluorocomplexometric method; Corning Analyser 940); urea (enzymatic, followed by the reaction of Berthelot); total protein (biuret method); triglycerides (fully enzymatic kinetic method); and creatinine kinase (according to the method of Oliver and Rosalki). Serum glutamate-oxalate transaminase and serum glutamate-pyruvate transaminase activities were measured at 25° with an automatic kinetic enzyme system (Vitatron AKES, Dieren, The Netherlands).

Hematology. Complete blood count (including hemoglobin, packed cell volume, WBC, and thrombocytes) was performed biweekly.

Vitamin E Determinations. For these assays, high-pressure liquid chromatography was used as follows. To 0.5 ml serum was added 0.5 ml 99% ethanol. After vortexing, 5 ml hexane

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were added and mixed. This mixture was spun down at 4000 rpm for 10 min at 4°C, after which 4 ml of the supernatant were dried under nitrogen at 35°C and the residue was dissolved in 200 μl hexane. Fifty μl of this mixture were injected into the high-pressure liquid chromatograph. The samples were assayed on a Partisil column (25 cm long, 0.4 cm internal diameter). The eluant, a mixture of 5% diisopropyl ether and n-hexane, was degassed before use. The flow rate was set at 2 ml/min. Vitamin E was detected with a Pye Unicam detector at 295 nm wavelength.

Cardiac Function (IFR). After the predetermined dose of 400 mg/sq m was reached or after 3 months, the rabbits were anesthetized with Hypnorm (1 mg/kg i.m.). After sufficient anesthesia was obtained, the animals were heparinized, thoracotomy was performed, and the beating heart was rapidly removed, attached to the Langendorff system, and perfused retrogradely (2). The standard perfusate was a modified Tyrode solution of the following composition (mmol): NaCl, 128; CaCl₂, 1.3; KCl, 4.7; MgCl₂, 1.0; NaH₂PO₄, 0.43; NaHCO₃, 20.2; glucose, 11, in deionized bidistilled water. After oxygenation by bubbling with 95% O₂ and 5% CO₂, the pH was 7.3 to 7.4. The perfusate temperature was 37°C, at a perfusion pressure of 70 cm water. After trimming of excess tissue, the mitral valve was made insufficient, and an atrioventricular block was applied. Two platinum eyelet electrodes were then sutured onto the heart and connected to a pulse generator. A displacement transducer was attached to the left ventricular apex, through which the apicobasal shortening was registered on a Hewlett Packard 78748A recorder. The plot of this shortening as a function of stimulation frequency is called a "Kruh curve" and is regarded as a reliable measure for contractility (2, 14). Next, the heart was perfused until a steady state was obtained. The IFR curve was obtained by stimulating the heart for 60 sec with a 10 ma-10 μsec pulse. The initial pulse interval, which was 500 msec, was decreased in 50-msec steps until no adequate reaction on pacing occurred. This procedure takes 10 min.

Light and Electron Microscopy of the Heart. Immediately after the cardiac function test, the hearts were perfused with 2.5% glutaraldehyde in buffered phosphate for 10 min. This procedure is known to result in well-fixed tissue for further microscopic analysis. Standardized sections taken from the left ventricular free wall and the interventricular septum were postfixed in 1% osmium tetroxide and embedded in Epon. Silver-gray sections were cut on a LKB ultramicrotome III, stained with uranyl acetate and lead citrate, and examined in a Philips EM 200 electron microscope. The remaining heart tissue was postfixed in 4% formaldehyde solution for light microscopic analysis. The tissues were embedded in paraffin, sectioned, stained with hematoxylin-eosin, and graded for severity of the lesions according to the following scale (27): Grade 0, no cardiomyopathy; Grade 1, foci of lesions in the left ventricular free wall; Grade 2, foci of lesions in the free wall and the interventricular septum; Grade 3, diffuse lesions in the left ventricular free wall and focal lesions in the interventricular septum; and Grade 4, diffuse lesions in the left ventricular free wall and interventricular septum. The mean score of damage was determined for each of the 4 treatment groups. After removal of the heart, material was collected. The liver, kidney, diaphragm, and aorta were fixed in 4% formaldehyde solution for histopathology. The tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistical analysis by single-factor analysis was applied to the chemical results, and the homogeneity of the contractility parameters in all groups together was tested by the method described by Kendal and Stuart (13).RESULTS

General Toxicity and Survival. Severe systemic toxicity was observed in the ADM-treated animals as well as in the groups treated with ADM plus vitamin E. Toxicity was expressed as severe weakness, weight loss, and characteristic periocular and middorsal alopecia. Animals in both ADM-treated groups showed progressive loss of appetite. After 70 days of treatment, 7 rabbits in the ADM-treated groups and 7 rabbits in the group given ADM in combination with vitamin E were still alive. The other animals died of progressive weight loss and pulmonary infections. One animal died intercurrent (Group 2) of the effects of a jump after an ADM injection; necropsy showed rupture of the stomach. No deaths occurred in the groups treated with vitamin E or 0.9% NaCl solution. The number of rabbits in each group was too small to permit determination of significant prolongation of survival time. The mean survival time and cumulative mortality in the 10th week are shown in Chart 1. In the seventh week, urine production of the ADM-treated rabbits increased temporarily, and the animals became dehydrated; all were given 100 ml 0.9% NaCl solution daily for 10 days.

Hematological Toxicity. WBC did not differ significantly between the 4 groups throughout the experiment. The mean total hemoglobin concentration and the packed cell volume dropped in all animals given ADM (Table 1). The mean thrombocyte count was significantly (p < 0.05) lower in the ADM-treated animals as well as in the groups treated with vitamin E or 0.9% NaCl solution. The number of rabbits in each group was too small to permit determination of significant prolongation of survival time. The mean survival time and cumulative mortality in the 10th week are shown in Chart 1. In the seventh week, urine production of the ADM-treated rabbits increased temporarily, and the animals became dehydrated; all were given 100 ml 0.9% NaCl solution daily for 10 days.

Biochemical Parameters. Urea and creatinine kinase con-
vitamin E Concentration. The initial mean plasma vitamin E concentration in the ADM-treated animals (Groups 1 and 2) was significantly lower than in the control rabbits at 8 and 12 weeks (Table 2). The mean total serum calcium concentration in the rabbits of Groups 1, 2, and 3 decreased in the course of the experiment; the divergence from control values only became significant (p < 0.05) at Week 12 (Table 3). At the end of the experiment, plasma triglyceride concentrations in both ADM-treated groups were 190% of values in the group not given ADM (p < 0.05).

**Table 1**

<table>
<thead>
<tr>
<th>Treatment time (wk)</th>
<th>Hemoglobin (g/l)</th>
<th>Packed cell vol. (%)</th>
<th>Thrombocyte counts (x10^9/cu mm)</th>
<th>Hemoglobin (g/l)</th>
<th>Packed cell vol. (%)</th>
<th>Thrombocyte counts (x10^9/cu mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.5 ± 0.3</td>
<td>40 ± 0.3</td>
<td>370 ± 91</td>
<td>6.9 ± 0.8</td>
<td>43 ± 0.5</td>
<td>360 ± 77</td>
</tr>
<tr>
<td>4</td>
<td>7.5 ± 0.6</td>
<td>37 ± 0.6</td>
<td>163 ± 74</td>
<td>7.4 ± 0.5</td>
<td>35 ± 0.3</td>
<td>200 ± 85</td>
</tr>
<tr>
<td>8</td>
<td>7.3 ± 0.5</td>
<td>34 ± 0.5</td>
<td>145 ± 103</td>
<td>6.3 ± 0.9</td>
<td>29 ± 0.5</td>
<td>215 ± 61</td>
</tr>
<tr>
<td>12</td>
<td>5.5 ± 0.6</td>
<td>28 ± 0.3</td>
<td>152 ± 75</td>
<td>6.0 ± 2.1</td>
<td>29 ± 0.9</td>
<td>280 ± 16</td>
</tr>
</tbody>
</table>

a Mean ± S.E. (n = 6 to 8).
b Significantly different from control (p < 0.05).

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total plasma protein (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM</td>
<td>62 ± 6</td>
</tr>
<tr>
<td>ADM + vitamin E</td>
<td>59 ± 9</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>69 ± 4</td>
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</table>

a Mean ± S.E.
b Significantly different from control (p < 0.05).

**Table 3**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Plasma calcium concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>ADM + vitamin E</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Control</td>
<td>3.4 ± 0.1</td>
</tr>
</tbody>
</table>

a Mean ± S.E. (n = 5 to 9).
b Significantly different from control (p < 0.05).

Vitamin E Protection Failure against ADM Cardiotoxicity

Homogenization of the sarcoplasm.

Rabbit hearts from the group treated with ADM plus vitamin E showed myofibrillar degeneration and necrosis as well. Four of 6 hearts showed irregularly shaped pale areas in the myocardium. There was no preferred localization of these lesions. All hearts in this group showed a disorganized myofibrillar pattern. Myocytolysis due to mononuclear phagocytes was seen in 5 of 6 hearts. Vaccuolation was more pronounced in the hearts of rabbits treated with ADM alone. The lesions were consistently accompanied by inflammatory infiltration of the myocardium, in all likelihood as a response to the degenerative changes and necrosis of the myocytes. Quantitative grading for severity of the lesions is shown in Table 4. No clear difference was found between the 2 ADM-treated groups (Groups 1 and 2). Hearts of the vitamin E-treated group showed only slight vacuolation of some myocytes (Fig. 1).
disintegrating segments, filamentous material, and degenerating mitochondria. Large areas of the sarcoplasm became filled with complex degenerated material ultimately resulting in “adria cells” (12). Early changes in the mitochondria appeared to be characterized by swelling and separation of cristae and condensation of the outer mitochondrial membrane. The mitochondria were relatively small and had electron-dense inclusions. Both ADM-treated groups showed separation of the intercalated disc, which was studded with filamentous material and concentric multilaminated bodies. This separation was not observed in the group given vitamin E alone.

Light Microscopy of the Liver, Kidney, Aorta, and Diaphragm. The light microscopic findings in the liver and kidney of the animals treated with ADM were similar to those in the animals treated with ADM plus vitamin E. The liver parenchyma showed centrolobular congestion. The kidneys showed distended tubules with necrosis, extensive thickening of the basement membrane of Bowman’s capsule, and calcification. In all cases, the tubuli contained protein casts. There was sporadic focal interstitial infiltration by mononuclear leukocytes. Light microscopy of the aorta showed sclerotic thickening of the intima in all vitamin E-treated animals; the diaphragm showed no abnormalities.

IFR of the Heart. In the IFR studies, the control animals showed a gradual increase of apicobasal shortening with increasing stimulation frequency. Maximal shortening occurred at a stimulation interval of 250 msec. The increase amounted to up to 120% of the displacement at the initial stimulation interval of 500 msec (Chart 2). The IFR of ADM-treated animals showed an impaired contractile function; maximum displacement occurred at a longer stimulation interval than in hearts of control rabbits. The IFR of hearts of animals given vitamin E additionally showed a similar curve, thus indicating that the coadministration of vitamin E had no beneficial effect on the contractile impairment induced by ADM. Hearts of animals treated with vitamin E alone showed a slight depression of contractile behavior. The homogeneity test of Kendall and Stuart showed a significant difference (p < 0.05) between the maximum contraction height values of Groups 1, 2, and 3 and the values of the control group. A slight difference was found between the stimulation frequency values at the maximum contraction height of Groups 1 and 2 and of Groups 3 and 4. No significant difference was observed between the animals in Groups 1 and 2.

DISCUSSION

The heart of the New Zealand White rabbit has been shown to be quite sensitive to ADM, developing well-circumscribed pathological changes after a cumulative drug dose of 400 mg/

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Severity Score Grade: No. of rabbits</th>
</tr>
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<tbody>
<tr>
<td>ADM</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>ADM + vitamin E</td>
<td>1 1 4</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>3 1 1 4</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 4

Cardiomyopathy score in the 4 treatment groups

Contingency table showing the incidence and severity of cardiomyopathy in the 4 treatment groups.

Values ± S.E.M.

Chart 2. IFR curves (Kruta curves) of animals in the various treatment groups. E, vitamin E. sq m (10). Vitamin E has been shown to have a protective effect on the isolated heart muscle perfused under hypoxic conditions followed by reoxygenation (8). Furthermore, Myers et al. (20) showed that coadministration of vitamin E prevented the formation of malondialdehyde, a breakdown product of lipid peroxides, and prolonged the survival of CD2F1 mice after a single dose of ADM.

In the present study, the pathological light and electron microscopic changes found in the rabbit heart after chronic administration of ADM were similar to those described in the literature (11). Development of these abnormalities was not prevented by supraphysiological plasma plateau levels of vitamin E (20 to 30 μM). Morphological analysis of the hearts treated with ADM (Groups 1 and 2) revealed changes similar to those found in other studies (12). The lesions were present throughout the analyzed area of the heart. Previous investigators reported a tendency for the lesions to develop in fibers around arteries and arterioles (27), but this was not confirmed by our findings in the present study. All stages in the development of the lesions were observed, including sarcoplasmic vacuolation, myofibrillar lysis, fiber death, phagocytosis of fibers, and replacement fibrosis. Because ADM-induced cardiotoxicity is clinically characterized by progressive cardiac failure, the contractile behavior of these hearts was studied as well as the morphology. The IFR of the hearts of the ADM-treated animals showed severe disturbances, and vitamin E had a small insignificant effect on the decline in contractile behavior. In the present study, plasma creatinine kinase activity was not significantly altered in the 3 treatment groups compared with that in the control animals. This is in agreement with the findings in earlier studies performed in rabbits (10) and other species (22).

Minor morphological changes were observed in the hearts of animals treated with vitamin E alone, although there was a
slight but insignificant change in the IFR compared to that of control hearts, possibly related to the pathological findings in the aorta. A cumulative ADM dose of 400 mg/sq m led to 50% mortality during the 10-week period of exposure. The cause of death was probably multifactorial. Treatment with ADM makes the animal susceptible to infections (9). Extracardiac toxicity effects were present in most of the ADM-treated rabbits, the most prominent being renal dysfunction. The effect of ADM on the kidney has been described in both animals and humans (5, 29). Additional s.c. administration of 0.9% NaCl solution in our series of experiments may have prolonged normal time in both ADM-treated animal groups rather than precipitate death among the animals receiving both ADM and vitamin E in the course of the seventh and eighth weeks. It is difficult to assess the degree to which the accumulation of fluids in the body cavities is ascribable to renal dysfunction, cardiac failure, hypoproteinemia (Groups 1 and 2), or vitamin E i.p. (Groups 2 and 3). Because of these changes, the body weight proved to be a poor parameter for toxicity in this study.

The mechanisms which could explain the protective effect of vitamin E on the heart observed by others (20) are unknown, but it has been postulated that membrane stabilization and antioxidation are involved (17). Vitamin E binds to sulfur-containing proteins enhancing membrane stability (18).

ADM-induced alterations in the membrane deserve further study in more depth. It has been shown that incubation of ADM in vitro with erythrocytes results in increased permeability of membranes (23). ADM reacts with lipid membranes, in particular the cardiolipids in mitochondrial membranes, and with glycoproteins in the membrane of the cardiac myocytes. Like other quinones, ADM is an effective chelator of divalent cations (28) which play a key role in the maintenance of structural and functional integrity (6, 15). The drug also interferes directly with the formation of high-energy phosphates necessary for the preservation of the integrity of the membrane of the myocyte (19). ADM can be converted to its semiquinone radical by either mitochondrial or microsomal metabolism. This semi-quinone can give rise to free radical formation, thus causing peroxidation of polyunsaturated fatty acids in the cellular and mitochondrial membrane (1, 26).

Since all of these influences could be responsible for the progressive impairment of cardiac function, it may be concluded that the elimination of one toxicity-inducing mechanism will not prevent the ultimate development of ADM cardiotoxicity. Reduction of the chelating properties of anthracyclines might offer an additional approach to the development of less cardiotoxic anthracyline analogs, as shown for the iron-containing agent quelamycin (7). In any case, the findings made in the present study resulted in only marginal protection. Hence, clinical use of vitamin E for the prevention of ADM-induced cardiotoxicity does not seem to be warranted at present.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Section of myocardium from a vitamin E-treated rabbit. Note the slight vacuolation of the myocytes (arrow). Toluidine blue, × 350.

Fig. 2. Electron micrograph of myocardium from a rabbit given ADM in combination with vitamin E. Myofibrils are widely separated and disintegrated. Sarcoplasm contains disorganized filaments and mitochondrial with electron-dense bodies. Uranyl acetate-lead citrate, × 7,600.

Fig. 3. Low-power magnification of cross-sectioned myocardial fiber, showing disorganized myofilaments in a rabbit given ADM plus vitamin E. Uranyl acetate-lead citrate, × 5,900.

Fig. 4. Note the distension of the sarcoplasmic reticulum (arrow) with separation of the mitochondrial cristae. DM plus vitamin E. Uranyl acetate-lead citrate, × 14,700.

Fig. 5. Longitudinal section through a myocardial fiber with abnormal electron-dense disintegrating mitochondria. DM plus vitamin E. Uranyl acetate-lead citrate, × 14,700.

Fig. 6. Longitudinally sectioned myocardial fiber showing dilation of the intercalating disc (arrow) which is studded with membranous material. Rabbit given ADM plus vitamin E. Uranyl acetate-lead citrate, × 14,700.
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